J. Food Sci. Nutr.
Vol. 5, No. 2, p. 86~92 (2000)

Preparation of a Large Quantity of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 Conjugated Linoleic Acid (CLA) Isomers from Synthetic CLA

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Abstract

Conjugated linoleic acid (CLA) refers to a collective term of positional and geometric isomers of linoleic acid, which are different in their biological activities. The predominant isomer of CLA in animal tissues is *cis*-9,*trans*-11; smaller amounts of *trans*-10,*cis*-12 also occurs. To develop a method for preparation of a large quantity of the relatively pure *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA isomers, CLA methyl ester (CLA-Me) was chemically synthesized from linoleic acid by the alkaline isomerization method. The synthetic CLA-Me, mainly composed of *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA, was dissolved in acetone, stored at -68°C for 1 day, and the supernatant (*cis*-9,*trans*-11 CLA-Me) was separated from the precipitate (*trans*-10,*cis*-12 CLA-Me). After the processes were repeated three times at -68°C, the whole processes were repeated three times at -71°C in order to increase the purity of these two isomers. The *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA isomers were further purified by the urea adduct. Purities of the *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me were 90.3 and 99.9%, respectively. This method could be employed for the preparation of a large quantity of highly purified *cis*-9,*trans*-11 CLA-Me or *trans*-10,*cis*-12 CLA-Me from synthetic CLA-Me.

Key words: conjugated linoleic acid (CLA), *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, low temperature precipitation, urea adduct

INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term of positional (9,11 and 10,12) and geometric (*cis, trans*; *trans, cis*; *cis, cis*; and *trans, trans*) isomers of octadecadienoic acid with a conjugated double bond system. Synthetic CLA exhibits a potent anticarcinogenic activity for the carcinogen-induced several animal models (1-6). Other biological activities such as the immune stimulation (7), body fat reduction (8,9) modulation of cholesterol content in blood (10) and growth stimulation (11), were evident. The synthetic CLA is composed of approximately 47% *cis*-9,*trans*-11 CLA, 49% *trans*-10,*cis*-12 CLA, and 4% of other isomers. CLA isomers incorporated in phospholipid fractions from the tissues of experimental animals intentionally fed the synthetic CLA are approximately 90% *cis*-9,*trans*-11 CLA and 10% *trans*-10,*cis*-12 CLA (12). Recently, it has been recognized that the *trans*-10,*cis*-12 CLA isomer is more effective for the reduction of mouse body fat than *cis*-9,*trans*-11 CLA isomer (13). Hence, the biological activities of the synthetic CLA might be reevaluated by these two individual CLA isomers. Given this information in hand, a large quantity of both *cis*-9,*trans*-11 CLA and *trans*-10, *cis*-12 CLA isomers is required.

Many scientists have carried out researches for the preparation of individual *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA isomers. The *cis*-9,*trans*-11 CLA is produced as a metabolite by anaerobic bacteria *B. fibrisolvens* (14) and *Lcatobacillus sp.* (15), but impossible to get a large quantity. Relatively pure (83%) *cis*-9,*trans*-11 CLA was chemically synthesized from the ricinolate methyl ester (16): however, this is a complicate and expensive procedure, and produces many oxidation byproducts. The *cis*-9,*trans*-11 CLA was selectively purified from synthetic CLA, using *Asp. niger via* (17), but the purity and quantity are not adequate for the biological activity test. Several studies were performed to obtain *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA by argentation chromatographic methods (18-22), but these were only analytical scales. All of these methods are not adequate for the production of *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA isomers for industrial scales.

To date, the most simple and inexpensive way to get both of these two isomers is to separate each individual compounds from synthetic CLA methyl ester (CLA-Me) by means of their different freezing temperatures in given solvents. Nichols et al. (23) was the first to prepare *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me from synthetic CLA by twenty-one fractional precipitation steps in methanol. Scholfield and Koritala (24) prepared *trans*-10,*cis*-12 CLA-Me in acetone by fractional precipitation at -57°C~-59°C. They obtained relatively pure *trans*-10,*cis*-12 CLA-Me, but the yield was very low (75%). These methods are too complicate and give a low

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yield. Hence, a simple and inexpensive method suitable for the mass production of these two CLA isomers should be investigated.

