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ARTICLES

Preparation of a *t*,*t* Conjugated Linoleic Acid Methylester (CLA-Me) Isomers Mixture from Synthetic CLA by Methylation with BF_3/Methanol

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Mixtures of *t*,*t* conjugated linoleic acid methylester (*t*,*t* CLA-Me) isomers were prepared from synthetic CLA, consisting of 47.8% *t*10,*c*12 CLA; 45.5% *c*9,*t*11 CLA; 2.0% *t*,*t* CLA; and 4.7% others, by methylation with BF_3/methanol (designated TT-TC/CT) in conjunction with purification at −68 °C for 24 h. The amount or composition of the TT-TC/CT was greatly affected by the concentration of BF_3 in methanol and the duration of methylation. The methylation of 50 mg of synthetic CLA for 30 min with 1 mL of 7.0% BF_3/methanol produced a TT-TC/CT (21.54 mg) with the composition of 1.3% *t*12,*t*14; 5.9% *t*11,*t*13; 42.7% *t*10,*t*12; 44.0% *t*9,*t*11; 5.0% *t*8,*t*10; and 1.1% *t*7,*t*9 CLA, whereas the methylation for 60 min with 14.0% BF_3/methanol produced a TT-TC/CT (28.62 mg) with the composition of *t*,*t* CLA isomers different from that of TT-TC/CT by methylation for 30 min with 7.0% BF_3/methanol. A large quantity of TT-TC/CT (14.15 g) with the composition similar to that of TT-TC/CT prepared from 50 mg of synthetic CLA was also prepared from 25 g of synthetic CLA. The purity of TT-TC/CT samples was greater than 98%. These results suggest that TT-TC/CT with a purity greater than 98% was easily prepared from synthetic CLA by BF_3-catalyzed methylation, and the amount and composition of *t*,*t* CLA isomers of TT-TC/CT samples could be controlled by methylation conditions.

KEYWORDS: Conjugated linoleic acid (CLA); *t*,*t* CLA isomers; BF_3; methylation

INTRODUCTION

CLA is a collective term for positional (7,9; 8,10; 9,11;10,-12; 11,13; and 12,14) and geometric (*t*,*c*; *c*,*t*; *c*,*c*; and *t*,*t*) isomers of octadecadienoic acid (C18:2) with a conjugated double bond system (*1*−*3*). Predominant CLA isomers in synthetic CLA, synthesized from linoleic acid by alkaline isomerization, are *t*10,*c*12 CLA and *c*9,*t*11CLA (designated *tc*/*ct* CLA): small amounts of *t*10,*t*12 CLA; *t*9,*t*11 CLA; and other isomers also occur (*4*, *5*).

Synthetic CLA exhibits a potent anticarcinogenic activity for the carcinogen-induced carcinogenesis in several animal models (*1*, *6*−*10*) and other biological activities (*11*−*15*). However, it has been revealed that individual CLA isomers exhibit different biological activities in animal and cancer cell studies. For example, Park et al. (*16*) reported that the *t*10,*c*12 CLA isomer is more effective for the reduction of mice body fat than the *c*9,*t*11 CLA isomer. The *t*10,*c*12 CLA also inhibits cell growth and secretion of insulin-like growth factor-II in Caco-2 cells (*17*). In addition, current studies show that a mixture of *t*,*t* CLA isomers, mainly composed of *t*10,*t*12 and *t*9,*t*11 CLA isomers, exhibited stronger cytotoxicity against *NCI*−*N87* gastric cancer cells than *tc*/*ct* CLA isomers, by inhibiting proliferation and modulating arachidonic acid metabolism (*18*). These suggest that biological activities of all individual CLA isomers must be evaluated. Given this information in hand, a substantial quantity of pure individual CLA isomers (*t*10,*c*12 CLA; *c*9,*t*11 CLA; *t*10,*t*12 CLA; *t*9,*t*11 CLA; and other CLA) is required for their biological activity tests.

The *c*9,*t*11 CLA is prepared by *Butyrivibrio fibrisolvens* (*19*), *Lactobacillus reuteri* (*20*), *Aspergillus niger via* (*21*) and

