A Novel Method for Solvent Fractionation of Two CLA Isomers

Hidetaka Uehara[a,*], Tomomi Suganuma[a], Satoshi Negishi[a], Satoru Ueno[b], and Kiyotaka Sato[b]

[a]Research Laboratory of Nisshin OilliO Group, Ltd., Yokosuka 239-0832, Japan, and
[b]Faculty of Applied Biological Science, Hiroshima University, Higashi-Hiroshima 739-8528, Japan

ABSTRACT: Conjugated linoleic acid (CLA) is commercially available as a mixture consisting of almost equal amounts of the *cis*-9,trans-11-CLA (*c*9,*t*11) and *trans*-10,*cis*-12-CLA (*t*10,*c*12) isomers. Separation of the two isomers is highly significant since each exhibits different biochemical properties. Highly efficient separation could be accomplished by crystallization in acetone (solvent) of the two CLA isomers (solutes) in the presence of medium-chain fatty-acid (MCFA) additives. The relative concentration ratios of the two CLA isomers in the solvent-crystallized materials varied depending on which MCFA were added. Addition of lauric and decanoic acids resulted in the crystals predominantly containing *t*10,*c*12, whereas octanoic acid yielded those predominantly containing *c*9,*t*11. We have confirmed that one-time solvent crystallization using decanoic acid and octanoic acid additives increased the *t*10,*c*12 and *c*9,t11 concentrations, and that repeated solvent crystallization resulted in the ratio of *c*9,*t*11 to *t*10,*c*12 of at least 4:96 or 98:2.

Paper no. J11243 in *JAOCS 83*, 261–267 (March 2006).

KEY WORDS: Conjugated linoleic acid, medium-chain fatty acid, solvent fractionation.

CLA is a collective term describing a mixture of positional and geometrical isomers of linoleic acid involving a conjugated double bond at various positions; it is one of the FA contained in meat and dairy products (1). CLA is manufactured industrially by alkali-induced conjugation of the linoleic acid-rich oils safflower oil and sunflower oil in the presence of propylene glycol and is obtained as a liquid mixture consisting of almost equivalent amounts of the isomers, *cis*-9,*trans*-11-CLA (*c*9,*t*11) and *trans*-10,*cis*-12-CLA (*t*10,*c*12) (2,3). CLA has various biochemical properties, such as a reduction in cancer incidence (4–6), a beneficial effect on atherosclerosis (7,8), a decrease in body fat content (9,10), and an improvement of immune function (11). Recently, it was reported that the *c*9,*t*11 isomer exhibited antitumor activity (12), whereas the *t*10,*c*12 isomer decreased body fat (13–15), increased energy expenditure (16), and suppressed the development of hypertension (17). These findings triggered investigations of isomer separation.

One of the well-known techniques to separate CLA isomers is the enzymatic (lipase) method (18–21). Lipase has high substrate specificity, and the isomers are separated because of the difference in the rate of enzymatic esterification or hydrolysis. For example, *Candida rugosa* lipase acted on *c*9,*t*11 more strongly than on *t*10,*c*12 (20,21). Although this is an excellent method to separate the isomers in terms of yield and purity (20,21), tedious tail-end procedures are required because the ester must be separated from the corresponding FA and alcohol after the enzymatic reaction, and a hydrolytic treatment is required again when the ester is used as a substrate. Another disadvantage of this method is that lipase is very expensive.

Although solvent crystallization has generally been used to purify fats and FA (22), conventional solvent fractionation methods were not applied to separate the two isomers because there may be little difference between the crystallization behaviors of the two isomers in the solution phase owing to subtle differences in solubility values of the two isomers. However, we found that the CLA isomers in an acetone solution could be separated efficiently by solvent fractionation in the presence of medium-chain FA. We wished to cause specific molecular interactions between CLA isomers and additives containing a –COOH group at one end and a short hydrocarbon chain at the other. The –COOH group interacts with the –COOH group of CLA, whereas the short chain interacts with the chain segment of the CLA between the –COOH group and the *cis/trans* double bond. For this reason, we chose MCFA additives, since similarity in chain length of the MCFA and the chain segments made of –COOH, hydrocarbon chain, and double bonds of the CLAs may cause specific molecular interactions. The present paper reports the effects of the chain length, content of MCFA, and temperature of crystallization on the efficiency of isomer separation in the fractionation processes.

