JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Methylation Methods for the Quantitative Analysis of Conjugated Linoleic Acid (CLA) Isomers in Various Lipid Samples

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Precise methylation methods for various chemical forms of conjugated linoleic acid (CLA), which minimize the formation of *t,t* isomers and allylmethoxy derivatives (AMD) with the completion of methylation, were developed using a 50 mg lipid sample, 3 mL of 1.0 N H_2SO_4/methanol, and/or 3 mL of 20% tetramethylguanidine (TMG)/methanol solution(s). Free CLA (FCLA) was methylated with 1.0 N H_2SO_4/methanol (55 °C, 5 min). CLA esterified in safflower oil (CLA-SO) was methylated with 20% TMG/methanol (100 °C, 5 min), whereas CLA esterified in phospholipid (CLA-PL) was methylated with 20% TMG/methanol (100 °C, 10 min), followed by an additional reaction with 1.0 N H_2SO_4/methanol (55 °C, 5 min). Similarly, CLA esterified in egg yolk lipid (CLA-EYL) was methylated by base hydrolysis, followed by reaction with 1.0 N H_2SO_4/methanol (55 °C, 5 min). These results suggest that for the quantitative analysis of CLA in lipid samples by GC, proper methylation methods should be chosen on the basis of the chemical forms of CLA in samples.

KEYWORDS: Conjugated linoleic acid (CLA); esterified CLA (ECLA); free CLA (FCLA); isomerization; methylation method

INTRODUCTION

Conjugated linoleic acid (CLA), which exhibits several significant biological activities in animals (*1*−*9*) and humans (*10*), is a collective term for positional (9,11; 10,12) and geometric (*c*,*t*; *t*,*c*; *c*,*c*; and *t*,*t*) isomers of octadecadienoic acid (C18:2) with a conjugated double-bond system (*2*). Among the isomers, *c*9,*t*11 CLA and *t*10,*c*12 CLA (*c*/*t* CLA) isomers are major and the rest are minor constituents.

Much concern about the determination of the *c*/*t* CLA isomers has been given, because they have different values for biological activity (*11*, *12*) and are contained in a variety of foods (*13*−*16*) and biological materials (*2*, *5*, *8*, *17*, *18*). CLA isomers are usually quantified by gas chromatographic (GC) analysis of their methyl esters prepared by acid- or base-catalyzed methylation methods (*18*−*24*). Because the two major CLA isomers are thermodynamically unstable, care should be paid for the methylation of the CLA isomers.

Acid-catalyzed methylation, which is suitable for the preparation of methyl esters of free fatty acids or fatty acids esterified in glycerides and phospholipids, easily isomerizes the *c*/*t* CLA to their corresponding *t*,*t* CLA isomers and/or converts to allylmethoxy derivatives (AMD). H_2SO_4/methanol, anhydrous HCl/methanol, or BF_3/methanol is a choice of acid catalysts for the methylation of esterified CLA (ECLA) and free CLA (FCLA) at 80−100 °C for 1 h (*24*), resulting in the extensive isomerization of the *c*/*t* CLA to *t*,*t* CLA isomers and conversion to AMD. When acid-catalyzed methylations are carried out at a low temperature (60 °C) using 4% HCl/methanol, or at room temperature using 14% BF_3/methanol with longer reaction time, the extent of isomerization and AMD formation is greatly reduced (*21*, *24*, *25*). At such a condition, methylation of CLA esterified in triglyceride (CLA-TG) and CLA esterified to phospholipid (CLA-PL) is not completed, resulting in inaccurate quantitation of CLA isomers. Meanwhile, tetramethylguanidine (TMG)- or $NaOCH_3$-catalyzed methylation methods can be applied only to ECLA such as CLA-TG and CLA-PL (*23*), but these methods have a problem for the methylation of FCLA, which is present in samples. Even though TMG has an astringent smell, it is more efficient for the methylation of ECLA than $NaOCH_3$.

Given this information, it is of great importance to standardize the methylation conditions, such as reaction time and temperature and concentration of acids in methanol, for given chemical forms of CLA to minimize the isomerization and the AMD formation and to complete the methylation. Methylation methods of CLA reported in the literature to date applied only to a

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10.1021/jf011185b CCC: $22.00 © 2002 American Chemical Society
Published on Web 02/02/2002

specific CLA sample; hence, systematic or integrated methylation methods are needed for the variety of chemical forms of CLA samples.

CLA is included in food extracts (*13−16*, *21*), dietary supplements (*10*, *27*), and other products (*2*, *17*, *18*, *28*) in a variety of chemical forms, including CLA-TG and CLA-PL. Commercial CLA, being used for the examination of its biological activity, is a FCLA form (*27*, *28*). Recently, researchers have been interested in the preparation of CLA-containing structured fats and oils (*29−32*) and the production of CLA-containing eggs from hens fed the commercial FCLA (*33*, *34*).

It is of interest to provide simple and precise methylation methods for the quantitative analysis of CLA isomers in specific CLA samples such as FCLA, structured fats and oils, phospholipids, and egg yolks. Hence, the objective of the present study is to develop methylation methods that minimize the formation of *t*,*t* CLA isomers and AMD, for a variety of chemical forms of CLA for quantitative analysis by GC. Four representative chemical forms of CLA, FCLA, CLA esterified in safflower oil (CLA-SO), CLA-PL, and CLA esterified in lipids of egg yolk (CLA- EYL), were used as samples for the methylation with 1.0 N H_2SO_4/methanol and/or 20% TMG/methanol. Lipozyme was used as a positive control agent.

MATERIALS AND METHODS

Materials. Linoleic acid (99.0%) and egg yolk lecithin (96.1%) were purchased from Dusan Laboratories (Yongin, Korea). H_2SO_4 (>98%) and HCl (>99%) were obtained from Aldrich Chemical Co. (Milwaukee, WI). BF_3 (14% in methanol), TMG, absolute methanol, and heptadecanoic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Lipozyme was obtained from Novo Nordisk (Bagsvaerd, Denmark). Methanolic HCl (1.0 N) solution was prepared by dissolving HCl in absolute methanol. A precoated silica gel 60 F245 plate (20 × 20 cm, alumina) was obtained from Merck (Darmstadt, Germany). All other chemicals used were of reagent grade.