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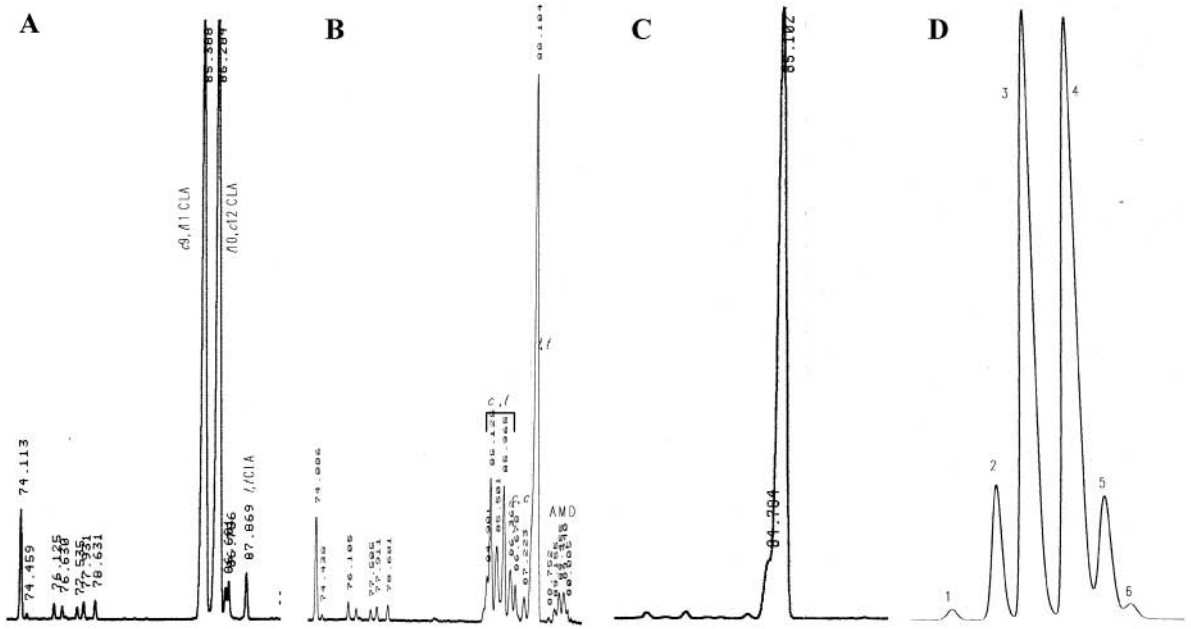

Figure 1. Typical GC chromatograms of synthetic CLA (**A**), TT-TC/CT sample prepared from panel **A** sample by methylation for 30 min with 1 mL of 14% BF_3/methanol (**B**), TT-TC/CT purified at −68 °C for 24 h (**C**), and Ag^+-HPLC chromatograms of panel **C** sample (**D**). Peak identification of panel **D**: 1, *t*12,*t*14 CLA; 2, *t*11,*t*13 CLA; 3, *t*10,*t*12 CLA; 4, *t*9,*t*11 CLA; 5, *t*8,*t*10 CLA; and 6, *t*7,*t*9 CLA. AMD of panel **B** represents allylic methoxy derivatives from CLA isomers.

mushroom (*22*) and from ricinolate methylester (*23*). In addition, a large quantity of *tc*/*ct* CLA isomers has been prepared from synthetic CLA by low-temperature precipitation with (*24*) or without (*25*) urea treatment. For the isomerization of *tc*/*ct* CLA to *t,t* CLA isomers, many investigators reported the formation of a minute quantity of the *t,t* CLA, as an artifact, from *c*9,*t*11 CLA or synthetic CLA during methylation by acid-catalyzed methylation in methanol (*4*, *5*). Chipault and Hawkins reported the conversion of *tc*/*ct* CLA-Me to the *t,t* isomers in the presence of iodine and CS_2 by light (*26*). They only investigated the conversion rate and the influence of solvents, iodine concentrations, temperatures, and light intensities for the isomerization of *tc*/*ct* CLA-Me to *t,t* CLA-Me by measuring specific infrared absorption signals responsible for *tc*/*ct* and/or *t,t* of the CLA molecule. However, no other report on the preparation of highly pure *t,t* CLA isomers has been seen in the literature to date.

In the present study, mixtures of highly pure *t,t* CLA-Me isomers were prepared from synthetic CLA by methylation with 14% BF_3/methanol in conjunction with low-temperature precipitation at −68 °C for 24 h. The effects of the concentration of BF_3 in methanol and the methylation time on the purity and composition of *t,t* CLA isomers were also examined. In comparison, purified *tc*/*ct* CLA fractions were used for the preparation of *t,t* CLA-Me isomer mixtures.

MATERIALS AND METHODS

Materials. Linoleic acid (95.0%) was obtained from Nu Check PREP Inc. (Elysian, MN). BF_3 (14% in methanol) was obtained from Sigma Chemical Co. (St. Louis, MO). Hexane, methanol, acetonitrile, and acetone were purchased from Aldrich Chemical Co. (Milwaukee, WI). Urea and KOH (Shinyo Chemical Co., Osaka, Japan) were used. All other reagents used were ACS grade.

Preparation of Synthetic CLA and *tc*/*ct* CLA-Me Fractions. Synthetic CLA was prepared from linoleic acid by alkaline isomerization method and methylated with 1.0 N H_2SO_4/methanol (*27*). The *t*10,*c*12 CLA-Me and *c*9,*t*11 CLA-Me isomer fractions were prepared from synthetic CLA-Me by low-temperature crystallization at both −68 and −71 °C in conjunction with urea treatment (*24*). The *tc*/*ct* CLA-Me fractions were hydrolyzed to their free forms by a conventional method.

Preparation of *t,t* CLA-Me Isomer Mixtures. The screw-capped test tube (10 mL), containing a 50 mg sample (synthetic CLA, *t*10,*c*12 CLA, or *c*9,*t*11 CLA) dissolved in 1 mL of various concentrations of BF_3 in methanol (0.5, 1.0, 3.5, 7.0, and 14.0%), was wrapped with aluminum foil and then heated in a boiling water bath for various reaction times (1, 5, 10, 30, and 60 min). The resultant, cooled to room temperature, was extracted with hexane (1 mL × 3), followed by washing with distilled water (1 mL × 3) and drying over Na_2SO_4 anhydrous. The hexane extract, dried under vacuum, was redissolved in acetone at a concentration of 50 mg/1.25 mL and purified by storing it at −68 °C for 24 h as described by Kim et al. (*24*). For the preparation of a large quantity of *t,t* CLA-Me isomer mixture, the screw-capped bottle (1 L), containing 25 g of synthetic CLA dissolved in 500 mL of 14% BF_3/methanol, was wrapped with aluminum foil and then heated in a boiling water bath for 30 min. The resultant was extracted with hexane (500 mL × 3) and then washed with distilled water (500 mL × 3). The hexane extract redissolved in acetone (625 mL) was purified at −68 °C for 24 h.