EXPERIMENTAL PROCEDURES

Materials. Commercially available CLA80HG (Nisshin OilliO Group, Ltd., Tokyo, Japan) was used as a starting CLA material. Octanoic (purity >99%), decanoic (purity >99%), lauric (purity >99%), myristic (purity >98%), and palmitic (purity >95%) acids, which were used as the additives in the solvent crystallization, and 14% methanolic boron trifluoride solution were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acetone (purity >99.5%), hexane (purity >96%), toluene (purity >99.5%), and sodium chlorate (purity >99.5%) were purchased from Kanto Chemical Co., INC. (Tokyo, Japan).

Precrystallization of CLA sample. CLA80HG contains *c*9,*t*11 (39.1%), *t*10,*c*12 (40.2%), other CLA isomers (2.6%), palmitic acid (5.0%), stearic acid (1.6%), oleic acid (8.9%), and

*To whom correspondence should be addressed at Research Laboratory of Nisshin OilliO Group, Ltd., 1-Banchi, Shinmei-cho, Yokosuka-shi, Kanagawa 239-0832 Japan. E-mail: h-uehara@nisshin-oillio.com

unknown (2.6%). Crystallization with acetone was therefore performed to reduce the content of saturated FA (precrystallization). CLA80HG (3,500 g) was dissolved in a volume of acetone of the same weight (4,425 mL). The solution was placed in a 10-L flask, and the mixture was cooled overnight under stirring in a programmed low-temperature thermostatic bath (TRL-P135; Thomas Kagaku Co., Ltd., Tokyo, Japan). The temperature of the bath was adjusted so that the liquid temperature gradually decreased to −15°C. The precipitated crystals and supernatant liquid were separated by filtration under reduced pressure by aspiration, and acetone in the crystals and the supernatant liquid was evaporated under reduced pressure.

Solvent fractionation for separation of CLA isomers. Small-scale and large-scale solvent fractionations were carried out. The former experiments were done to search for optimal crystallization conditions to obtain better separation efficiency at three typical ratios of CLA-mix and FA additives (FA): CLA-mix/FA = 8:2, 5:5, and 2:8. The latter experiments were done to find specific combinations of CLA-mix and MCFA with different CLA-mix/MCFA concentration ratios in accordance with the results of the small-scale experiments.

We used a 100-mL sample bottle with a stopper for the small-scale experiments. A total of 10 g of CLA-mix/MCFA mixture with different concentration ratios was mixed with 38 mL (30 g) of acetone and stirred in the container at 50°C. The sample bottles were placed in a cooling thermostatic bath (SYS05009; Tokyo Rika-kikai Co., Ltd., Tokyo, Japan) overnight without stirring. The bath was adjusted to a specific cooling temperature for every combination of CLA-mix and FA so that the yield of isolated crystals was 20 to 30% for every CLA-mix/FA mixture. The crystallized samples and the supernatant liquid were separated by filtration under reduced pressure by aspiration. Filtration was carried out in a low-temperature thermostatic chamber (PG-2KP, Espec Corp., Osaka, Japan). Filtration was done at the same temperature of cooling. Acetone in the crystals and supernatant liquid was evaporated under reduced pressure.

In the large-scale experiments, we used a 1-L three-necked flask in which a total of 100 g of CLA-mix/FA mixture at different concentration ratios was mixed with 379 mL (300 g) of acetone. The flask containing the test sample mixture was placed in the programmed low-temperature thermostatic bath and cooled under stirring. The cooling temperature was chosen so that every CLA-mix and MCFA combination could yield optimal fractionation efficiency. The crystallized samples and the supernatant liquid were separated in the same manner as that used in the small-scale experiment.

Method for analysis of CLA isomers. CLA isomers were analyzed by GC after treatment with boron trifluoride for methyl esterification (23).

To 25 mg of the test sample were added 1 mL of toluene and 2 mL of a methanolic boron trifluoride solution. The mixture was then heated to 40°C for 10 min to complete the reaction. After the reaction had been terminated by adding 3 mL of a saturated aqueous sodium chloride solution, the solution was cooled in an ice-water bath. The resulting FAME were extracted with 2 mL of hexane. The hexane extract was dried with sodium sulfate.

CLA methyl esters were analyzed on a Shimadzu model GC-2010 gas chromatograph (Kyoto, Japan) equipped with an FID. Capillary columns DB-23 (0.25 mm × 30 m; Agilent Technologies, Inc., Palo Alto, CA) were used for the analysis. The column temperature was raised from 130 to 220°C at a rate of 2°C/min, and the final temperature was maintained for 3 min. The temperature of the column inlet and detector was set at 250°C. The carrier gas was helium with a head pressure of 114.7 kPa. The split ratio was 1:100.