In the present study, a simple and inexpensive method was established to prepare a large quantity of highly pure *cis*-9, *trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me from synthetic CLA-Me. The CLA-Me was chemically-synthesized from linoleic acid (95% purity), and fractionated and purified in acetone by a low-temperature precipitation in conjunction with urea adduct.

MATERIALS AND METHODS

Materials

Linoleic acid (95%) was prepared from safflower oil according to the method described Kim et al. (25). Safflower oil was obtained from Cheiljedang Co. (Inchen, Korea). Acetone, hexane, ethanol, isopropanol, ethylacetate, and methanol were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Ethylene glycol (Sigma Chemical Company, MO, USA), and KOH and urea (Shinyo Chemical Co., Osaka, Japan) were used. All other reagents used were ACS grade.

Synthesis of conjugated linoleic acid (CLA)

A round bottom flask (3 L) containing ethylene glycol (1 L) was maintained at 190°C for 10 min, followed by cooling down to 165°C. After carefully adding KOH (250 g) to the round bottom flask, the bottle was heated to 180°C. The linoleic acid (500 ml) was slowly added and maintained at 180°C with swirling frequently. One hour later, the reactant was cooled down to the room temperature over a running tap water. All of the steps were performed under nitrogen. Methanol (500 ml) and then 6 N HCl (1 L) were added to the reactant. Synthetic CLA was extracted with hexane (500 ml × 2) and washed with distilled water (250 ml × 3). The hexane extract was dried over Na_2SO_4 anhydrous. Synthetic CLA was obtained by removing the hexane solvent under vacuum.

Methylation of CLA

CLA-Me was prepared by a slight modification of H_2SO_4-catalyzed methyl esterification method. CLA (100 g) dissolved in 300 ml of 0.05 N H_2SO_4/ methanol was heated in a boiling water bath for 10 min. The reactant was cooled down to room temperature, followed by adding distilled water (100 ml) and extracting with hexane (300 ml × 3). The hexane extract was washed with distilled water (100 ml × 3) and then dried over Na_2SO_4 anhydrous. CLA-Me was recovered from the hexane extract by removing the hexane under vacuum.

Separation of the *cis*-9,*trans*-11 CLA-Me from *trans*-10, *cis*-12 CLA-Me by a low-temperature precipitation

Organic solvents employed

The solvents employed were acetone, isopropanol, hexane, and ethyl acetate. CLA-Me (10 g) sample dissolved in each solvent (120 ml) was stored in a deep freezer (-71°C, Korye Instrument Co., Seoul, Korea) for 35 days to separate *trans*-10, *cis*-12 CLA-Me from *cis*-9,*trans*-11 CLA-Me by precipitation. The purity of the *cis*-9,*trans*-11 CLA-Me (supernatant) and *trans*-10,*cis*-12 CLA-Me (precipitate) was analyzed at a 5-day interval by GC analysis.

Separation of the isomers in acetone

The *cis*-9,*trans*-11 CLA-Me (supernatant) was separated from the *trans*-10,*cis*-12 CLA-Me (precipitate) by a low-temperature precipitation of CLA-Me (100 g) dissolved in acetone (1200 ml) at -68°C and -71°C. After the sample was stored at a given temperature for one day, the precipitate was separated from the supernatant by filtration. The precipitation and separation process were repeated 3 times at both -68°C and -71°C in acetone with the concentration of 100 g/1200 ml. The concentrations of *cis*-9,*trans*-11 CLA-Me and *trans*-10, *cis*-12 CLA-Me in the resulting supernatant and precipitate were determined by GC as described below.

Purification of *cis*-9,*trans*-11 CLA-Me and *trans*-10, *cis*-12 CLA-Me by urea inclusion

Urea (100 g) and ethanol (2000 ml) were added to 50 g sample of the *cis*-9,*trans*-11 CLA-Me or the *trans*-10,*cis*-12 CLA-Me fraction. Reaction mixture was refluxed for 60 min. The reactant was cooled down to room temperature, followed by stored at 4°C for 24 hr to form urea adduct. The *cis*-9,*trans*-11 CLA-Me and *trans-10,cis*-12 CLA-Me were purified by removing the urea adduct through filtration.

