# A Recommended Esterification Method for Gas Chromatographic Measurement of Conjugated Linoleic Acid

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**ABSTRACT:** The effect of the methylation method on isomerization of conjugated linoleic acid (CLA) in gas chromatographic analysis was studied. Among methylation methods examined, the magnitude of isomerization of CLA was greatest with $BF_3$ catalyst, followed by HCl and $H_2SO_4$ catalyst. Short-time methylation did not extensively change the CLA composition in all methods, and *c,t* and *t,c* isomers were essentially maintained, while the appearance of *t,t* isomers and unknown peaks was practically restricted. After 120 min of methylation, there was essentially no conversion in the $H_2SO_4$ method, in contrast to a marked change in the $BF_3$ method. The antioxidants butylated hydroxytoluene, ascorbic acid, β-carotene, and α-tocopherol did not suppress conversion, while dimethylsulfoxide (DMSO) and dimethylformamide (DMF) attenuated the changes in CLA composition. Suppression was more effective in the $H_2SO_4$ method than in the $BF_3$ method. Thus, methylation with $H_2SO_4$ in the presence of a proper amount of DMSO or DMF is recommended for esterification of CLA.

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**KEY WORDS:** $BF_3$, conjugated linoleic acid, gas chromatography, HCl, $H_2SO_4$, isomerization, methylation.

Conjugated linoleic acid (CLA) is a generic term for geometric and positional isomers of octadecadienoic acid. Linoleic acid (LA) has double bonds at the 9- and 12-positions with *cis* configuration, while CLA is a mixture of various octadecadienoic acids with a conjugated double bond in *cis* or *trans* configuration, such as 9*c*,11*c*; 10*c*,12*c*; 9*t*,11*t*; 10*t*,12*t*; 9*t*,11*c*; 9*c*,11*t*; 10*c*,12*t*; and 10*t*,12*c*. Recently, it was shown that 8,10 and 11,13 isomers of CLA were found in French cheeses (1). CLA exists mainly in foodstuffs of ruminant origin, such as milk, cheese, yogurt, and beef (2,3). It is also found in human milk (4). The major isomer of CLA present in these foodstuffs is 9*c*,11*t* isomer while others constitute only a small percentage of total CLA (5).

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Recently, the powerful anticarcinogenic effect of CLA has gained attention, especially for the prevention of mammary cancer. Ip *et al.* (6) reported that feeding diets with CLA at doses as low as 0.5% inhibited 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary carcinogenesis, and that only the 9*c*,11*t* isomer was incorporated into the liver (7) at a higher rate in neutral lipids than in phospholipids (8). In addition, CLA has diverse physiological effects, such as antiatherogenic activity (9), reduction of prostaglandin $E_2$ production (10), stimulation of interleukin-2 production and proliferation of mouse lymphocytes (11), regulation of immunoglobulin production (12), and reduction of body fat (13). However, there is little information on the mechanism by which CLA exerts these physiological effects. It is also unknown which isomer is the active principle. To analyze such issues, establishment of a method for accurate analysis of CLA is a prerequisite.

Gas chromatography (GC) is routinely used for the analysis of fatty acids because of its rapidity, high resolution, and sensitivity (14). For GC analysis, fatty acids should be converted to more nonpolar and volatile derivatives, such as fatty acid methyl esters, and $BF_3$ is one of the most popular methylating agents.

Werner *et al.* (15) reported that CLA endured conversion from *c,t* or *t,c* isomers to *t,t* isomers or unknown substances during the $BF_3$ methylation procedure. A similar phenomenon also was reported for oleic acid (16), and artifacts were produced from some lipids during $BF_3$ methylation (17). Recently, a method for identification of CLA by silver-ion high-performance liquid chromatography was presented (18,19). This method can separate isomers efficiently, but it is not easy. GC analysis is still a practical method, but optimization of esterification is required. Esterification of free fatty acids is complete in an hour. However, transesterification of triglycerides needs 2 h to complete the reaction. Because fatty acids in foodstuffs exist as triglycerides, it is necessary to examine the influence of the methylation method on the composition of CLA.