RESULTS AND DISCUSSION

After the precrystallization process, the CLA mixtures in the supernatant liquid contained *c*9,*t*11 (40.5%), *t*10,*c*12 (41.4%), and other CLA isomers (2.7%), palmitic acid (2.8%), stearic acid (0.7%), and oleic acid (9.8%); this supernatant liquid was called CLA-mix. The isomeric CLA content in the precipitated crystals was not greatly different from that in the solution (crystal yield 6.6%; FA composition 15.8% *c*,9,*t*11 and 19.6% *t*10,*c*12; isomer ratio *c*9,*t*11/*t*10,*c*12 = 45:55).

Figure 1 presents three typical data plots of the GC analyses of (i) starting CLA-mix sample, (ii) crystallized materials obtained by the solvent crystallization at –30°C using the CLA-mix and octanoic acid additive with a mixing ratio of 8:2, and (iii) crystallized materials obtained by the solvent crystallization at –32°C using the CLA-mix and decanoic acid with a mixing ratio of 5:5, the latter two of which were obtained in the small-scale experiments. The peaks at retention times around 27 min correspond to the two CLA isomers. In Figure 1A, the starting CLA-mix sample had two peaks with almost the same height, indicating almost equal amounts of the two CLA isomers. In addition, the peaks for palmitic, stearic, and oleic acids appear at retention times of 15 to 23 min. It is notable that the *c*9,*t*11 peak was higher than that of *t*10,*c*12 when octanoic acid was added, as shown in Figure 1B, whereas the opposite result was observed in Figure 1C in which decanoic acid was added. In both cases, the GC peaks of palmitic acid, oleic acid, and the added MCFA appeared at the appropriate times according to previously reported times for standards. Relative concentration ratios of the two CLA isomers were calculated from the corresponding GC peak areas.

Table 1 shows the results of the small-scale experiments carried out with CLA-mix/FA ratios of (i) 8:2, (ii) 5:5, and (iii) 2:8. The experiments were repeated twice for every condition, and the average values are listed in Table 1. At the 8:2 CLA-mix/FA ratio (Table 1, part a), the decanoic and palmitic acid additives did not change the concentration ratios of *c*9,*t*11 to *t*10,*c*12, but the myristic and lauric acid additives changed the concentration ratios of *c*9,*t*11 to *t*10,*c*12 toward a *t*10,*c*12-rich direction. The addition of octanoic acid, however, remarkably increased the concentration of *c*9,*t*11, in contrast with the other four FA additives. With the CLA-mix/FA ratio of 5:5 (Table 1,