Gas chromatography

The *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me were analyzed by GC (Hewlett packard 5890; Bellefonte, PA) equipped with flame ionization detector (FID) and Supelcowax-10 capillary column (60 m × 0.32 mm, i.d.). Carrier gas was N_2. Oven temperature increased from 50°C to 200°C at a rate of 10°C/min. Injector and detector temperatures were 240°C and 260°C, respectively. The composition of the isomers was calculated by the peak area ratios counted by the Integrator (Hewlett-Packard 3396 Series III, Bellefonte, PA).

RESULTS

Composition of CLA-Me isomers

Fig. 1 shows GC chromatograms of linoleic acid prior to and after alkaline isomerization. This alkaline isomerization method converted most linoleic acid to CLA. Based on the area counts of individual CLA isomers, the synthetic CLA consisted of 47.2% *cis*-9,*trans*-11 CLA-Me, 50.7% *trans*-10,*cis*-12 CLA-Me, 1.2% *trans*, *trans*-CLA-Me, and 0.9% other CLA isomers. The *trans*-9,*trans*-11 CLA-Me and *trans*-10,*trans*-12 CLA-Me were not separated each other (eluted at a same retention time) by this analytical condition. These results are in agreement with reports that synthetic CLA contains approximately 48% of each *cis*-9,*trans*-11 CLA-Me and *trans*-10, *cis*-12 CLA-Me, and minor isomers (2).

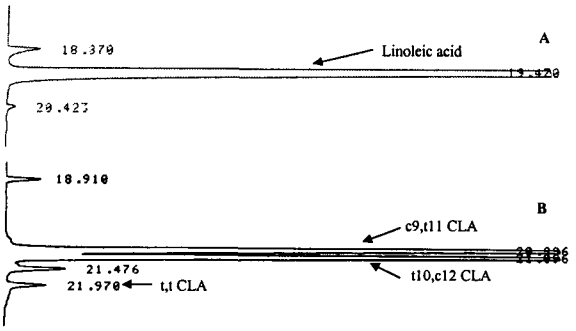

Fig. 1. GC chromatograms of linoleic acid prior to (A) and after alkaline isomerization (B).

Separation of *cis*-9,*trans*-11 CLA-Me from *trans*-10, *cis*-12 CLA-Me by a low-temperature precipitation

Selection of solvent

The temperature and duration for the completion of the precipitation of *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me are not identical with each other in a given solvent. Hence, CLA-Me (100 g) dissolved in 1200 ml of various solvents (acetone, isopropanol, hexane, or ethyl acetate) was stored at -71°C for 35 days to separate *cis*-9,*trans*-11 CLA-Me from *trans*-10,*cis*-12 CLA-Me. The compositions of *cis*-9, *trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me in supernatant (Fig. 2) and precipitate (Fig. 3) were analyzed at a 5-day interval over a period of 35 days by GC. The major CLA isomers detected in supernatant and precipitate were *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me, respectively, for all solvents tested. No change in the composition of *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me was seen, respectively, in supernatant and precipitate for over a period of 35 days. At 35 days, the composition of the *cis*-9,*trans*-11 CLA-Me in supernatant was 78.4% in acetone, 77.6% in isopropanol, 62.7% in hexane, and 55.6% in ethyl acetate (Fig. 2), whereas the composition of the *trans*-10,*cis*-12 CLA-Me in precipitate was 94.2% in acetone and 93.7% isopropanol (Fig. 3).

The separation of the precipitate formed in acetone was much easier than that of the precipitate formed in isopropanol, due to the formation of emulsion in isopropanol. The precipitate was not formed in hexane and ethyl acetate, and so these solvents were not adequate to separate CLA isomers. Based on these data, the best condition for the preparation of the *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me isomers by a low-temperature precipitation is to store the synthetic CLA at -71°C for 5 days in acetone.