In the present study, we examined the effect of the methylation method on the composition of CLA to establish accurate GC analysis.

## MATERIALS AND METHODS

*Reagents.* CLA was prepared either from linoleic acid (LA) with a purity of more than 99% (Sigma, St. Louis, MO) or from a fatty acid mixture from high-linoleic safflower oil (Linoru Oil, Nagoya, Japan). For esterification, $BF_3$–methanol complex (Tokyo Kasei Industry, Tokyo, Japan), HCl (Wako Pure Chemicals, Osaka, Japan), and $H_2SO_4$ (Nacalai Tesque, Kyoto, Japan) were used for GC analysis. Butylated hydroxytoluene (BHT, analytical grade) (Sigma), ascorbic acid (AsA) (Wako Pure Chemicals), β-carotene (ICN Biomedicals, Aurora, OH), and α-tocopherol (Toc) (Eizai, Tokyo, Japan) were used as antioxidants. Dimethylsulfoxide (DMSO) and dimethylformamide (DMF) (Sigma, analytical grade) were used to inhibit the conversion of CLA. Other reagents were of analytical grade.

*Preparation of CLA*. CLA was prepared by the method of Ip *et al*. (6). Sodium hydroxide (1.5/g) and 29 g ethyleneglycol were placed in a three-necked flask, and 5.0 g of LA was dissolved. The solution was then heated for 2 h at 180°C under a $N_2$ stream and cooled to room temperature. The pH was then adjusted to 4.0 with 1 N HCl. CLA was extracted two times with 50 mL hexane from the reaction mixture. The hexane layer was washed with 25 mL of 5% NaCl solution to remove impurities. After removing water with 3 Å molecular sieves, the solution was filtered through a Buchner funnel. The hexane was removed in a rotary vacuum evaporator, and the sample was kept at −30°C until analyzed.

*Methylation procedure*. For the methylation of CLA, three commonly used methods were adopted. The $BF_3$ method used was that of Morrison and Smith (20). The $BF_3$–methanol complex was added to a final $BF_3$ concentration of 14%, and the solution was heated on a dry block at 70°C, shaken every 30 min, and cooled to room temperature. To know the effect of antioxidants on isomerization of CLA, methylation was performed in the presence of 1 mM antioxidants. Lipids extracted with hexane were washed with distilled water, followed by centrifugation at 1,000 × *g* for 5 min. The hexane layer was used for GC analysis. Methylation, either with HCl or with $H_2SO_4$, was performed by the method of Stoffel *et al*. (21) or Luddy *et al*. (22), respectively. In these methods, 5% HCl or 0.87% $H_2SO_4$ dissolved in methanol was used and heated at 100°C, respectively. CLA was extracted by the procedure described in the $BF_3$ method.

*Analysis of fatty acid composition.* Fatty acid methyl esters were analyzed with a GC-17A (Shimadzu, Kyoto, Japan) and a Supelco wax-10 fused-silica capillary column (Supelco, Bellefonte, PA) (60 m × 0.32 mm i.d.). The column temperature was kept at 150°C for 2 min, then raised to 220°C at a rate of 3°C/min and held for 15 min. Identification of individual CLA isomers was done by comparing the equivalent chain lengths (ECL) (5) with GC–mass spectrometry (GC–MS) (JEOL Auto MS 50, Tokyo, Japan).

*Statistical analysis.* Data were analyzed using Student's *t*-test in each experiment.

## RESULTS AND DISCUSSION

*Effect of methylation method and reaction time.* To establish the most suitable methylation method for CLA analysis, CLA was methylated by three different methods with $BF_3$, $H_2SO_4$, or HCl as the catalyst. In the $BF_3$ method, there was a time-dependent change in the CLA composition. Thus, during heating for 5 min, *c,t* and *t,c* isomers constituted more than 80%, while the other isomers were below 3% (Fig. 1). When reacted for 60 min, the proportion of *t,t* isomers markedly increased, and two unknown peaks appeared. GC–MS analysis of peaks 6 and 7 indicated that they were not CLA (data not shown). These response patterns were also observed in the other methods. The time courses of changes in CLA compositions for different methylation procedures are shown in Figure 2.