GC Analysis of CLA-Me Isomers. The purity of CLA-Me isomers mixture was analyzed by GC (Hewlett-Packard 5890, Avondale, PA) equipped with a FID and a CP-Sil 88 capillary column (100 m × 0.25 mm, i.d., 0.2 μm film thickness; Chrompack, Bridgewater, NJ). The column temperature was held at 70 °C for 5 min, followed by increasing to 175 °C at a rate of 13 °C/min, and 30 min later, it was increased to 215 °C at a rate of 4 °C/min. The carrier gas used was N_2. The injector and detector temperatures were 240 and 260 °C, respectively. The composition of individual CLA isomers, identified by the report of Sehat et al. (*28*), was calculated by the peak area ratio of the given CLA isomer to that of total CLA isomers.

HPLC Analysis of CLA-Me Isomers. Ag^+-HPLC separation of the CLA isomers was carried out by a Young-Lin HPLC system (Anyang, Korea), equipped with a M930 solvent delivery system, UV detector (M720) operated at 233 nm, and an operating system (Autochro-win version 2.0). Two ChromSpher 5 lipids analytical silver-impregnated columns (4.6 mm i.d. × 250 mm stainless steel, 5 μm particle size, Chrompack) were connected for the analysis of samples. The mobile phase was 0.1% acetonitrile in hexane and operated

Table 1. Purity of *t,t* CLA-Me Isomer Mixtures Prepared by Methylation with 14% BF_3/Methanol for 30 min[a]

t,t CLA-Me isomer mixture[b]	*t,t* CLA isomers (%)[c]	others (%)[d]
TT-TC/CT	60.2 ± 1.5a[e]	39.8 ± 1.7a
TT-TC	61.8 ± 1.8a	38.2 ± 2.5a
TT-CT	62.4 ± 2.0a	37.6 ± 1.4a

[a] Sample was analyzed by GC (**Figure 1B**). [b] TT-TC/CT was prepared from 50 mg of synthetic CLA, containing 47.8% *t*10,*c*12 and 45.5% *c*9,*t*11 CLA, as shown in **Figure 1A**. TT-TC and TT-CT were prepared from 50 mg each of *t*10,*c*12 CLA (99.1%) and *c*9,*t*11 CLA (91.0%), respectively. [c] Percentage of the relative peak area of *t,t* CLA isomers to total peak area of fatty acids, including *t,t* CLA isomers and allylic methoxy derivatives. [d] Others included allylic methoxy derivatives and fatty acids except for *t,t* CLA isomers. [e] Means ± SD of three experimental data. Means with different letters in the same column represent a significant difference at $p < 0.05$ by Duncan's multiple test.

Table 2. Purity of the *t,t* CLA-Me Isomer Mixtures Purified by Low-Temperature Precipitation[a]

t,t CLA-Me isomer mixture	*t,t* CLA isomers (%)[b]	others (%)[c]
TT-TC/CT	98.9 ± 0.1b[d]	1.1 ± 0.1a
TT-TC	99.5 ± 0.1a	0.5 ± 0.1b
TT-CT	98.8 ± 0.1b	1.2 ± 0.1a

[a] The *t,t* CLA-Me isomer mixtures obtained from **Table 1** or **Figure 1B** were purified at −68 °C for 24 h and then analyzed by GC (**Figure 1C**). [b] Percentage of the relative peak area of *t,t* CLA isomers to total peak area of fatty acids, including *t,t* CLA isomers. [c] Others included fatty acids except for *t,t* CLA isomers. [d] Mean ± SD of three experimental data. Means with different letters in the same column represent a significant difference at $p < 0.05$ by Duncan's multiple test.

isocratically at a flow rate of 1.0 mL/min. The flow was initiated 0.5 h prior to sample injection. CLA isomers of samples were identified by the report of Sehat et al. (*2*).

RESULTS

Composition of *t,t* CLA Isomers in the *t,t* CLA-Me Isomer Mixtures Prepared from Synthetic CLA and *tc*/*ct* CLA Fractions. A small quantity of *t,t* CLA-Me isomer mixtures was prepared from 50 mg of synthetic CLA, *t*10,*c*12 CLA, or *c*9,*t*11 CLA. Synthetic CLA (47.8% *t*10,*c*12 CLA; 45.5% *c*9,*t*11 CLA; 2.0% *t,t* CLA; and 4.7% others; **Figure 1A**), *t*10,*c*12 CLA (99.1%), and *c*9,*t*11 CLA (91.0%) samples were methylated for 30 min with 1 mL of 14% BF_3/methanol, which is an optimal methylation condition determined (data not shown). GC analysis revealed that the resultants contained impurities including unisomerized *tc*/*ct* and *c,c* CLA isomers remaining in samples (**Figure 1B**). When calculated by the peak area basis, the purities of the *t,t* CLA-Me isomer mixtures prepared from synthetic CLA, *t*10,*c*12 CLA, and *c*9,*t*11 CLA (designated to TT-TC/CT, TT-TC, and TT-CT, respectively) were 60.2, 61.8, and 62.4%, respectively (**Table 1**); however, they were further increased to 98.9, 99.5, and 98.8%, respectively, by purification at −68 °C for 24 h (**Figure 1C**, **Table 2**).