Preparation of FCLA, CLA-SO, and CLA-PL. FCLA was chemically synthesized from linoleic acid by alkaline isomerization according to the method of Kim et al. (*35*). CLA-SO and CLA-PL were prepared by Lipozyme-catalyzed transesterification of the CLA methyl ester (CLA-Me) to SO and egg yolk lecithin, respectively, and then purified according to the method described by Park et al. (*31*).

Preparation of CLA-EYL. Eggs were collected from laying hens fed the NRC normal diet supplemented with 2.5% FCLA, synthesized from SO according to the method described by Kim et al. (*35*), for 4 weeks in a temperature- and humidity-controlled facility at the Department of Animal Science, Gyeongsang National University, Chinju, Korea. The total egg yolk lipid was extracted from the egg yolk with a hexane/2-propanol (3:2, v/v) mixture (*33*). Free fatty acids in the total lipid were removed by saponification, followed by extraction with hexane (*36*). An aliquot amount of the total lipid was dissolved in 10 mL of hexane and then stored at −20 °C until use.

Hydrolysis of CLA-SO, CLA-PL, and CLA-EYL Samples. One milliliter of ethanolic KOH solution (1.0 N KOH in 90% ethanol) was added to the screw-cap test tube (15 mL) containing sample (∼100 mg). The test tube was filled with nitrogen and capped tightly with a Teflon-lined screw cap. The test tube was wrapped with aluminum foil to prevent light exposure and then heated in a water bath (80 °C) for 1 h and cooled to room temperature. The reaction mixture, mixed with distilled water (3 mL), was extracted with hexane (3 mL × 3) to remove unsaponifiable substances. The aqueous layer was acidified with 6.0 N HCl (1 mL), vortexed for 30 s, and extracted with hexane (3 mL × 3). The hexane extract was washed with distilled water (3 mL × 3), dried over sodium sulfate anhydrous, and rotorevaporated in a water bath (40 °C).

Methylation of FCLA. Methanolic H_2SO_4 (0.05 and 1.0 N), HCl (0.05 and 1.0 N), and BF_3 (0.7 and 14%) solutions were used for the methylation of FCLA and other free fatty acids. An acid solution (3 mL) was added to a screw-cap test tube (15 mL), containing sample

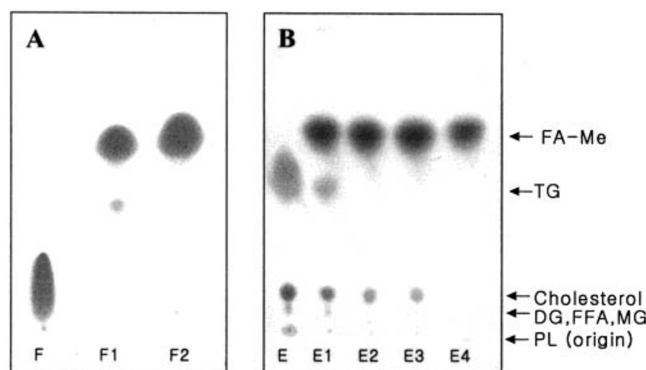

Figure 1. Completion of the methylation of FCLA (A) and CLA-EYL (B) samples methylated by various acid- or base-catalyzed methods. Methylation condition for (A): F, FCLA (control); F1, 0.05 N H_2SO_4/methanol (100 °C, 5 min); and F2, 1.0 N H_2SO_4/methanol (55 °C, 5 min). Methylation condition for (B): E, CLA-EYL (control); E1, 1.0 N H_2SO_4/methanol (100 °C, 30 min); E2, 20% TMG/methanol (100 °C, 20 min); E3, 20% TMG/methanol (100 °C, 10 min), followed by reaction with 1.0 N H_2SO_4/methanol (55 °C, 5 min); and E4, base hydrolysis, followed by methylation with 1.0 N H_2SO_4/methanol (55 °C, 5 min).

(50 mg) with or without heptadecanoic acid (1 mg) as the internal standard, and vortexed for 30 s. The test tube was heated in a water bath (100 or 55 °C) for various reaction times (5, 10, or 20 min). After the test tube was cooled to room temperature, CLA-Me was extracted with hexane (3 mL × 3) by hand-shaking for 1 min. The hexane extract was washed with 1.0 N NaOH in 50% ethanol (3 mL × 2) and with distilled water (3 mL × 3). The sample was dried over sodium sulfate anhydrous and analyzed by the GC described below.

For Lipozyme-catalyzed methylation of FCLA, Lipozyme (10% H_2O) and hexane (2 mL) were added to the screw-cap test tube containing FCLA (50 mg) in methanol (33 μL). The sample test tube, filled with nitrogen and capped, was incubated in a shaking incubator (60 °C, 170 rpm) for 2 h. CLA-Me was extracted and purified according to the procedure described in the acid-catalyzed methylation method.

Methylation of ECLA. ECLA (CLA-SO, CLA-PL, and CLA-EYL) was methylated by a direct acid- or base-catalyzed methylation method or a two-step methylation method: base hydrolysis followed by acid-catalyzed methylation. Sample (50 mg) in a screw-cap test tube containing heptadecanoic acid or its methyl ester (1 mg) was methylated for various reaction times with 3 mL of 1.0 N H_2SO_4/methanol in a boiling water bath (100 °C) or with 3 mL of 20% TMG/methanol in a water bath (55 or 100 °C). For a two-step methylation, ECLA sample was hydrolyzed to FCLA by base treatment as described above, followed by methylation with 3 mL of 1.0 N H_2SO_4/methanol (55 °C, 5 min), or was methylated with 3 mL of 20% TMG/methanol (100 °C, 10 min), followed by reaction with 3 mL of 1.0 N H_2SO_4/methanol (55 °C; 5, 10, or 20 min). After methylation, sample was purified as described above.

Determination of the Completion of Methylation and Hydrolysis. The completion of methylation and hydrolysis of CLA samples was determined by a precoated silica gel TLC analysis. FCLA, CLA-SO, and CLA-EYL samples were developed with a hexane/ethyl acetate (5:1, v/v), whereas CLA-PL sample was developed with a chloroform/methanol/acetate/water (170:30:20:7, v/v/v/v) mixture. The TLC plate was visualized with 5% phosphomolybdic acid in ethanol.

Analysis of CLA Isomers by GC. Composition of CLA-Me isomers was analyzed by GC (Hewlett-Packard 5890) equipped with an FID and a fused silica capillary column Supelcowax-10 (60 m × 0.32 mm, i.d., 25 μm film thickness). Oven temperature was increased from 180 to 200 °C at a rate of 2 °C/min and then held for 30 min. Injection port and detector temperatures were 240 and 260 °C, respectively. Nitrogen (99.9%) was used as a carrier gas with a flow rate of 2 mL/min. CLA-Me isomers of samples were identified by comparison of the relative retention time of the standard run of CLA-Me isomers (*2*, *35*).