For the first 5 min of reaction, the CLA composition was essentially comparable among the three methods; the proportion of *c,t* and *t,c* isomers was above 80%, and unknown peaks were practically negligible. The most marked changes in proportions of CLA isomers were observed in the $BF_3$ method. Heating for 120 min resulted in a considerable increase in the *t,t* isomers, and they reached 36% of total CLA at the expense of *c,t* and *t,c* isomers, which were decreased to 39%. The percentage of unknown substances increased until 30 min of methylation and then reached a plateau. In the HCl method, few changes occurred until after 30 min of reaction; thereafter, drastic changes in CLA proportions were observed, and unknown substances were generated up to 60 min. The *t,t* isomers increased continuously throughout 120 min of methylation. The *t,t* isomers increased to 24% with a decrease in the total amount of *c,t* and *t,c* isomers to 55%. Changes in the CLA proportions in the $H_2SO_4$ method were relatively smaller than those observed in other methods. After

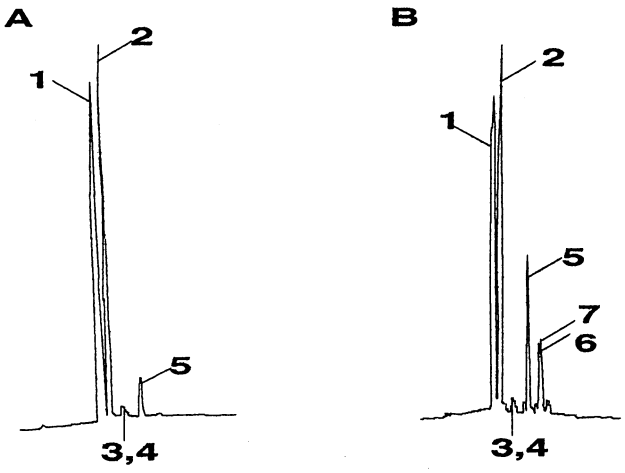


**FIG. 1.** Gas chromatogram of conjugated linoleic acid after 5 (A) or 60 (B) min of methylation by the $BF_3$ method. (1) 9*c*,11*t* and 9*t*,11*c*; (2) 10*c*,12*t* and 10*t*,12*c*; (3) 9*c*,11*c*; (4) 10*c*,12*c*; (5) 9*t*,11*t* and 10*t*,12*t*; (6) unknown peak 1; (7) unknown peak 2.

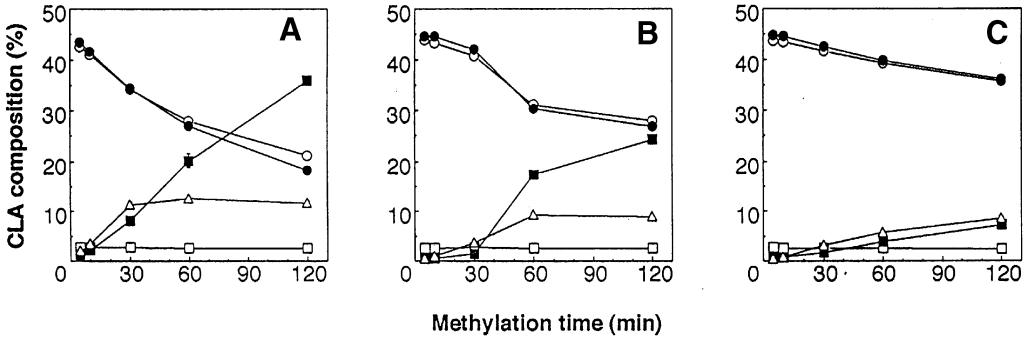


**FIG. 2.** Effect of methylation method and reaction time on conjugated linoleic acid (CLA) composition. Values are means ± SD for three measurements. –○– 9*c*,11*t* and 9*t*,11*c*; –●– 10*c*,12*t* and 10*t*,12*c*; –□– 9*c*,11*c* and 10*c*,12*c*; –■– 9*t*,11*t* and 10*t*,12*t*; –△– unknown peaks. (A) $BF_3$; (B) HCl; (C) $H_2SO_4$.