Ag^+-HPLC analysis revealed that the purified TT-TC/CT, TT-TC, and TT-CT samples, shown in **Figure 1C** or **Table 2**, were found to be a mixture of positional isomers of *t,t* CLA with a conjugated double bond at C12,C14; C11,C13; C10,-C12; C9,C11; C8,C10; and C7,C9 positions (**Figure 1D**). In TT-TC/CT, TT-TC, and TT-CT samples, *t*10,*t*12 CLA (37.3−41.8%) and *t*9,*t*11 CLA (41.1−44.2%) were the major isomers, whereas *t*12,*t*14 CLA (0.2−1.1%); *t*11,*t*13 CLA (7.0−9.4%); *t*8,*t*10 CLA (5.7−9.7%); and *t*7,*t*9 CLA (0.1−0.7%) isomers were minor constituents (**Table 3**). Interestingly, the composition of the *t*12,*t*14 CLA; *t*11,*t*13 CLA; *t*10,*t*12 CLA; *t*9,*t*11 CLA; *t*8,*t*10 CLA; and *t*7,*t*9 CLA isomers symmetrically decreased, centered from *t*10, *t*12 CLA and *t*9,*t*11 CLA isomers to the direction of *t*12,*t*14 CLA and *t*7,*t*9 CLA, respectively. However, no distinctive difference in the composition of major *t,t* CLA isomers, *t*10,*t*12 CLA and *t*9,*t*11 CLA, was seen between TT-TC/CT and TT-TC or TT-CT.

Effect of BF_3 Concentration in Methanol on the Content of *t,t* CLA Isomers. TT-TC/CT, TT-TC, and TT-CT, prepared from 50 mg samples by methylation for 30 min with various BF_3 concentration in methanol, were purified at −68 °C and then analyzed by Ag^+-HPLC. **Table 4** shows the changes in the content of *t,t* CLA isomers in the TT-TC/CT sample. The 3.5% BF_3/methanol completed the methylation of the synthetic CLA. The total amount of *t,t* CLA isomers was increased by an increase BF_3 concentration: 1.09, 1.83, 8.75, 21.54, and 28.28 mg by 0.5, 1.0, 3.5, 7.0, and 14.0% BF_3/methnol, respectively. The amounts of *t*10,*t*12 CLA and *t*9,*t*11 CLA isomers in the synthetic CLA sample were 0.38 and 0.62 mg, respectively, and they were rapidly elevated to 9.20 and 9.47 mg, respectively, by 7.0% BF_3/methanol, after which they were not much. Meanwhile, *t*11,*t*13 and *t*8,*t*10 CLA isomers, which have a conjugated double bond closer to the conjugated double bond of *t*10,*t*12 CLA and *t*9,*t*11 CLA isomers, respectively, than *t*12,*t*14 and *t*7,*t*9 CLA isomers, started to be produced by 1.0% BF_3/methanol and *t*12,*t*14 and *t*7,*t*9 CLA isomers by 3.5% BF_3/methanol. A TT-TC/CT (28.28 mg), consisting of *t*12,*t*14 CLA (0.73 mg); *t*11,*t*13 CLA (3.02 mg); *t*10,*t*12 CLA (10.38 mg); *t*9,*t*11 CLA (10.81 mg); *t*8,*t*10 CLA (2.68 mg); and *t*7,*t*9 CLA (0.66 mg), was prepared by methylation for 30 min with 14% BF_3/methanol. Methylation for 30 min with 3.5% BF_3/methanol produced a TT-TC/CT (8.75 mg) containing *t*12,*t*14 CLA (0.10 mg); *t*11,*t*13 CLA (0.25 mg); *t*10,*t*12 CLA (4.02 mg); *t*9,*t*11 CLA (4.21 mg); *t*8,*t*10 CLA (0.14 mg); and *t*7,*t*9 CLA (0.03 mg), which is quite a different composition from that by methylation for 30 min with 14% BF_3/methanol. Similar results were observed from purified TT-TC and TT-CT (data not shown).

Compositional changes in *t,t* CLA isomers of the TT-TC/CT sample by BF_3 concentration were calculated from the data of **Table 4** (**Figure 2**). Synthetic CLA (50 mg) contained 1.0 mg of *t,t* CLA isomers, consisting of 38.0% *t*10,*t*12 CLA and 62.0% *t*9,*t*11 CLA. The compositions of *t*10,*t*12 CLA and *t*9,*t*11 CLA were changed to 45.9 and 48.1%, respectively, by 3.5% BF_3/methanol, after which they were linearly decreased to 36.7 and 38.2%, respectively, by 14% BF_3/metnanol. The changes in composition of *t*10,*t*12 and *t*9,*t*11 CLA isomers concomitantly produced other *t,t* isomers: 1.1% *t*12,*t*14 CLA; 2.9% *t*11,*t*13 CLA; 1.6% *t*8,*t*10 CLA; and 0.3% *t*7,*t*9 CLA by 3.5% BF_3/methanol. The compositions of the four *t,t* CLA isomers were further increased to 2.6, 10.7, 9.5, and 2.3%, respectively, by 14.0% BF_3/methanol.