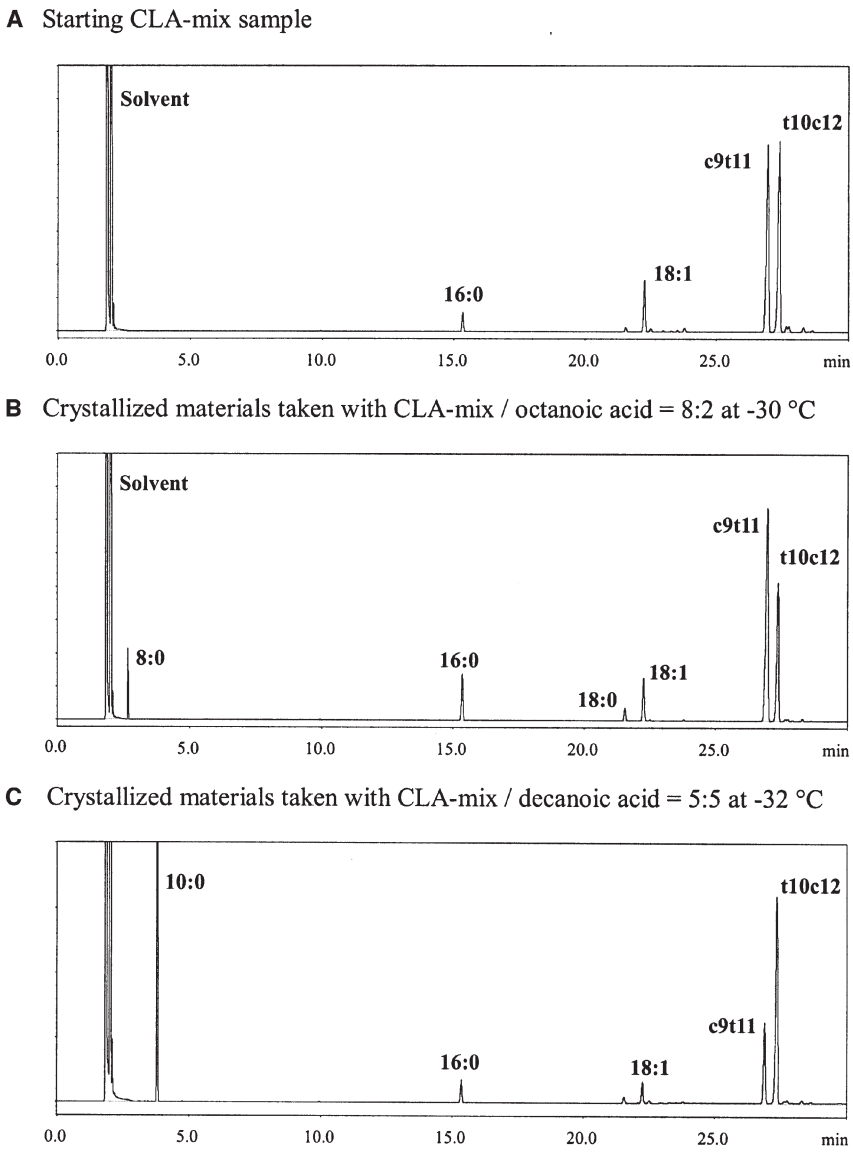


FIG. 1. Three typical data plots of the GC analyses of (A) starting CLA-mix sample, (B) crystallized materials taken with CLA-mix/octanoic acid = 8:2 at –30°C, (C) crystallized materials taken with CLA-mix/decanoic acid = 5:5 at –32°C.

part b), adding decanoic acid increased the concentration of *t*10,*c*12 at the expense of *c*9,*t*11. However, the additions of lauric, myristic, and palmitic acids did not change the *c*9,*t*11/*t*10,*c*12 ratio. Adding octanoic acid slightly increased the relative concentration of *c*9,*t*11, but the change was not as pronounced as that observed in Table 1, part a. Table 1, part c indicates that the *c*9,*t*11/*t*10,*c*12 concentration ratio did not change with the lauric, myristic, and palmitic acid additives.

The small-scale experiments summarized in Table 1 indicated that the separation efficiency of the two CLA isomers was most manifest for *c*9,*t*11 and *t*10,*c*12 with the additions of octanoic acid and decanoic acid, respectively. Large-scale solvent crystallization was then carried out under more precise conditions using the addition of these two acids.

Table 2 shows the effects of the addition of decanoic acid on the separation efficiency of the two CLA isomers carried out with CLA-mix/decanoic acid ratios of 8:2, 7:3, 6:4, 5:5, 4:6, 3:7 and 2:8 in the large-scale experiments. A *c*9,*t*11/*t*10,*c*12 ratio of 22:78 was obtained at CLA-mix/decanoic acid ratios of 6:4, 5:5, and 4:6. At the same time, the *t*10,*c*12 recovery yields were larger for the CLA-mix/decanoic acid ratios of 6:4, 5:5, and 4:6 than at the other ratios. Considering these results together with those of the small-scale examination, we concluded that the isomer ratio of the solvent-crystallized materials depended on the ratio of decanoic acid to CLA-mix.

Table 3 presents the results of solvent fractionation using the octanoic acid additive; the CLA-mix/octanoic acid ratios were 9:1, 85:15, 8:2, and 7:3. We did not perform experiments

TABLE 1
Effects of the Chain Length of Added FA (small-scale experiments)