120 min of methylation, *t,t* isomers increased to 8%, while the sum of *c,t* and *t,c* isomers decreased to 71%. The unknown peaks also continuously increased during the methylation, but the total level observed in the $H_2SO_4$ method was lower than in the other two methods. However, the percentages of total CLA and of unknown peaks were not significantly different among the three different methods regardless of reaction time (data not shown). The minor component, the proportion of the *c,c* isomers, was constant in all methods.

*Methylation of CLA prepared from high-linoleic safflower oil.* The $BF_3$ and $H_2SO_4$ methods were used for the methylation of CLA prepared from the fatty acid mixture from high-linoleic safflower oil. After 60 min of reaction, unknown peaks represented 10% of the total fatty acids in the $BF_3$ method, while it was suppressed to 7% in the $H_2SO_4$ method (Fig. 3).

*Influence of antioxidants on CLA isomerization.* To avoid isomerization of CLA, four antioxidants (BHT, AsA, β-carotene, and Toc) were added to the reaction mixture at a concentration of 1 mM. Without antioxidants, obvious isomerization was observed after 120 min of methylation in all procedures employed. However, no detectable preventive effects of these antioxidants were seen in the compositional change of CLA (Fig. 4 ).

*Influence of DMSO and DMF on CLA isomerization.* DMSO and DMF were also tested to determine whether they could suppress isomerization of CLA. DMSO and DMF were added to the reaction mixture at the level of one-third of the reaction mixture. Both reagents exhibited a characteristic effect on the isomerization for the $BF_3$ and $H_2SO_4$ methods (Fig. 5A, B). The sum of *c,t* and *t,c* isomers increased, and *t,t* isomers or unknown peaks decreased as DMSO or DMF was added. At the highest concentration of DMSO or DMF em-

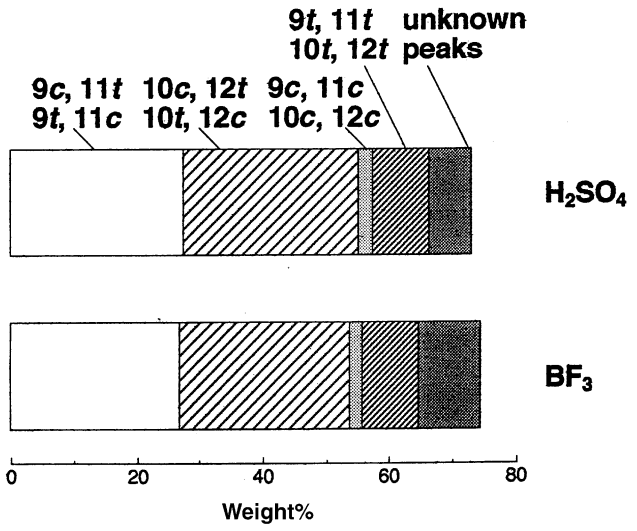


**FIG. 3.** Difference in CLA composition of alkali-isomerized safflower oil between $BF_3$ and $H_2SO_4$ methods. Reaction time was 120 min. See Figure 2 for abbreviation.