Effect of Methylation Time on the Content of *t,t* CLA-Me Isomers. Methylation time also greatly affected the content of *t,t* CLA isomers in the TT-TC/CT, prepared by methylation of synthetic CLA (50 mg) with 1 mL of 14.0% BF_3/methanol (**Table 5**). The 5 min reaction completely methylated the synthetic CLA, but only 19.1% of the CLA was converted to *t,t* CLA isomers, resulting in the production of a TT-TC/CT (8.99 mg), consisting of *t*12,*t*14 CLA (0.07 mg); *t*11,*t*13 CLA (0.26 mg); *t*10,*t*12 CLA (4.20 mg); *t*9,*t*11 CLA (4.31 mg); and *t*8,*t*10 CLA (0.15 mg). The 60 min reaction led to the conversion of 60.9% CLA to *t,t* CLA isomers (28.62 mg), consisting of

Table 3. Composition of *t,t* CLA Isomers in the Purified *t,t* CLA-Me Isomer Mixtures[a]

purified *t,t* CLA-Me isomer mixtures[b]	*t,t* CLA isomer (%)[c]							
	12,14	11,13	10,12	9,11	8,10	7,9	others[d]	total
TT-TC/CT	0.3 ± 0.1b[e]	7.0 ± 0.3a	40.1 ± 1.0a	43.3 ± 0.7a	8.0 ± 0.4a	0.7 ± 0.1a	0.6 ± 0.1a	100
TT-TC	0.2 ± 0.1b	7.3 ± 0.2a	41.8 ± 0.9a	44.2 ± 0.8a	5.7 ± 0.2b	0.1 ± 0.1b	0.7 ± 0.1a	100
TT-CT	1.1 ± 0.1a	9.4 ± 0.4a	37.3 ± 0.7a	41.1 ± 1.0a	9.7 ± 0.3a	0.7 ± 0.1a	0.7 ± 0.2a	100

[a] The sample was analyzed by Ag^+-HPLC (**Figure 1D**). [b] Purified *t,t* CLA-Me isomer mixtures were obtained from **Table 2** or **Figure 1C**. [c] Percentage of the relative peak area of given *t,t* CLA isomer to total peak area of CLA isomers. [d] Others included CLA isomers except for *t,t* CLA isomers. [e] Means ± SD of three experimental data. Means with different letters in the same column represent a significant difference at $p < 0.05$ by Duncan's multiple test.

Table 4. Effects of BF_3 Concentration in Methanol on the Yield of *t,t* CLA Isomers in TT-TC/CT Sample[a]

t,t CLA isomer	concentration (% BF_3/methanol)					
	0[b]	0.5	1.0	3.5	7.0	14.0
12,14	–[c]	Tr[d]	Tr	0.10 ± 0.01c[e]	0.29 ± 0.01b	0.73 ± 0.04a
11,13	Tr	Tr	0.02 ± 0.01d	0.25 ± 0.01c	1.27 ± 0.06b	3.02 ± 0.15a
10,12	0.38 ± 0.02e	0.43 ± 0.02e	0.79 ± 0.04d	4.02 ± 0.22c	9.20 ± 0.47b	10.38 ± 0.52a
9,11	0.62 ± 0.03e	0.66 ± 0.03e	1.01 ± 0.05d	4.21 ± 0.15c	9.47 ± 0.40b	10.81 ± 0.55a
8,10	Tr	Tr	0.01 ± 0.01d	0.14 ± 0.01c	1.08 ± 0.05b	2.68 ± 0.12a
7,9	–	Tr	Tr	0.03 ± 0.01c	0.23 ± 0.01b	0.66 ± 0.03a
total yield (mg)	1.00 ± 0.05e	1.09 ± 0.05e	1.83 ± 0.09d	8.75 ± 0.44c	21.54 ± 1.08b	28.28 ± 2.41a

[a] TT-TC/CT sample, prepared from synthetic CLA (50 mg) by methylation for 30 min with 1 mL of various concentration of BF_3 in methanol, was purified at −68 °C for 24 h and then analyzed by Ag^+-HPLC (**Figure 1D**). The completion of sample methylation was determined by TLC, using a silica gel precoated aluminum plate developed with hexane:ethyl acetate (5:1,v/v). The methylation was completed by more than 3.5% BF_3/methanol. The recovery of *t,t* CLA isomers from TT-TC/CT samples was greater than 95%. The amount of each *t,t* CLA isomer was calculated by the given peak area ratio to total peak areas. The total yield (mg) was calculated by considering the percentage of synthetic CLA converted to *t,t* CLA (60.2%), purity of TT-TC/CT after purification (98.9%), and percentage of recovery (>95%). [b] Content of *t,t* CLA isomers present in original synthetic CLA sample, which was determined by the method of Park et al. (*27*). [c] Not detected. [d] Tr means less than 0.01 mg. [e] Mean ± SD of three experimental data. Means with different letters in the same row represent a significant difference at $p < 0.05$ by Duncan's multiple test.