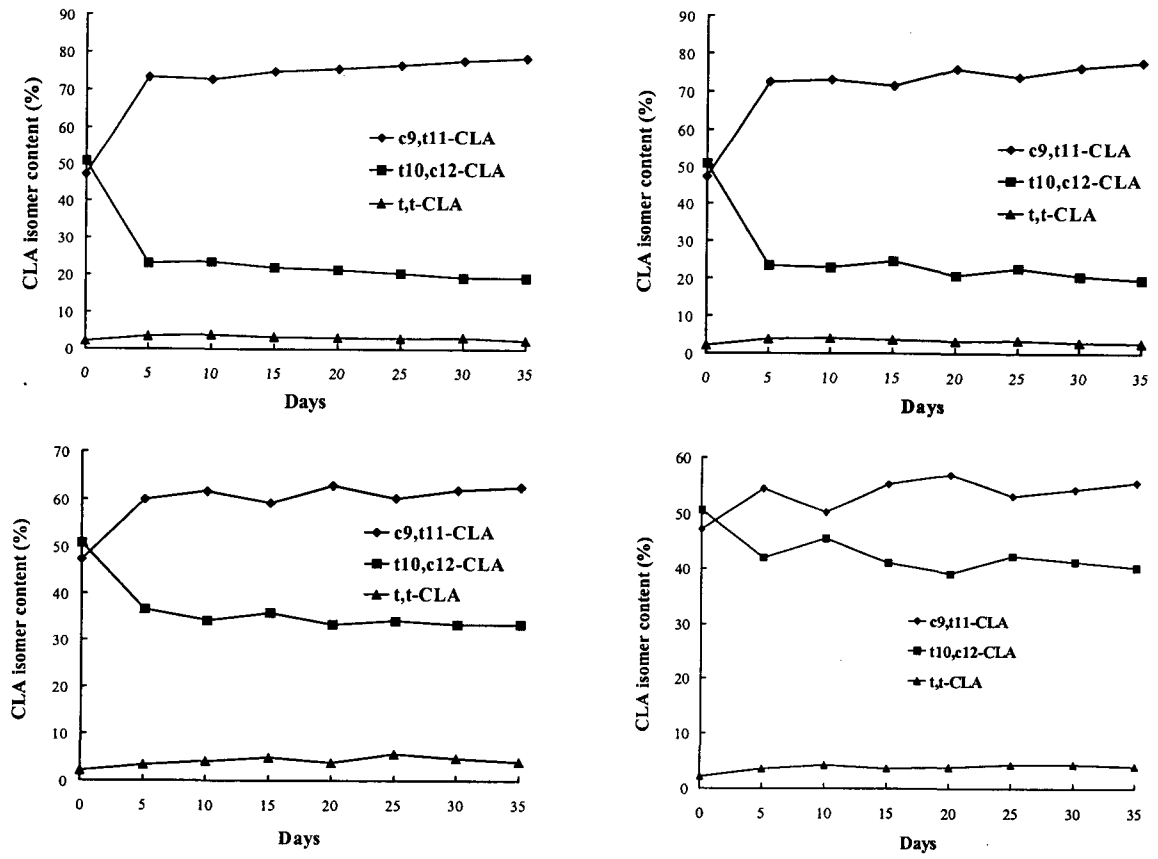

Fig. 2. Distribution of *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me to the supernatant prepared by storing CLA-Me solution at -71°C. Panel identification according to the solvent used: top left, acetone; top right, isopropanol; bottom left, hexane; and bottom right, ethylacetate.

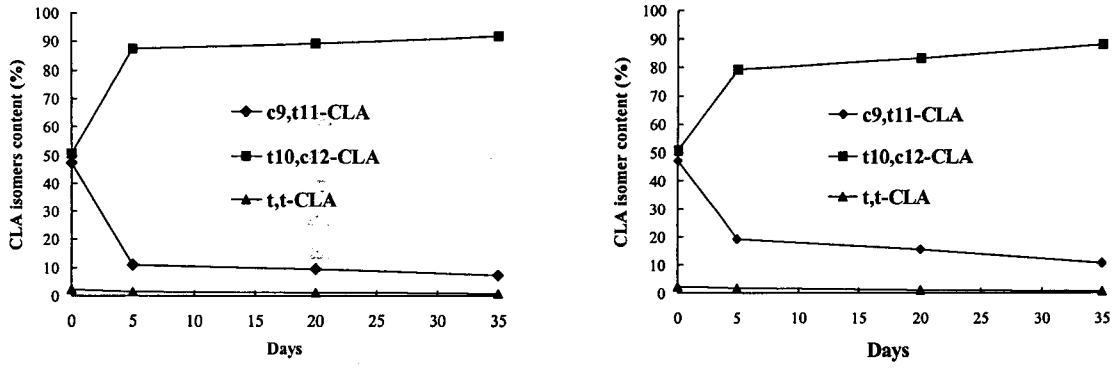

Fig. 3. Distribution of the *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me to the precipitate prepared by storing CLA-Me solution at -71°C. Panel identification according to the solvent used: left, acetone; and right, isopropanol.