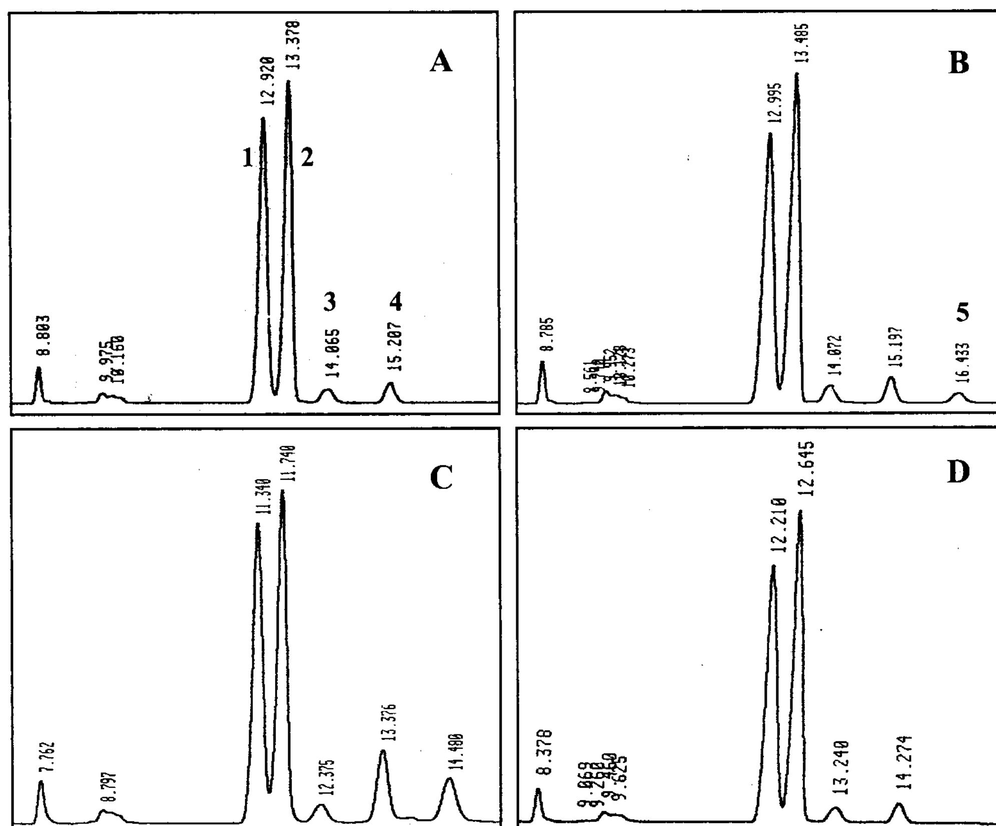

Figure 2. Typical GC chromatograms of the FCLA (50 mg) methylated under various conditions. Methylation conditions: (A) 1.0 N H_2SO_4/methanol (55 °C, 5 min); (B) 0.05 N H_2SO_4/methanol (100 °C, 5 min); (C) 1.0 N H_2SO_4/methanol (100 °C, 5 min); (D) Lipozyme. Peaks: 1, *c*9,*t*11 CLA; 2, *t*10,*c*12 CLA; 3, others (*c*9,*c*11 CLA and *c*10,*c*12 CLA); 4, *t*10,*t*12 CLA and *t*9,*t*11 CLA; and 5, AMD.

RESULTS

For the quantitative analysis of CLA by GC, CLA samples must be completely methylated without forming any artifacts. For this purpose, methylation methods that minimize the formation of *t,t* CLA isomers and AMD with the completion of methylation were developed for given CLA samples

Methylation of FCLA Sample. Figure 1A shows the completion of FCLA methylation. The FCLA (50 mg) was completely methylated with 3 mL of 1.0 N H_2SO_4/methanol (55 °C, 5 min), whereas it was not completely methylated with 3 mL of 0.05 N H_2SO_4/methanol (100 °C, 5 min). As compared to the results by Lipozyme-catalyzed methylation (**Figure 2D**), which did not use any acid during methylation, the former methylation condition did not produce *t,t* isomers and AMD (**Figure 2A**), but the latter produced AMD (**Figure 2B**). Substantial amounts of these artifacts were formed with 3 mL of 1.0 N H_2SO_4/methanol (100 °C, 5 min) (**Figure 2C**), which is a stronger methylation condition than those used for others tested. These results indicate that 3 mL of 1.0 N H_2SO_4/methanol (55 °C, 5 min) condition is suitable for the methylation of 50 mg of FCLA without forming any artifacts.

Table 1 shows the composition of the FCLA methylated by various reaction conditions. Lipozyme-catalyzed methylation revealed that the FCLA sample contains 45.4% *c*9,*t*11 CLA, 48.0% *t*10,*c*12 CLA, 3.6% *t,t* CLA, and 3.0% other CLA isomers and does not contain AMD. As compared to the data for the sample methylated with Lipozyme, *t,t* isomers and AMD were not formed by the methylation with 1.0 N H_2SO_4/methanol (55 °C, 5−20 min); however, they were formed during reaction at 100 °C for 5 min with 1.0 N H_2SO_4/methanol. At 100 °C, these artifacts were formed by methylation even at the concentration of 0.05 N H_2SO_4 in methanol, which did not complete the methylation of FCLA (50 mg) as shown in **Figure 1A**. HCl-catalyzed methylation methods revealed results similar to those by H_2SO_4-catalyzed methylation but produced more *t,t* isomers and AMD. BF_3-catalyzed methylation conditions tested produced higher *t,t* isomers and AMD than other acid-catalyzed methylation conditions tested. Consequently, the condition of 1.0 N H_2SO_4/methanol (55 °C, 5 min) is suitable for the methylation of FCLA and revealed that the FCLA sample contains 45.3% *c*9,*t*11 CLA, 47.9% *t*10,*c*12 CLA, 3.6% *t,t* CLA, and 3.2% others. This agrees with the result from Yamasaki et al., who reported that H_2SO_4/methanol is recommended for the esterification of CLA (*20*).