	FA additives				
	Octanoic	Decanoic	Lauric	Myristic	Palmitic
(a) CLA-mix/FA = 8:2					
Cooling temperature (°C)	–30	–30	–29	–18	6
Yield (%)	21.3	35.4	13.8	17.5	13.8
MCFA (%)	3.1	17.3	41.8	47.6	68.2
*c*9,*t*11-CLA (%)	53.2	34.5	13.4	16.1	11.6
*t*10,*c*12-CLA (%)	25.0	34.2	27.4	21.7	12.2
*c*9,*t*11/*t*10,*c*12 ratio	68:32	50:50	33:67	43:57	49:51
(b) CLA-mix/FA = 5:5					
Cooling temperature (°C)	–42	–35	–10	6	21
Yield (%)	17.2	35.1	11.8	19.9	21.3
MCFA (%)	22.8	51.1	59.6	86.8	82.6
*c*9,*t*11-CLA (%)	35.3	10.9	14.4	4.0	6.5
*t*10,*c*12-CLA (%)	26.2	29.8	17.0	4.7	6.8
*c*9,*t*11/*t*10,*c*12 ratio	57:43	27:73	46:54	46:54	49:51
(c) CLA-mix/FA = 2:8					
Cooling temperature (°C)	—[a]	–20	0	15	29
Yield (%)	—	25.1	39.1	42.0	36.1
MCFA (%)	—	90.5	91.9	92.8	91.7
*c*9,*t*11-CLA (%)	—	3.0	2.8	2.3	2.8
*t*10,*c*12-CLA (%)	—	4.5	3.3	2.6	2.9
*c*9,*t*11/*t*10,*c*12 ratio	—	40:60	46:54	47:53	49:51

[a]Data not available for –42°C. MCFA, medium-chain FA.

TABLE 2
Effects of the Ratio of CLA-mix/Decanoic Acid (large-scale experiment)

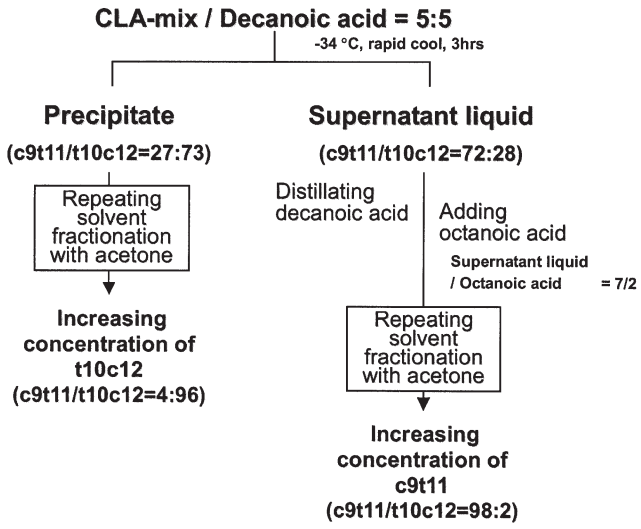
	Ratios of CLA-mix/decanoic acid						
	8:2	7:3	6:4	5:5	4:6	3:7	2:8
Cooling temperature (°C)	–32	–32	–31	–32	–29	–24	–20
Yield (%)	27.6	24.0	27.4	30.1	37.6	22.2	29.0
Decanoic acid (%)	17.3	31.8	36.7	52.3	70.1	83.6	90.2
*c*9,*t*11-CLA (%)	33.2	13.5	11.4	8.5	5.3	4.1	3.0
*t*10,*c*12-CLA (%)	32.8	40.8	40.8	30.9	19.2	8.3	4.6
*c*9,*t*11/*t*10,*c*12 ratio	50:50	25:75	22:78	22:78	22:78	33:67	39:61
*t*10,*c*12 recovery yield (%)[a]	27	34	45	45	44	15	16

[a]The recovery yield was calculated as the amount of *t*10,*c*12 contained in the crystals relative to that contained in the test sample (CLA-mix/decanoic acid mixture).

TABLE 3
Effects of the Ratio of CLA-mix/Octanoic Acid (large-scale experiment)

	Ratios of CLA-mix/octanoic acid			
	9:1	85:15	8:2	7:3
Cooling temperature (°C)	–24	–26	–28	–30
Yield (%)	20.5	38.2	49.3	35.4
Decanoic acid (%)	3.0	5.6	10.8	14.0
*c*9,*t*11-CLA (%)	56.1	45.9	40.8	41.7
*t*10,*c*12-CLA (%)	18.0	32.0	33.3	29.4
*c*9,*t*11/*t*10,*c*12 ratio	76:24	59:41	55:45	59:41
*c*9,*t*11 recovery yield (%)[a]	32	51	62	52

[a]The recovery yield was calculated as the amount of *c*9,*t*11 contained in the crystals relative to that contained in the test sample (CLA-mix/octanoic acid mixture).