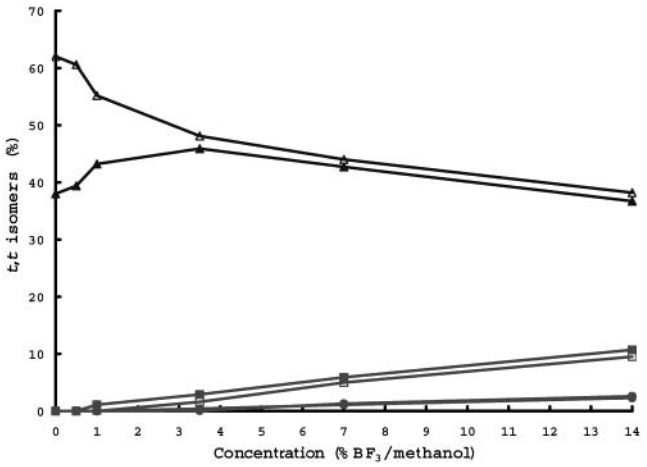

Figure 2. Effects of BF_3 concentration in methanol on the composition of *t,t* CLA isomers in the TT-TC/CT sample. The compositional data of *t,t* CLA isomers were derived from **Table 4**. Line identification: closed circles, *t*12,*t*14 CLA; closed squares, *t*11,*t*13 CLA; closed triangles, *t*10,*t*12 CLA; open triangles, *t*9,*t*11 CLA; open squares, *t*8,*t*10 CLA; and open circles, *t*7,*t*9 CLA.

*t*12,*t*14 CLA (1.66 mg); *t*11,*t*13 CLA (4.21 mg); *t*10,*t*12 CLA (8.50 mg); *t*9,*t*11 CLA (8.79 mg); *t*8,*t*10 CLA (3.92 mg); and *t*7,*t*9 CLA (1.54 mg).

Figure 3 shows the compositional changes of *t,t* CLA isomers in the TT-TC/CT sample derived from **Table 5**. The synthetic CLA contained 1.0 mg of *t,t* CLA isomers with a composition of 38.0% *t*10,*t*12 CLA and 62.0% *t*9,*t*11 CLA isomers. The 5 min methylation changed the composition of *t*10,*t*12 and *t*9,*t*11 CLA isomers to 46.7 and 47.9%, respectively, and they decreased to 36.7 and 38.2%, respectively, by 30 min methylation. When the composition of the *t*10,*t*12 and *t*9,*t*11 CLA isomers decreased by methylation, other *t,t* CLA isomers were produced as follows: *t*11,*t*13 and *t*8,*t*10 CLA isomers by 1 min and then *t*12,*t*14 CLA and *t*7,*t*9 CLA by 5 min. The composition of the latter four isomers was further elevated by 30 min: 2.6% *t*12,*t*14; 10.7% *t*11,*t*13; 9.5% *t*8,*t*10; and 2.3% *t*7,*t*9 CLA isomers.

Production of a Large Quantity of TT-TC/CT. As seen in the above results from the effects of BF_3 concentration in methanol and methylation time on the formation of *t,t* CLA isomers, the composition of *t,t* CLA isomers in the TT-TC/CT sample closely resembled that of the TT-TC or TT-CT sample. It is much easier and more simple to prepare a large quantity of TT-TC/CT rather than TT-TC or TT-CT; hence, in this experiment, a *t,t* CLA-Me isomer mixture was prepared from synthetic CLA (25 g) by methylation for 30 min with 500 mL of 14% BF_3/methanol. The purity of TT-TC/CT was 60.6%; however, it was further increased to 98.5% by purification at −68 °C for 24 h, resulting in 14.15 g of TT-TC/CT.

Ag^+ HPLC analysis revealed that the purified TT-TC/CT sample was found to be a mixture of *t*12,*t*14 CLA (2.3%); *t*11,*t*13 CLA (9.7%); *t*10,*t*12 CLA (38.0%) and *t*9,*t*11 CLA (39.3%); *t*8,*t*10 CLA (9.0%); and *t*7,*t*9 CLA (1.7%) isomers. The *t*10,*t*12 CLA and *t*9,*t*11 CLA were the major isomers, whereas *t*12,*t*14 CLA; *t*11,*t*13 CLA; *t*8,*t*10 CLA; and *t*7,*t*9 CLA isomers were minor constituents.

DISCUSSION

The present study has revealed that a mixture of *t,t* CLA-Me isomers (28.28 mg), consisting of *t*12,*t*14 (2.6%); *t*11,*t*13 (10.7%); *t*10,*t*12 (36.7%); *t*9,*t*11 (38.2%); *t*8,*t*10 (9.5%); and *t*7,*t*9 (2.3%), was easily prepared from the synthetic CLA (50 mg) by two steps: the methylation for 30 min with 1 mL of 14% BF_3/methanol, followed by purifying at −68 °C for 24 h. A similar mixture of *t,t* CLA-Me isomers (14.15 g) was also