Methylation of CLA-SO and CLA-PL Samples. Table 2 shows the composition of CLA-Me isomers of CLA-SO methylated by various methods. Lipozyme-catalyzed methylation revealed that the CLA-SO contains 45.3% *c*9,*t*11 CLA, 47.6% *t*10,*c*12 CLA, 4.0% *t,t* CLA, and 3.1% others. This composition was similar to that methylated by base hydrolysis, followed by reaction with 1.0 N H_2SO_4/methanol (55 °C, 5 min), which completely methylated 50 mg CLA-SO samples (data not shown). These two methods did not produce AMD. CLA-SO was also methylated with 3 mL of 20% TMG/methanol (100 °C, 5−20 min) or 3 mL of 20% TMG/methanol (55 °C, 10−30 min), resulting in the completion of methylation (data not shown). Reaction temperature (55 or 100 °C) and reaction time (5−30 min) of the 20% TMG/methanol methods did not affect the formation of *t,t* isomers and AMD, as compared to the results from Lipozyme-catalyzed methylation. Hence, for the convenience of analysis, the condition of 20% TMG/methanol (100 °C, 5 min) is suitable for the methylation of CLA-SO (**Figure

Table 1. Composition of CLA Isomers in the FCLA[a] Methylated by Various Methods

methylation method[b]			CLA isomer (%)					
catalyst	reaction temp (°C)	reaction time (min)	*c*9,*t*11	*t*10,*c*12	*t*,*t*[c]	others[d]	AMD (%)	*c*9,*t*11/*t*10,*c*12
Lipozyme	60	120	45.4 ± 0.3[e]	48.0 ± 0.2	3.6 ± 0.1[f,A]	3.0 ± 0.1	ND[g]	0.9
1.0 N H_2SO_4	55	5	45.3 ± 0.4	47.9 ± 0.3	3.6 ± 0.2[A]	3.2 ± 0.1	ND	0.9
1.0 N HCl			45.5 ± 0.3	47.8 ± 0.5	3.7 ± 0.1[A]	3.0 ± 0.2	ND	1.0
14% BF_3			45.1 ± 0.5	47.5 ± 0.4	3.7 ± 0.3[A]	3.0 ± 0.2	0.7 ± 0.1	0.9
1.0 N H_2SO_4	55	10	45.5 ± 0.3	47.9 ± 0.4	3.6 ± 0.2[A]	3.0 ± 0.1	ND	0.9
1.0 N HCl			45.4 ± 0.4	47.8 ± 0.5	3.8 ± 0.2[A]	3.0 ± 0.1	ND	0.9
14% BF_3			44.9 ± 0.5	47.4 ± 0.4	3.8 ± 0.1[A]	2.8 ± 0.3	1.1 ± 0.1	0.9
1.0 N H_2SO_4	55	20	45.4 ± 0.4	47.8 ± 0.4	3.7 ± 0.2[A]	3.1 ± 0.1	ND	0.9
1.0 N HCl			45.3 ± 0.5	47.7 ± 0.5	3.7 ± 0.2[A]	3.0 ± 0.3	0.3 ± 0.1	0.9
14% BF_3			44.5 ± 0.5	46.9 ± 0.3	3.8 ± 0.2[A]	3.0 ± 0.2	1.8 ± 0.1	0.9
0.05 N H_2SO_4	100	5	45.0 ± 0.5	47.2 ± 0.5	3.7 ± 0.1[A]	2.9 ± 0.2	1.2 ± 0.1	1.0
0.05 N HCl			44.7 ± 0.4	46.8 ± 0.3	3.8 ± 0.1[A]	3.0 ± 0.2	1.7 ± 0.1	1.0
0.7% BF_3			44.8 ± 0.5	47.1 ± 0.4	3.8 ± 0.1[A]	3.1 ± 0.4	1.2 ± 0.1	1.0
1.0 N H_2SO_4	100	5	42.6 ± 0.4	44.8 ± 0.4	4.8 ± 0.2[B]	3.0 ± 0.3	4.8 ± 0.1	1.0
1.0 N HCl			42.5 ± 1.2	44.9 ± 0.7	4.7 ± 0.1[B]	3.0 ± 0.2	4.9 ± 0.1	0.9
14% BF_3			33.1 ± 1.3	33.4 ± 0.5	20.8 ± 1.1[C]	3.1 ± 0.3	9.6 ± 0.2	1.0

[a] FCLA sample was prepared from linoleic acid by alkaline isomerization. [b] Three milliliters of methanolic acid catalytic solution was used for the methylation of FCLA (50 mg). [c] The *t*,*t* isomers mean a mixture of *t*9,*t*11 CLA and *t*10,*t*12 CLA, which are not separated by the GC system used. [d] Others represent CLA isomers except for the two *c*/*t* CLA and *t*,*t* CLA isomers listed in this table. [e] Mean ± SD for three experimental data. [f] Means with different capital superscript letters in the same column represent significantly different from the mean methylated by Lipozyme at $p < 0.05$ by *t*-test. [g] Not detected.

Table 2. Composition of CLA Isomers in the CLA-SO[a] Methylated by Various Methods

methylation method[b]			CLA isomer (%)					
catalyst	reaction temp (°C)	reaction time (min)	*c*9,*t*11	*t*10,*c*12	*t*,*t*[c]	others[d]	AMD (%)	*c*9,*t*11/*t*10,*c*12
Lipozyme	60	120	45.3 ± 0.2[e]	47.6 ± 0.3	4.0 ± 0.1	3.1 ± 0.1	ND[f]	1.0
Base hydrolysis + 1.0 N H_2SO_4[g]	55	5	45.4 ± 0.4	47.6 ± 0.2	4.0 ± 0.1	3.0 ± 0.2	ND	1.0
20% TMG	100	5	45.2 ± 0.5	47.6 ± 0.3	4.2 ± 0.2	3.0 ± 0.1	ND	0.9
		10	45.4 ± 0.3	47.6 ± 0.3	4.0 ± 0.2	3.0 ± 0.2	ND	1.0
		20	45.4 ± 0.2	47.6 ± 0.4	4.0 ± 0.3	3.0 ± 0.2	ND	1.0
20% TMG	55	10	45.4 ± 0.3	47.6 ± 0.4	4.0 ± 0.2	3.0 ± 0.2	ND	1.0
		20	45.3 ± 0.4	47.5 ± 0.2	4.1 ± 0.1	3.1 ± 0.1	ND	1.0
		30	45.2 ± 0.2	47.6 ± 0.5	4.2 ± 0.4	3.0 ± 0.2	ND	0.9

[a] CLA-SO sample was prepared from SO by transesterification of CLA-Me with Lipozyme. [b] Three milliliters of methanolic acid catalytic solution was used for the methylation of CLA-SO (50 mg). [c] The *t*,*t* isomers mean a mixture of *t*9,*t*11 CLA and *t*10,*t*12 CLA, which are not separated by the GC system used. [d] Others represent CLA isomers except for the two *c*/*t* CLA and *t*,*t* CLA isomers listed in this table. [e] Mean ± SD for three experimental data. [f] Not detected. [g] Sample was hydrolyzed with base, followed by methylation with 3 mL of 1.0 N H_2SO_4 (55 °C, 5 min).