SCHEME 1

using concentrations of octanoic acid higher than 40% because efficient fractionation was not expected based on the results in Table 3 and because the solvent crystallization temperature was decreased below –40°C, which is not practical. We found that

TABLE 4
Effects of Cooling Temperature

	Cooling temperature	
	–32°C	–34°C
Yield of isolated crystals (%)	30.1	52.5
Decanoic acid (%)	52.3	53.2
*c*9,*t*11-CLA (%)	8.5	10.8
*t*10,*c*12-CLA (%)	30.9	28.5
*c*9,*t*11/*t*10,*c*12 ratio in crystals	22:78	27:73
*t*10,*c*12 recovery yield in crystals (%)	45	72
*c*9,*t*11/*t*10,*c*12 ratio in supernatant liquid	61:39	72:28

the *c*9,*t*11 isomer increased at the expense of *t*10,*c*12 only with the CLA-mix/octanoic acid ratio of 9:1. Under this condition, the *c*9,*t*11/*t*10,*c*12 ratio varied between 55:45 and 76:24, when the starting material of the CLA mixture varied from one sample to the others. The minor components such as oleic acid or unknown polymerized substances included in the CLA mixture may thus disturb the solvent fractionation.

Finally, Table 4 presents the effects of final temperature of the cooling procedure on the separation efficiency, using decanoic acid at a CLA-mix/decanoic acid ratio of 5:5. The yield of isolated crystals increased from 30 to 53%, and the

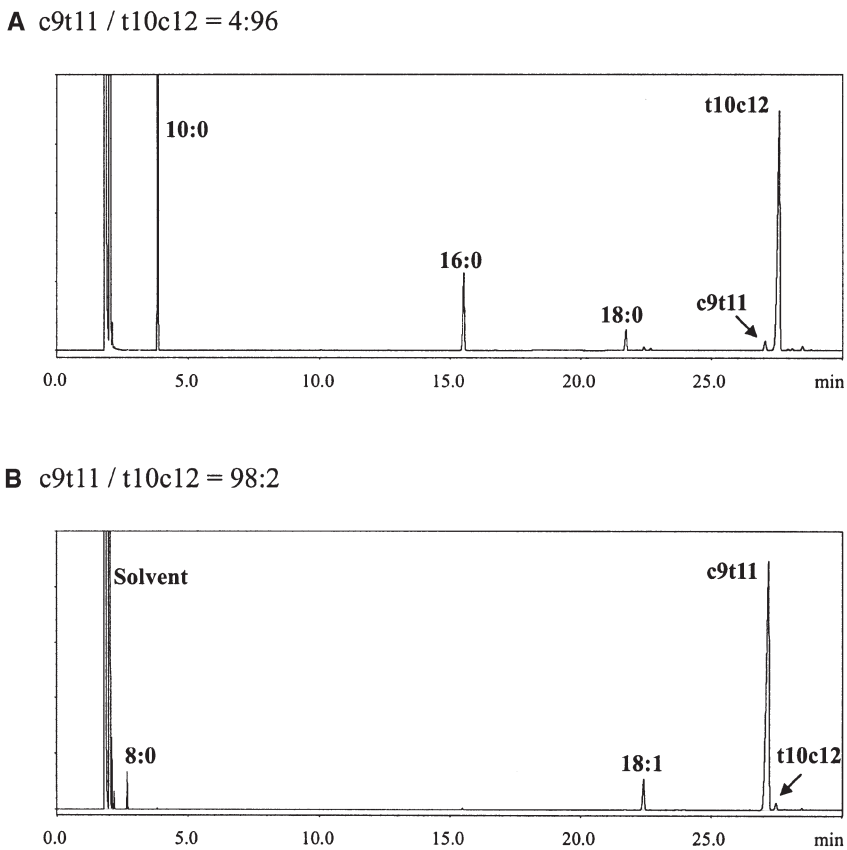


FIG. 2. GC analysis data plots with the c9,t11/t10,c12 ratio of 4:96 (A) and 98:2 (B).

*c*9,*t*11/*t*10,*c*12 ratio changed from 22:78 to 27:73 when the final temperature decreased from −32 to −34°C. The *t*10,*c*12 recovery yield increased from 45 to 72% because of the increased yield of isolated crystals. As a result, the *c*9,*t*11/*t*10,*c*12 ratio in the supernatant liquid increased from 61:39 to 72:28 as the final temperature was decreased. This indicates that the rate of solvent crystallization was increased by decreased final temperature, influencing the extent of the crystallized materials and relative concentration ratio of the two CLA isomers.