Separation in acetone

The separation of *cis*-9,*trans*-11 CLA-Me from *trans*-10, *cis*-12 CLA-Me in acetone (-71°C) was effectively completed in 5 days, implying that the precipitation of *trans*-10,*cis*-12 CLA-Me might be completed within 5 days. Fig. 4 shows the composition of the *cis*-9,*trans*-11 CLA-Me and *trans*-10, *cis*-12 CLA-Me in the supernatant from the synthetic CLA in acetone (100 g/1200 ml) (-71°C) stored for over period of 2 days. The sample stored for one day contained 80.4% *cis*-9, *trans*-11 CLA-Me and 18.1% *trans*-10,*cis*-12 CLA-Me. No further difference in the composition of CLA isomers was seen between the duration of storages (1 day vs 2 days). These indicate that one storage day of the synthetic CLA-Me in acetone at -71°C was enough to separate the *cis*-9,*trans*-11 CLA-Me from *trans*-10,*cis*-12 CLA-Me.

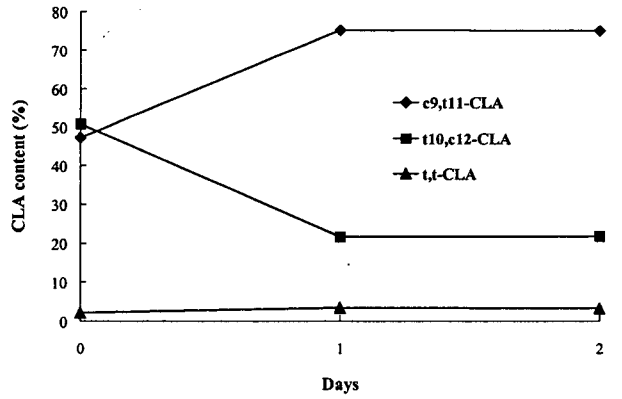

Fig. 4. Effect of the storage duration at -71°C on the distribution of the *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA in the supernatant prepared by storing CLA-Me in acetone.

Optimum temperature for the separation of *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me in acetone

The two isomers of CLA-Me were satisfactorily separated in acetone one day after storage at -71°C. To investigate effective temperature for the separation of the two isomers in acetone, the synthetic CLA-Me (100 g) dissolved in acetone (1200 ml) was stored at various temperature (-60°C, -65°C, -68°C, -71°C, -75°C, and -80°C) for one day. The precipitate of *trans*-10,*cis*-12 CLA-Me was not formed at -60°C, but it was formed at -65°C, -68°C, 71°C, -75°C and -80°C. The precipitates formed were not different in their appearances; however, the highest composition of *trans*-10,*cis*-12 CLA-Me was observed in the precipitate formed at -68°C (Fig. 5). The composition of *cis*-9,*trans*-11 CLA-Me in supernatant (Fig. 5, upper) and *trans*-10,*cis*-12 CLA-Me (Fig. 5, bottom) was 79.1% and 90.5%, respectively, stored at -68°C for one day. Temperature lower than -75°C was not adequate for the separation