3A) and revealed that the CLA-SO sample is composed of 45.2% *c*9,*t*11 CLA, 47.6% *t*10,*c*12 CLA, 4.2% *t*,*t* CLA, and 3.0% other isomers (**Table 2**), the composition of which closely resembles that of FCLA used for CLA-SO synthesis (**Table 1**).

CLA-PL was methylated with four different methods. They included base hydrolysis, followed by methylation with 3 mL of 1.0 N H_2SO_4/methanol (55 °C, 5−20 min); methylation with 3 mL of 20% TMG/methanol (100 °C, 10 min), followed by an additional reaction with 3 mL of 1.0 N H_2SO_4/methanol (55 °C, 5−20 min); methylation with 3 mL of 20% TMG/methanol (100 °C, 10−30 min); and methylation with 3 mL of H_2SO_4/methanol (100 °C, 10−30 min). All of these methods completely methylated a 50 mg CLA-PL sample (data not shown), and the composition of CLA isomers and AMD is shown in **Table 3**. The methylation methods of H_2SO_4/methanol (100 °C, 10−30 min) produced a significant amount of *t*,*t* isomers and AMD, but the other methods did not produce any artifacts and also did not alter the composition of CLA isomers. Considering time, cost, and the production of *t*,*t* isomers and AMD, CLA-PL should be methylated with 20% TMG/methanol, followed by an additional reaction with 1.0 N H_2SO_4/methanol (55 °C, 5 min) (**Figure 3B**), resulting in the composition of 48.2% *c*9,*t*11 CLA, 47.8% *t*10,*c*12 CLA, 1.8% *t*,*t* CLA, and 2.2% others.

Methylation of CLA-EYL Sample. Figure 1B shows the completion of methylation of CLA-EYL sample, containing 65% CLA-TG, 28% CLA-PL, 5% cholesterol, and 2% others. This sample (50 mg) was completely methylated only by base hydrolysis, followed by an additional reaction with 3 mL of 1.0 N H_2SO_4/methanol (55 °C, 5 min) as shown in **Figure 1B**, lane E4, but not completed by other methods tested as shown in **Figure 1B**, lanes E1−E3. The phospholipid fraction of the CLA-EYL sample was relatively resistant to 1.0 N H_2SO_4/methanol (100 °C, 30 min; **Figure 1B**, lane E1) or 20% TMG/methanol (100 °C, 20 min; **Figure 1B**, lane E2) for methylation. Moreover, a trace amount of the phospholipid fraction of the sample remained when methylated with 3 mL of 20% TMG/methanol (100 °C, 10 min), followed by methylation with 3 mL of 1.0 N H_2SO_4/methanol (55 °C, 5 min) (**Figure 1B**, lane E3), which is an optimal methylation method for CLA-TG.