Table 5. Effect of Methylation Time on the Yield of *t,t* CLA Isomers in TT-TC/CT Sample[a]

	methylation time (min)					
t,t CLA isomer	0[b]	1	5	10	30	60
12,14	–[c]	–	0.07 ± 0.01d[d]	0.15 ± 0.01c	0.73 ± 0.04b	1.66 ± 0.07a
11,13	Tr[e]	0.04 ± 0.01e	0.26 ± 0.01d	0.60 ± 0.03c	3.02 ± 0.15b	4.21 ± 0.21a
10,12	0.38 ± 0.02f	0.72 ± 0.03e	4.20 ± 0.21d	6.91 ± 0.35c	10.38 ± 0.52a	8.50 ± 0.43b
9,11	0.62 ± 0.03f	0.91 ± 0.05e	4.31 ± 0.22d	7.18 ± 0.36c	10.81 ± 0.55a	8.79 ± 0.45b
8,10	Tr	Tr	0.15 ± 0.01d	0.44 ± 0.02c	2.68 ± 0.12b	3.92 ± 0.20a
7,9	–	–	Tr	0.10 ± 0.01c	0.66 ± 0.03b	1.54 ± 0.08a
total yield (mg)	1.00 ± 0.05e	1.67 ± 0.08d	8.99 ± 0.45c	15.38 ± 0.77b	28.28 ± 2.41a	28.62 ± 1.43a

[a] TT-TC/CT sample, prepared from synthetic CLA (50 mg) by methylation with 1 mL of 14% BF_3 in methanol for various times, was purified at −68 °C for 24 h and then analyzed by Ag^+-HPLC (**Figure 1D**). The completion of sample methylation was determined by the TLC method described in **Table 4**. More than 5 min of reaction completed the methylation. The recovery of *t,t* CLA isomers from TT-TC/CT samples was greater than 95%. The amount of each *t,t* CLA isomer was calculated by the given peak area ratio to total peak areas. The total yield (mg) was calculated by considering the percentage of synthetic CLA converted to *t,t* CLA (60.2%), purity of TT-TC/CT after purification (98.9%), and percentage of recovery (>95%). [b] Content of *t,t* CLA isomers present in original synthetic CLA sample, which was determined by the method of Park et al. (27). [c] Not detected. [d] Mean ± SD of three experimental data. Means with different letters in the same row represent a significant difference at $p < 0.05$ by Duncan's multiple test. [e] Tr means less than 0.01 mg.

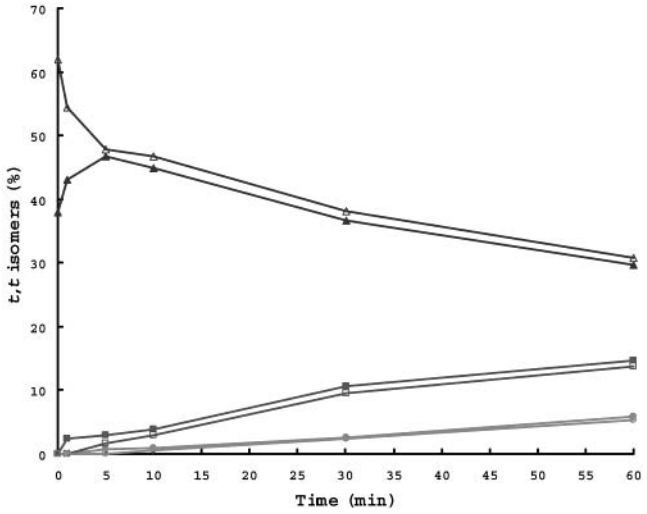

Figure 3. Effect of methylation time on the composition of the *t,t* CLA isomers of the TT-TC/CT sample. The compositional data of *t,t* CLA isomers were derived from **Table 5**. Line identification: closed circles, *t*12,*t*14 CLA; closed squares, *t*11,*t*13 CLA; closed triangles, *t*10,*t*12 CLA; open triangles, *t*9,*t*11 CLA; open squares, *t*8,*t*10 CLA; and open circles, *t*7,*t*9 CLA.

prepared from 25 g of synthetic CLA by methylation for 30 min with 500 mL of 14% BF_3/methanol, followed by purifying at −68 °C for 24 h. Although a minute quantity of *t,t* CLA was produced from *c*9,*t*11 CLA or synthetic CLA as an artifact during methylation with strong acid in methanol (*4*, *5*), this is the first that a substantial amount of highly pure *t,t* CLA-Me isomer mixtures (greater than 98%) was purposely prepared from synthetic CLA.

A mixture of *t,t* CLA-Me isomers with similar *t,t* CLA-Me isomer compositions of TT-TC/CT could also be prepared from a purified *c*9,*t*11 CLA or *t*10,*c*12 CLA fraction. To do this, four additional steps were required to obtain the purified *c*9,*t*11 CLA or *t*10,*c*12 CLA fraction from synthetic CLA: the methylation of synthetic CLA, precipitation and separation at both −68 and −71 °C, purification with urea, and base hydrolysis (*24*). This means that the preparation of the *t,t* CLA-Me isomer mixture from the purified *c*9,*t*11 CLA or *t*10,*c*12 CLA fraction is a more difficult and time-consuming process than that from synthetic CLA. It is also considered that the removal of impurities was very difficult from the *c*9,*t*11 CLA-Me or *t*10,*c*12 CLA-Me fraction by the low-temperature precipitation at both −68 and −71 °C (*24*), but it was easily removed from the *t,t* CLA-Me isomer fraction by storing it at −68 °C. Hence, a mixture of *t,t* CLA-Me isomer mixture could be directly prepared from *c*9,*t*11 CLA-Me or *t*10,*c*12 CLA-Me fraction without purification. However, this process is still more complicated than that of synthetic CLA.