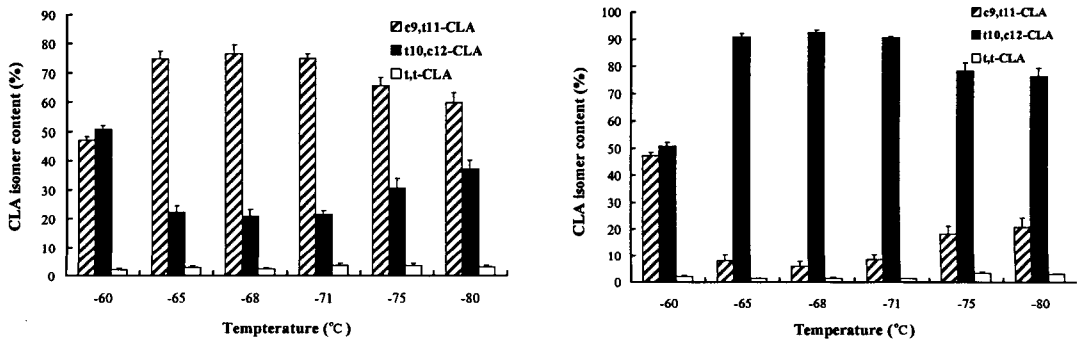

Fig. 5. Distribution of the *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me to the supernatant (top panel) and precipitate (bottom panel) prepared by storing CLA-Me solution for 1 day at given temperature. Separation was repeated consecutively three times.

of the two isomers because of coprecipitation of *cis*-9,*trans*-11 CLA-Me with *trans*-10,*cis*-12 CLA-Me.

As the results, the precipitation of the *trans*-10,*cis*-12 CLA-Me in acetone was completed at -68°C; however, the precipitation of *cis*-9,*trans*-11 CLA-Me in supernatant began at -75°C, indicating that freezing temperatures of these two isomers are different in acetone by 4~7°C. Thus, the purity of the two isomers increased, when the two isomers were separated consecutively three times at both -68°C and then -71°C by storage for one day each (Fig. 6). The purity of *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me prepared at -68°C was 80.7% and 91.1%, respectively, whereas the purity of *cis*-9, *trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me prepared at both -68°C and -71°C increased to 87.3% and 92.5%, respectively.

Purification of *cis*-9,*trans*-11 CLA-Me and *trans*-10, *cis*-12 CLA-Me by the urea inclusion

The *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me fractions prepared by a low-temperature precipitation contained some impurities such as minor fatty acids derived from the linoleic acid sample used for CLA-Me synthesis and oxidative byproducts produced during the synthesis of CLA-Me (Fig. 1, 7A, and 8A). The CLA isomers dissolved in acetone were treated with urea to remove these impurities by the formation of urea adducts. As results seen in Fig. 7B and 8B, most fatty acids and oxidative products present in the *cis*-9, *trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me fractions were removed.

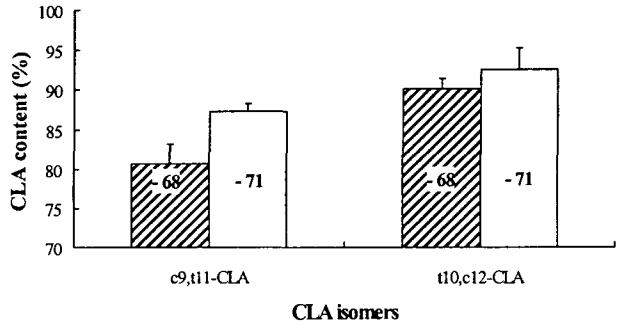

Fig. 6. Increase in the purity of the *cis*-9,*trans*-11 CLA (supernatant) and *trans*-10,*cis*-12 CLA (precipitate) by purifying CLA-Me (100 g CLA-Me/1200 ml acetone) at -68°C, followed by at -71°C, three times each.

Table 1 shows the purity of *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me isomers purified by a sequential procedures of a low-temperature precipitation and urea inclusion. Synthetic CLA-Me contained 47.2% *cis*-9,*trans*-11 CLA-Me and 50.7% *trans*-10,*cis*-12 CLA-Me. The purity of the *cis*-9, *trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me increased to 87.3% and 92.5%, respectively, when separated in acetone three times each at both -68°C and -71°C in the order. The purity of *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me further increased to 90.3% and 99.9% by the urea treatment.