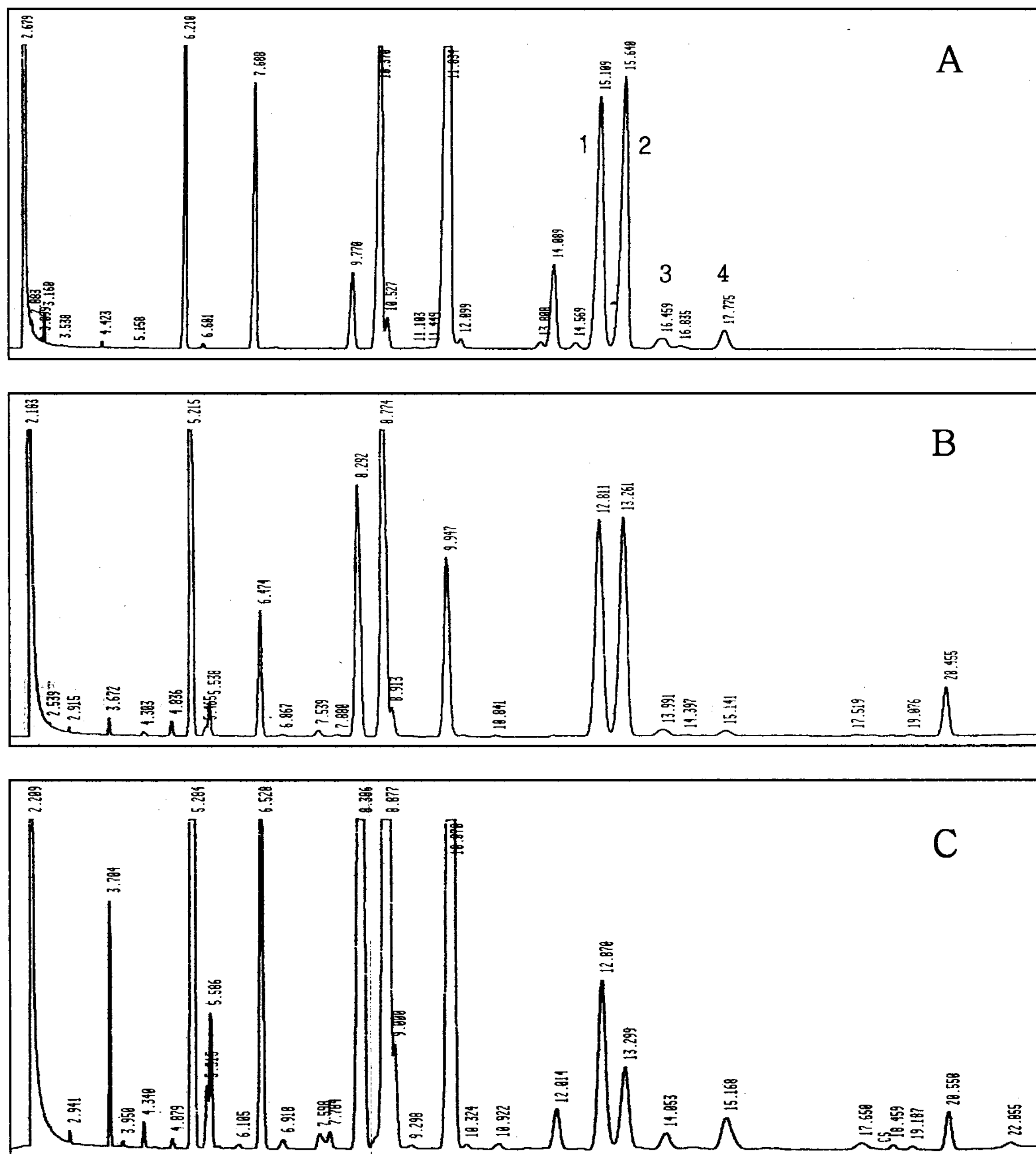

Figure 3. Typical GC chromatograms of CLA-SO, CLA-PL, and CLA-EYL samples methylated by their optimal methods as shown in Table 5: (A) CLA-SO methylated with 20% TMG/methanol (100 °C, 5 min); (B) CLA-PL methylated with 20% TMG (100 °C,10 min), followed by treatment with 1.0 N H_2SO_4/methanol (55 °C, 5 min); (C) CLA-EYL hydrolyzed with base, followed methylation with 1.0 N H_2SO_4/methanol (55 °C, 5 min). Peaks: 1, *c*9,*t*11 CLA; 2, *t*10,*c*12 CLA; 3, others (*c*9,*c*11 CLA and *c*10,*c*12 CLA); 4, *t*10,*t*12 CLA; and *t*9,*t*11 CLA.

These results suggest that the CLA-EYL sample (50 mg) could be methylated with base hydrolysis, followed by an additional reaction with 3 mL of 1.0 N H_2SO_4/methanol (55 °C, 5 min).

Table 4 shows the composition of CLA isomers in the CLA-EYL sample methylated by base hydrolysis, followed by reaction at 55 °C with 3 mL of 1.0 N H_2SO_4/methanol for 5 min. The CLA-EYL sample methylated for 5 min with the two-step method contained 52.2% *c*9,*t*11 CLA, 27.7% *t*10,*c*12 CLA, 13.8% *t,t* CLA, and 6.3% others (**Figure 3C**), a composition similar to that from methylation with Lipozyme. A reaction time of up to 20 min did not affect the composition of CLA isomers and the formation of AMD. The methylation condition of H_2SO_4/methanol (100 °C, 10−30 min) altered the composition of CLA isomers, but other methods, 20% TMG/methanol (100 °C, 10−30 min) or 20% TMG/methanol (100 °C, 10 min), followed by methylation with 1.0 N H_2SO_4/methanol (55 °C, 5 min) did not. However, the last three methods did not complete methylation (**Figure 1B**).

Quantitative Analysis of CLA Isomers in Lipid Samples. **Table 5** shows quantitative analysis data of FCLA, CLA-SO, CLA-PL, and CLA-EYL samples methylated with their optimal methylation conditions determined in this study (**Figures 2A** and **3**). The FCLA sample (1.0 g) contained 450.8 mg of *c*9,*t*11 CLA, 474.8 mg of *t*10,*c*12 CLA, 37.4 mg of *t,t* CLA, and 33.0 mg of others. Total CLA contents in CLA-SO, CLA-PL, and CLA-EYL were found to be 232.6, 186.0, and 43.6 mg/g of sample, respectively. The CLA-SO sample (1.0 g) contained 105.6 mg of *c*9,*t*11 CLA (45.4%), 110.7 mg of *t*10,*c*12 CLA (47.6%), 9.4 mg of *t,t* CLA (4.0%), and 6.9 mg of others (3.0%). Similarly, CLA-PL contained 89.7 mg of *c*9,*t*11 CLA (48.2%),

Table 3. Composition of CLA Isomers in the CLA-PL[a] Methylated by Various Methods

methylation method			CLA isomer (%)					
catalyst	reaction temp (°C)	reaction time (min)	*c*9,*t*11	*t*10,*c*12	*t*,*t*[b]	others[c]	AMD (%)	*c*9,*t*11/*t*10,*c*12
Lipozyme	60	120	48.3 ± 0.2[d]	47.8 ± 0.2	1.8 ± 0.1[e,A]	2.1 ± 0.1	ND[f]	1.0
Base hydrolysis + 1.0 N H_2SO_4[g]	55	5	48.4 ± 0.3	47.7 ± 0.5	1.8 ± 0.1^{A}	2.1 ± 0.2	ND	1.0
		10	48.3 ± 0.4	47.8 ± 0.3	1.8 ± 0.2^{A}	2.1 ± 0.1	ND	1.0
		20	48.4 ± 0.3	47.7 ± 0.4	1.7 ± 0.2^{A}	2.2 ± 0.3	ND	1.0
20% TMG + 1.0 N H_2SO_4[h]	55	5	48.2 ± 0.6	47.8 ± 0.3	1.8 ± 0.1^{A}	2.2 ± 0.1	ND	1.0
		10	48.5 ± 0.4	47.8 ± 0.4	1.7 ± 0.1^{A}	2.0 ± 0.1	ND	1.0
		20	48.4 ± 0.5	47.8 ± 0.5	1.7 ± 0.2^{A}	2.1 ± 0.2	ND	1.0
20% TMG	100	10	48.4 ± 0.3	47.8 ± 0.3	1.8 ± 0.2^{A}	2.0 ± 0.1	ND	1.0
		20	48.4 ± 0.2	47.7 ± 0.5	1.8 ± 0.1^{A}	2.1 ± 0.2	ND	1.0
		30	48.2 ± 0.4	47.7 ± 0.2	1.9 ± 0.1^{A}	2.2 ± 0.1	ND	1.0
1.0 N H_2SO_4	100	10	39.4 ± 0.5	38.2 ± 0.4	11.0 ± 0.1^{B}	2.2 ± 0.2	9.2 ± 0.2	1.0
		20	36.4 ± 0.3	35.0 ± 0.2	16.8 ± 0.8^{C}	2.4 ± 0.2	9.4 ± 0.3	1.0
		30	33.0 ± 0.8	31.0 ± 0.5	24.5 ± 1.1^{D}	2.4 ± 0.2	9.1 ± 0.1	1.1

[a] CLA-PL samples were synthesized from egg yolk lecithin by transesterification of CLA-Me with Lipozyme. [b] The *t*,*t* isomers means a mixture of *t*9,*t*11 CLA and *t*10,*t*12 CLA, which are not separated by the GC system used. [c] Others represent CLA isomers except for the two *c*/*t* CLA and *t*,*t* CLA isomers listed in this table. [d] Mean ± SD for three experimental data. [e] Means with different capital superscript letters in the same column represent significantly different from the mean methylated by Lipozyme at $p < 0.05$ by *t*-test. [f] Not detected. [g] Sample was hydrolyzed with base, followed by methylation with 3 mL of 1.0 N H_2SO_4 (55 °C, 5–20 min). [h] Sample was methylated with 3 mL of 20% TMG (100 °C, 10 min), followed by an additional reaction with 3 mL of 1.0 N H_2SO_4 (55 °C, 5–20 min).