The composition of *t,t* CLA isomers in TT-TC/CT was greatly affected by methylation conditions with BF_3/methanol (**Tables 4** and **5**). The TT-TC/CT sample (21.54 mg), prepared by methylation for 30 min with 7.0% BF_3/methanol, contained 1.3% *t*12,*t*14; 5.9% *t*11,*t*13; 42.7% *t*10,*t*12; 44.0% *t*9,*t*11; 5.0% *t*8,*t*10; and 1.1% *t*7,*t*9 CLA, whereas the TT-TC/CT sample (28.62 mg) by methylation for 60 min with 14.0% BF_3/methanol contained 5.8% *t*12,*t*14; 14.7% *t*11,*t*13; 29.7% *t*10,*t*12; 30.7% *t*9,*t*11; 13.7% *t*8,*t*10; and 5.4% *t*7,*t*9 CLA isomers. This suggests that the designed *t,t* CLA isomer mixture with a specified composition could be prepared by controlling the methylation conditions. It is of significance to note that the degree of the isomerization of *tc*/*ct* CLA in synthetic CLA was not further increased by stronger methylation conditions than 30 min methylation with 14.0% BF_3/methanol.

The composition of major *t,t* CLA isomers, *t*10, *t*12 CLA and *t*9,*t*11 CLA, in TT-TC/CT, prepared by relatively mild methylation condition such as 30 min with 3.5% BF_3/methanol (**Figure 2**) or 5 min with 14.0% BF_3/methanol (**Figure 3**), changed to approximately 47% from 38.0 and 62.0%, respectively. Furthermore, the strong methylation condition, like the 30 min with 14.0% BF_3/methanol, led the composition of *t*10,*t*12 CLA and *t*9,*t*11 CLA to approximately 37%, resulting in the production of other *t,t* CLA isomers: 2.6% *t*12,*t*14; 10.7% *t*11,*t*13; 9.5% *t*8,*t*10; and 2.3% *t*7,*t*9 CLA (**Figures 2** and **3**). This clearly shows that the migration of double bonds occurred from C10,C12 to C9,C11 positions and vice versa by the mild methylation conditions, whereas by the strong conditions, the migration occurred from C10,C12 and C9,C11 toward the methyl terminal (C11,C13 and C12,C14) and/or carboxyl terminal (C8,C10 and C7,C9) of the CLA molecule. This double bond migration was further supported by the fact that the composition of *t,t* CLA isomers in TT-TC/CT, prepared from synthetic CLA consisting of 47.8% *t*10,*c*12 CLA and 45.5% *c*9,*t*11 CLA, resembled that of TT-TC and TT-CT, prepared from 99.1% *t*10,*c*12 CLA and 91.0% *c*9,*t*11 CLA, respectively (**Table 3**). At the present time, the direction of the double bond migration along with the chain of CLA molecules is not clear. These results are different from the reports that the methylene-interrupted double bonds in linoleic acid (C9, C12) migrated

to both directions along with the molecule of the linoleic acid at a similar ratio under alkaline conditions at high temperature, resulting in similar composition of *c*9,*t*11 and *t*10,*c*12 CLA isomers (*4*, *5*). Therefore, it is impossible to prepare a pure single *t,t* CLA isomer by the methylation of synthetic CLA or *tc*/*ct* CLA fractions with BF_3/methanol. Thus, the pure single *t,t* CLA isomers could be prepared by collecting each isomer peak eluted when separated TT-TC/CT by Ag^+-HPLC.

In the present study, HCl- or H_2SO_4-catalyzed methylation with various concentrations in methanol and various reaction times was also applied to synthetic CLA for the preparation of *t,t* CLA-Me isomer mixtures, resulting in a similar composition obtained from BF_3-catalyzed methylation. However, 1 N HCl-catalyzed or 1 N H_2SO_4-catalyzed methylation required a longer reaction time, at least 1 h, than the BF_3/methanol-catalyzed methylation conditions tested. Moreover, 2 N HCl-catalyzed or 2 N H_2SO_4-catalyzed methylation for 1 h altered the composition of *t,t* CLA isomers. Hence, the HCl- or H_2SO_4-catalyzed methylation method is inadequate for the preparation of *t,t* CLA isomer mixtures, due to slow isomerization and/or additional reactions such as chlorination or sulfonation on the double bonds (*5*).

In conclusion, a TT-TC/CT sample, consisting of *t*12,*t*14; *t*11,*t*13; *t*10,*t*12; *t*9,*t*11; *t*8,*t*10; and *t*7,*t*9 CLA isomers, was simply prepared from synthetic CLA by methylation with 14% BF_3/methanol, followed by purification at −68 °C for 24 h. The amount and composition of *t,t* CLA isomers in TT-TC/CT could be controlled by methylation conditions.

ABBREVIATIONS USED

CLA, conjugated linoleic acid; *t,t* CLA-Me, *t,t* conjugated linoleic acid methylester; *tc*/*ct* CLA, *t*10,*c*12 CLA and *c*9,*t*11 CLA; TT-TC/CT, a mixture of *t,t* CLA-Me isomers prepared from synthetic CLA; TT-TC, a mixture of *t,t* CLA-Me isomers prepared from *t*10,*c*12 CLA fraction; TT-CT, a mixture of *t,t* CLA-Me isomers prepared from *c*9,*t*11 CLA fraction; FID, flame ionization detector; GC, gas chromatography; HPLC, high-performance liquid chromatography.

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