DISCUSSION

A method for the preparation of a large quantity of highly pure *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me was established by a low-temperature precipitation in conjunction with urea adduct. For the reduction of the cost, inexpensive linoleic acid (approximately 95% purity) was used for the preparation of synthetic CLA-Me. The synthetic CLA-Me contained approximately 47.2% of *cis*-9,*trans*-11 CLA-Me and 50.7% of *trans*-10,*cis*-12 CLA-Me. The purity of *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me increased to 90.3% and 99.9%, respectively, when purified in acetone at a ratio of 100 g/1200 ml by a low-temperature precipitation at both -68°C and -71°C in conjunction with urea treatment. This

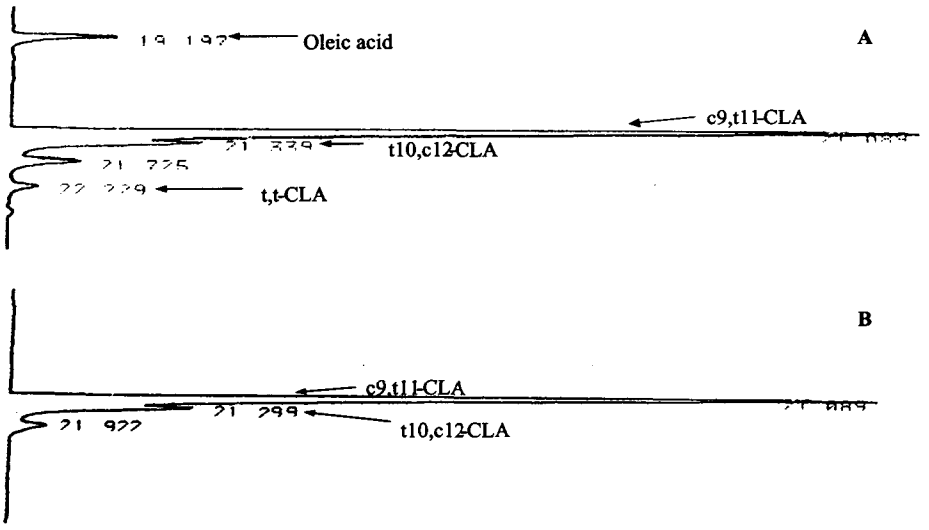

Fig. 7. GC chromatograms of the *cis*-9,*trans*-11 CLA-Me (supernatant) purified by precipitation of CLA-Me (Fig.1, B) in acetone at both -68°C and -71°C prior to (A) and after urea treatment (B).

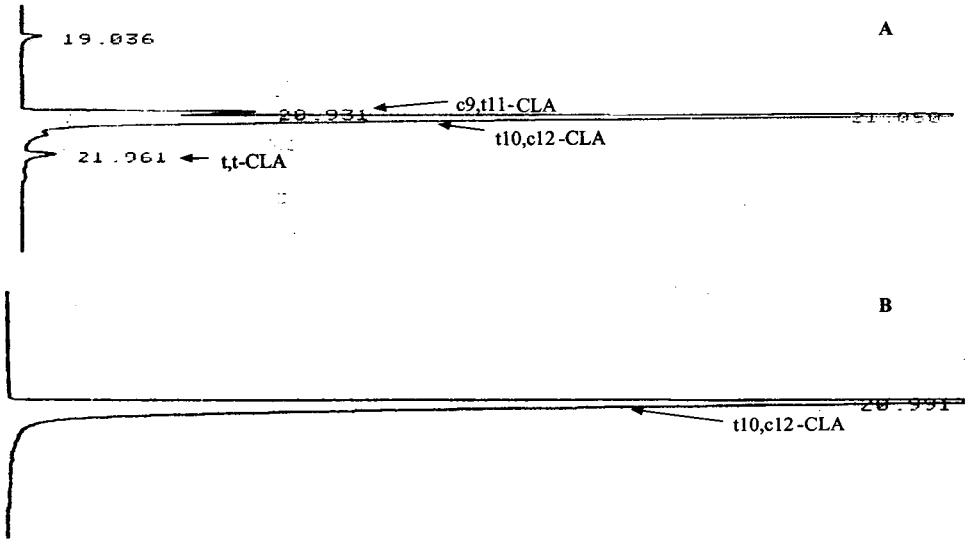

Fig. 8. GC chromatograms of the *trans*-10,*cis*-12 CLA-Me (precipitate) purified by precipitation of CLA-Me (Fig.1, B) in acetone at both -68°C and -71°C prior to (A) and after urea treatment (B).