Table 4. Composition of CLA Isomers in the CLA-EYL[a] Methylated by Various Methods

methylation method[b]			CLA isomer (%)					
catalyst	reaction temp (°C)	reaction time (min)	*c*9,*t*11	*t*10,*c*12	*t*,*t*[c]	others[d]	AMD (%)	*c*9,*t*11/*t*10,*c*12
Lipozyme	60	120	52.3 ± 0.3[e]	27.8 ± 0.4	13.7 ± 0.4[f,A]	6.2 ± 0.3	ND[g]	1.9
Base hydrolysis + 1.0 N H_2SO_4[h]	55	5	52.2 ± 0.3	27.7 ± 0.3	13.8 ± 0.1^{A}	6.3 ± 0.2	ND	1.9
		10	52.3 ± 0.4	27.7 ± 0.2	14.0 ± 0.2^{A}	6.0 ± 0.2	ND	1.9
		20	52.5 ± 0.3	27.8 ± 0.4	13.6 ± 0.4^{A}	6.1 ± 0.3	ND	1.9
20% TMG + 1.0 N H_2SO_4[i]	55	5	52.2 ± 0.2	27.4 ± 0.2	13.9 ± 0.2^{A}	6.5 ± 0.1	ND	1.9
		10	52.8 ± 0.4	27.6 ± 0.2	13.6 ± 0.2^{A}	6.0 ± 0.2	ND	1.9
		20	52.7 ± 0.4	27.6 ± 0.4	13.6 ± 0.4^{A}	6.1 ± 0.3	ND	1.9
20% TMG	100	10	51.9 ± 0.5	27.3 ± 0.5	14.1 ± 0.3^{A}	6.7 ± 0.2	ND	1.9
		20	51.8 ± 0.1	27.2 ± 0.3	14.2 ± 0.1^{A}	6.8 ± 0.2	ND	1.9
		30	51.4 ± 0.4	26.3 ± 0.4	15.8 ± 0.4^{B}	6.5 ± 0.1	ND	2.0
1.0 N H_2SO_4	100	10	43.5 ± 0.3	21.8 ± 0.2	20.0 ± 0.4^{B}	6.3 ± 0.3	8.4 ± 0.3	2.0
		20	37.8 ± 0.4	21.1 ± 0.1	25.3 ± 0.2^{C}	6.0 ± 0.2	9.8 ± 0.2	1.8
		30	33.1 ± 0.6	19.3 ± 0.4	30.2 ± 0.5^{D}	6.0 ± 0.4	11.4 ± 0.4	1.7

[a] CLA-EYL samples were derived from laying hens fed 2.5% dietary CLA for 6 weeks. [b] Fifty milligrams of CLA-EYL sample was used for methylation. [c] The *t*,*t* isomers mean a mixture of *t*9,*t*11 CLA and *t*10,*t*12 CLA, which are not separated by the GC system used. [d] Others represent CLA isomers except for the two *c*/*t* CLA and *t*,*t* CLA isomers listed in this table. [e] Mean ± SD for three experimental data. [f] Means with different capital superscript letters in the same column represent significantly different from the mean methylated by Lipozyme at $p < 0.05$ by *t*-test. [g] Not detected. [h] Sample was hydrolyzed with base, followed by methylation with 3 mL of 1.0 N H_2SO_4 (55 °C, 5–20 min). [i] Sample was methylated with 3 mL of 20% TMG (100 °C, 10 min), followed by an additional reaction with 3 mL of 1.0 N H_2SO_4 (55 °C, 5–20 min).

Table 5. Content of CLA Isomers in FCLA, CLA-SO, CLA-PL, and CLA-EYL Samples Methylated by Their Optimal Methylation Methods

		CLA isomer (mg/g)						
sample[a]	methylation method[b]	*c*9,*t*11	*t*10,*c*12	*t*,*t*[c]	others[d]	AMD (%)	total (mg/g)	*c*9,*t*11/*t*10,*c*12
FCLA	1.0 N H_2SO_4 (55 °C, 5 min)	450.8 ± 2.4[e]	474.8 ± 3.2	37.4 ± 1.6	33.0 ± 1.8	ND[f]	996.0 ± 3.3	0.95
CLA-SO	20% TMG (100 °C, 5 min)	105.6 ± 2.3	110.7 ± 1.7	9.4 ± 0.6	6.9 ± 0.4	ND	232.6 ± 5.9	0.95
CLA-PL	20% TMG (100 °C, 10 min) + 1.0 N H_2SO_4 (55 °C, 5 min)	89.7 ± 0.6	88.7 ± 0.3	3.5 ± 0.1	4.1 ± 0.1	ND	186.0 ± 2.7	1.01
CLA-EYL	base hydrolysis + 1.0 N H_2SO_4 (55 °C, 5 min)	22.8 ± 0.3	12.1 ± 0.2	6.0 ± 0.3	2.7 ± 0.2	ND	43.6 ± 3.3	1.88

[a] Sample sources are shown in Tables 1–4. Fifty milligram sample was used for the methylation. [b] Methylation method used was the optimal condition for each samples determined in this study. [c] The *t*,*t* isomers means a mixture of *t*9,*t*11 CLA and *t*10,*t*12 CLA, which were not separated by the GC system used. [d] Others represent CLA isomers except for the two *c*/*t* CLA and *t*,*t* CLA isomers listed in this table. [e] Mean ± SD for three experimental data. [f] Not detected.