Based on these experimental results, we concluded that the optimal conditions for efficient fractionation of the two CLA isomers were decanoic and octanoic acids as the additives and a CLA-mix/decanoic acid ratio of 5:5. Scheme 1 depicts a typical example of continuous solvent fractionation processes. After the first solvent fractionation at −34°C, the *c*9,*t*11/*t*10,*c*12 ratios are 27:73 in the precipitate and 72:28 in the supernatant liquid. The precipitate is subjected to further solvent fractionation by adding acetone. It is worth noting that the optimal fractionation temperature increases with increasing concentration of *t*10,*c*12 from −27 (first repeat) to −26°C (second repeat). The *c*9,*t*11/*t*10,*c*12 concentration ratio became 4:96 when solvent fractionation was repeated twice, as evidenced by the GC analysis results in Figure 2A. Decanoic acid was removed from the supernatant liquid by distillation at 100 to 150°C, and octanoic acid was added to the distilled supernatant liquid at ambient temperature at a supernatant liquid/octanoic acid ratio of 7:2. Further solvent fractionation at −30°C then changed the *c*9,*t*11/*t*10,*c*12 concentration ratio from 72:28 (initial), to 90:10 (first repeat) and 98:2 (second repeat), as evidenced by the GC analysis results in Figure 2B.

A unique property of the solvent fractionation reported in the present work is the use of additives in the solvent crystallization of CLA isomers that cannot be separated by the more usual methods without the additives because of similarity in their solubilities. The MCFA additives can easily be distilled by commonly used techniques. Starting from material with the same additive (decanoic acid), *t*10,*c*12 was rich in the precipitate and *c*9,*t*11was rich in the supernatant liquid after the initial solvent fractionation, as demonstrated in Scheme 1 and Table 4. Further solvent fractionation of the precipitate (supernatant liquid) can purify *t*10,*c*12 (*c*9,*t*11), and eventually it is easy to achieve *c*9,*t*11/*t*10,*c*12 ratios of either 1:99 or 99:1. Based on the findings that the *t*10,*c*12 isomer crystallized concurrently with MCFA and that the *c*9,*t*11 isomer crystallized without a concomitant crystallization of FA, we speculate that MCFA, in particular decanoic acid, interact more strongly with the *t*10,*c*12 isomer than with the *c*9,*t*11 isomer. It is also possible that the position of the *cis*-double bond in the CLA isomers may greatly affect isomer separation because the isomer composition of the deposited crystals depended on the chain length of the added FA.