Table 1. Purity of the *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me prepared by a sequential separation and purification of the synthetic CLA-Me

Treatment	*cis*-9,*trans*-11 CLA	*trans*-10,*cis*-12 CLA
CLA-Me[1)]	47.2	50.7
Low-temperature precipitation[2)]	87.3	92.5
Urea treatment[3)]	90.3	100

[1)]CLA-Me was chemically synthesized from linoleic acid (95%) by alkaline isomerization at 180°C.
[2)]CLA-Me dissolved in acetone (100 g/1200 ml) was stored at -68°C for one day, followed by separating the *cis*-9,*trans*-11 CLA-Me (supernatant) from *trans*-10,*cis*-12 CLA-Me (precipitate). The two isomers were further purified by storing at -71°C for one day. The precipitation and separation process at -68°C and -71°C was repeated three times.
[3)]Two isomers separated by a low-temperature precipitation was purified by the urea treatment.

whole process takes 7 days; hence, this method could be employed for the preparation of a large quantity (more than 1 kg/day) of each highly purified *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me.

The important factors affecting the fractionation of the two isomers from synthetic CLA at a low temperature were the solvent, the temperature, and the concentration of CLA-Me sample. Among the solvents tested, acetone was the most effective for the precipitation of *trans*-10,*cis*-12 CLA-Me at -68°C～-71°C. Efficacy of isopropanol was similar to that of acetone, but the *trans*-10,*cis*-12 CLA-Me was emulsified without forming precipitate, resulting in the difficulties for its separation. The *trans*-10,*cis*-12 CLA-Me was not precipitate at -68°C～-71°C in hexane or ethylacetate. The temperature was a very important factor for the precipitation of *trans*-10, *cis*-12 CLA-Me in acetone, whereas duration (1～35 days) at given temperature did not affect the precipitation.

In acetone, the precipitate of *trans*-10,*cis*-12 CLA-Me was not formed at -60°C～-65°C, but it was nearly completed at -68°C in one day. However, *cis*-9,*trans*-11 CLA-Me started to precipitate in acetone at -75°C. Hence, a promising method to get relatively pure CLA isomers from the synthetic CLA is to separate the *trans*-10,*cis*-12 CLA-Me from *cis*-9,*trans*-11 CLA-Me in acetone by precipitation at -68°C and in turn, to remove the residual isomer from each *trans*-10,*cis*-12 CLA-Me and *cis*-9,*trans*-11 CLA-Me by precipitation at -71°C. These processes resulted in 87.3% *cis*-9,*trans*-11 CLA-Me and 92.5% *trans*-10,*cis*-12 CLA-Me.

Another important factor is the concentration of CLA-Me in acetone. The concentration of 100 g CLA-Me/1200 ml acetone was the most promising for the separate these isomers rather than other concentration (100 g CLA-Me/600 ml or 100 g CLA-Me/2400 ml) (data not included in this paper).

Most impurities, such as saturated fatty acids, oleic acid, oxidation by-products, *trans,trans*-CLA isomers and *cis,cis*-CLA isomers contained in the *trans*-10,*cis*-12 CLA-Me fractions obtained from a low-temperature precipitation were completely removed by the urea treatment. However, *cis,cis*-CLA isomer, contained in the *cis*-9,*trans*-11 CLA-Me fraction was not removed. Since the linoleic acid sample used in this study contained relatively small amount of impurities, only single urea treatment was enough to remove most of the impurities; however, if the linoleic acid sample contains high amount of impurities, especially saturated fatty acids, two or three times of the urea treatment would be necessary to remove most of the impurities.

In conclusion, the *cis*-9,*trans*-11 CLA-Me (supernatant) from *trans*-10,*cis*-12 CLA-Me (precipitate) were separated at -68°C and -71°C from synthetic CLA-Me dissolved in acetone (100 g/1200 ml). The purities of the *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me were 87.3 and 92.5%, respec-

tively. These two isomers were further purified in ethanol (100 g/3000 ml) containing 200 g urea by refluxing for one hr, followed by storing at 4°C for one day, resulting in highly pure *cis*-9,*trans*-11 CLA-Me (90.3%) and *trans*-10,*cis*-12 CLA-Me (99.9%). This method simply and inexpensively produces highly pure and relatively large amount of *cis*-9,*trans*-11 CLA-Me and *trans*-10,*cis*-12 CLA-Me.

ACKNOWLEDGEMENTS

This study was supported by Interdisciplinary Research Grant of KOSEF (1999-2-220-004-5).

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(Received March 14, 2000)