88.7 mg of *t*10,*c*12 CLA (47.7%), 3.5 mg of *t*,*t* CLA (1.9%), and 4.1 mg of others (2.2%). Finally, the CLA-EYL sample contained 22.8 mg of *c*9,*t*11 CLA (52.3%), 12.1 mg of *t*10,*c*12 CLA (27.7%), 6.0 mg of *t*,*t* CLA (13.8%), and 2.7 mg of others (6.2%). Unlike CLA-SO and CLA-PL, the CLA-EYL sample contained a higher composition of *c*9,*t*11 CLA than of *t*10,*c*12 CLA.

DISCUSSION

Because natural materials contain a variety of chemical forms (FCLA and ECLA) and several isomers (mainly, the *c*/*t* CLA) of CLA, the chemical form and amount of CLA samples are important factors to be considered during methylation. In addition, the reaction time, reaction temperature, and acid or

base concentration in methanol are considered for the given chemical form and amount of CLA.

Much more care should be paid for the methylation of FCLA than for ECLA because FCLA is methylated only by acid catalysis, which easily decomposes *c*/*t* CLA isomers. FCLA (50 mg) was completely methylated with 1.0 N H_2SO_4/methanol (3 mL, 55 °C, 5 min), without forming any artifacts (**Figure 2A** and **Table 1**). Completion of the methylation is hardly achieved at <50 °C, but much more easily achieved at a higher temperature, especially in a boiling water bath (100 °C). Such a high temperature substantially enhanced decomposition of *c*/*t* CLA isomers to their corresponding *t*,*t* CLA isomers and AMD (**Figure 2C** and **Table 1**). The isomerization of *c*/*t* CLA isomers to the *t*,*t* CLA isomers was affected by the functions of reaction temperature and 1.0 N H_2SO_4 concentration in methanol, whereas the formation of AMD from *c*/*t* CLA isomers was affected only by reaction temperature (**Table 1**). BF_3 is a stronger Lewis acid, increasing in amount of *t*,*t* isomers and AMD. Hence, this BF_3 could be a useful agent for the mass production of the *t*,*t* isomers, which exhibit biological activities (*11*).

ECLA samples, CLA-SO, CLA-PL, and CLA-EYL, required higher H_2SO_4 concentration in methanol and/or longer reaction time for the completion of methylation than FCLA, which produced substantial amounts of *t*,*t* isomers and AMD artifacts (**Tables 2**−**4**). Thus, the satisfied methylation of ECLA was achieved by 20% TMG/methanol at 100 °C or by base treatment, followed by methylation with 1.0 N H_2SO_4/methanol. Extraction of CLA-Me from the ECLA sample methylated with 20% TMG/methanol is very difficult to complete by the conventional method, requiring care to avoid incorrect quantitation of CLA isomers. Hence, the extraction required additional steps of washing once or twice with saturated NaCl solution, followed by washing three times with distilled water. On the other hand, as seen in the methylation of CLA-PL (**Table 3**), the extraction is easily achieved by the conventional method used in this study, when CLA-PL, methylated with 20% TMG/methanol (100 °C, 10 min), is reacted for an additional 5 min at 55 °C with 3 mL of 1.0 N H_2SO_4/methanol.

It is of interest to figure out the true composition or amount of CLA isomers in CLA samples. A significant amount of *t*,*t* CLA and other CLA isomers was contained in the FCLA sample methylated by the optimal methylation condition, 1.0 N H_2SO_4/methanol (55 °C, 5 min). A similar amount of these artifacts was also found in the FCLA sample methylated with Lipozyme. Hence, the *t*,*t* CLA and other CLA isomers are not produced by the methylation, but they might originate from the FCLA synthesized by alkaline isomerization. This is further supported by the fact that the ratio of *c*9,*t*11 CLA to *t*10,*c*12 CLA in the FCLA samples methylated with optimal methylation condition and Lipozyme was identical to 0.9 (**Table 1**).

The area ratio of *c*9,*t*11 CLA to *t*10,*c*12 CLA in the FCLA, CLA-SO, and CLA-PL samples, methylated by various reaction conditions, was 1.0 (**Tables 1**−**3**). Similarly, as seen in **Table 4**, the area ratio in the CLA-EYL sample was also not altered by various reaction conditions. This suggests that the isomerization and decomposition rates of *c*9,*t*11 CLA are not different from those of *t*10,*c*12 CLA isomers during methylation.

As seen in **Table 5**, the ratios of *c*9,*t*11 CLA to *t*10,*c*12 CLA amount in CLA-SO and CLA-PL were found to be 0.95 and 1.01, respectively, whereas the ratio in CLA-EYL was 1.88. These results indicate that similar amounts of the *c*/*t* CLA isomers were incorporated into CLA-SO and CLA-PL, but the amount of *c*9,*t*11 CLA incorporated into CLA-EYL was 1.88 times greater than that of *t*10,*c*12 CLA. This could be, in part, due to preferential incorporation of *c*9,*t*11 CLA into phospholipid of EYL by acyltransferase in the liver of hens. No difference in the composition of the *t*,*t* CLA isomers in CLA-SO and CLA-PL was seen; however, the composition in CLA-EYL was higher than that in CLA-SO and CLA-PL (**Tables 2**−**5**). The reason is unclear at the present time, but it could be, in part, caused by the fact that the interfering substances in CLA-EYL were coeluted with *t*,*t* CLA isomers, indicating that special care should be taken for the quantitative analysis.

Some CLA samples contain a substantial amount of CLA, which is enough for quantitative determination by GC; however, many other samples contain a minute amount of CLA, which is not enough for GC analysis. In the latter case, CLA samples are first concentrated by argentation chromatography and then the chemical forms of CLA are determined, followed by methylation with the proper method developed here.

ABBREVIATIONS USED

CLA, conjugated linoleic acid; CLA-Me, CLA methyl ester; FCLA, free CLA; ECLA, esterified CLA; CLA-SO, CLA esterified in safflower oil; CLA-TG, CLA esterified in glyceride; CLA-PL, CLA esterified in phospholipid; CLA-EYL, CLA esterified in egg yolk lipid; *c*/*t* CLA, *c*9,*t*11 CLA and *t*10,*c*12 CLA; AMD, allylmethoxyl derivative; SO, safflower oil; TMG, tetramethylguanidine; FID, flame ionization detector.

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Received for review September 5, 2001. Revised manuscript received November 19, 2001. Accepted November 20, 2001. This study was in part supported by grants from the Korean Administration of Human Health and Welfare (HMP-98-F-5-0019) and the Korea Science and Engineering Foundation (2000-1-22000-011-3).

JF011185B