REFERENCES

1. Kepler, C.R., K.P. Hirons, J.J. MacNeill, and S.B. Tove, Intermediates and Products of the Biohydrogenation of Linoleic Acid by *Butyrovibrio fibrisolvens, J. Biol. Chem. 241*:1350–1354 (1966).
2. Rainer, L., and C.J. Heiss, Conjugated Linoleic Acid: Health Implications and Effects on Body Composition, *J. Am. Diet Assoc. 104*:963–968 (2004).
3. Nagao, K., and T. Yanagita, Conjugated Fatty Acids in Food and Their Health Benefits, *J. Biol. Chem. 100*:152–157 (2005).
4. Pariza, M.W., CLA, a New Cancer Inhibitor in Dairy Products, *Bull. Int. Dairy Fed. 257*:29–30 (1991).
5. Ip, C., S.F. Chin, J.A. Scimeca, and M.W. Pariza, Mammary Cancer Prevention by Conjugated Dienoic Derivatives of Linoleic Acid, *Cancer Res. 51*:6118–6124 (1991).
6. Ha, Y.L., N.K. Grimm, and M.W. Pariza, Anticarcinogens from Fried Ground Beef: Heat-Altered Derivatives of Linoleic Acid, *Carcinogenesis 8*:1881–1887 (1987).
7. Lee, K.N., D. Kritchevsky, and M.W. Pariza, Conjugated Linoleic Acid and Atherosclerosis in Rabbits, *Atherosclerosis 108*:19–25 (1994).
8. Nicolosi, R.J., E.J. Rogers, D. Kritchevsky, J.A. Scimeca, and P.J. Huth, Dietary Conjugated Linoleic Acid Reduces Plasma Lipoproteins and Early Aortic Atherosclerosis in Hypercholesterolemic Hamsters, *Artery 22*:266–277 (1997).
9. Park, Y., K.J. Albright, W. Liu, J.M. Storkson, M.E. Cook, and M.W. Pariza, Effect of Conjugated Linoleic Acid on Body Composition in Mice, *Lipids 32*:853–858 (1997).
10. Ostrowska, E., M. Muralitharan, R.F. Cross, D.E. Bauman, and F.R. Dunshea, Dietary Conjugated Linoleic Acids Increase Lean Tissue and Decrease Fat Deposition in Growing Pigs, *J. Nutr. 129*:2037–2042 (1999).
11. Sugano, M., A. Tsujita, M. Yamasaki, M. Noguchi, and K. Yamada, Conjugated Linoleic Acid Modulates Tissue Levels of Chemical Mediators and Immunoglobulins in Rats, *Lipids 33*:521–527 (1998).
12. Ha, Y.L., J.M. Storkson, and M.W. Pariza, Inhibition of Benzo(a)pyrene-Induced Mouse Forestomach Neoplasia by Conjugated Dienoic Derivatives of Linoleic Acid, *Cancer Res. 50*:1097–1101 (1990).
13. Park, Y., K.J. Albright, J.M. Storkson, W. Liu, M.E. Cook, and M.W. Pariza, Changes in Body Composition in Mice During Feeding and Withdrawal of Conjugated Linoleic Acid, *Lipids 34*:243–248 (1999).
14. Park, Y., K.J. Albright, J.M. Storkson, W. Liu, and M.W. Pariza, Evidence That the *trans*-10,*cis*-12 Isomer of Conjugated Linoleic Acid Induces Body Composition Changes in Mice, *Ibid. 34*:235–241 (1999).
15. De Deckere, E.A., J.M. Van Amelsvoort, G.P. McNeill, and P. Jones, Effect of Conjugated Linoleic Acid (CLA) Isomers on Lipid Levels and Peroxisome Proliferation in the Hamster, *Br. J. Nutr. 82*:309–317 (1999).
16. Nagao, K., Y.-M. Wang, N. Inoue, S.-Y. Han, Y. Buang, T. Noda, N. Kouda, H. Okamatsu, and T. Yanagita, The 10*trans*,12*cis* Isomer of Conjugated Linoleic Acid Promotes Energy Metabolism in OLETF Rats, *Nutrition 19*:652–656 (2003).
17. Nagao, K., N. Inoue, Y.-M. Wang, J. Hirata, Y. Shimada, T. Nagao, T. Matsui, and T. Yanagita, The 10*trans*,12*cis* Isomer of Conjugated Linoleic Acid Suppresses the Development of Hypertension in Otsuka Long–Evans Tokushima Fatty Rats, *Biochem. Biophys. Res. Commun. 306*:134–138 (2003).
18. Haas, M.J., J.K.G. Kramer, G. McNeill, K. Scott, T.A. Foglia, N. Sehat, J. Fritsche, M.M. Mossoba, and M.P. Yurawecz, Lipase-Catalyzed Fractionation of Conjugated Linoleic Acid Isomers, *Lipids 34*:979–987 (1999).
19. McNeill, G.P., C. Rawlins, and A.C. Peilow, Enzymatic Enrichment of Conjugated Linoleic Acid Isomers and Incorporation into Triglycerides, *J. Am. Oil Chem. Soc. 76*:1265–1268 (1999).
20. Nagao, T., Y. Shimada, Y. Yamauchi-Sato, T. Yamamoto, M. Kasai, K. Tsutsumi, A. Sugihara, and Y. Tominaga, Fractionation

and Enrichment of CLA Isomers by Selective Esterification with *Candida rugosa* Lipase, *Ibid*. *79*:303–308 (2002).
21. Yamauchi-Sato, Y., T. Nagao, T. Yamamoto, T. Terai, A. Sugihara, and Y. Shimada, Fractionation of Conjugated Linoleic Acid Isomers by Selective Hydrolysis with *Candida rugosa* Lipase, *J. Oleo Sci. 52*:367–374 (2003).
22. Krishnamurthy, R., and M. Kellens, Fractionation and Winterization, in *Bailey's Industrial Oil & Fat Products*, 5th edn., Vol. 4, edited by Y.H. Hui, John Wiley & Sons, New York, 1996, pp. 301–337.
23. Kamegai, T., M. Kasai, and I. Ikeda, Improved Method for Preparation of the Methyl Ester of Conjugated Linoleic Acid, *J. Oleo Sci. 50*:237–241(2001).

[Received September 23, 2005; accepted December 7, 2005]