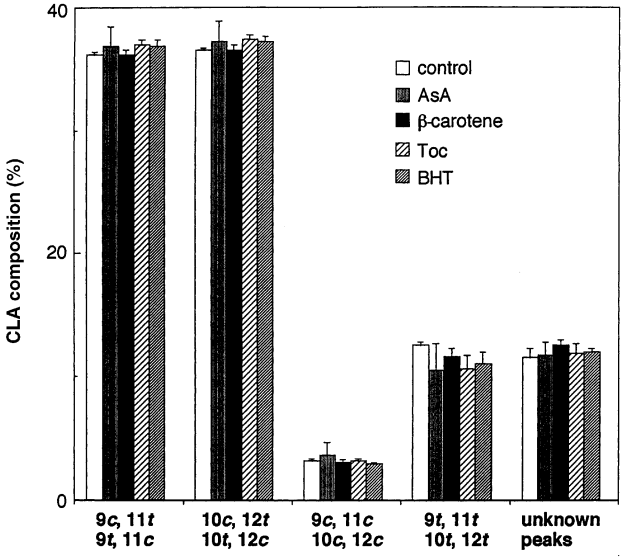


**FIG. 4.** Effect of antioxidants on isomerization of conjugated linoleic acid during methylation. Values are means ± SD for three measurements. Reaction time was 120 min. AsA, ascorbic acid; Toc, tocopherol; BHT, butylated hydroxytoluene. See Figure 2 for other abbreviation.

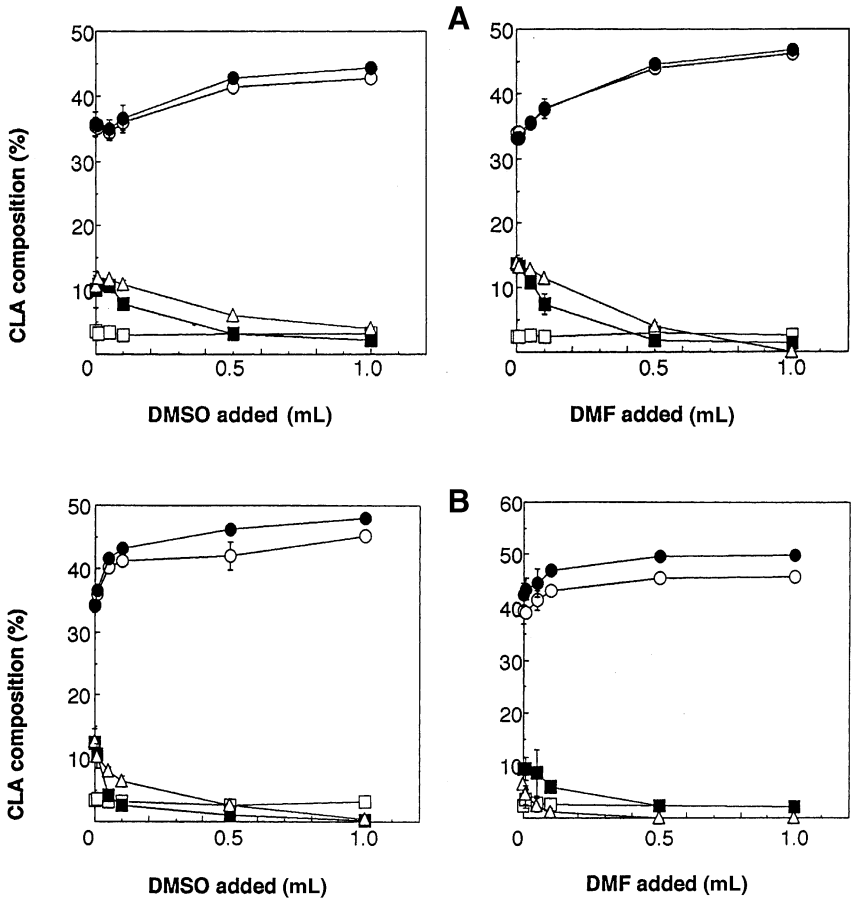


**FIG. 5.** (A) Effect of dimethylsulfoxide (DMSO) and dimethylformamide (DMF) on CLA composition during methylation catalyzed by $BF_3$. (B) Effect of DMSO and DMF on CLA composition during methylation catalyzed by $H_2SO_4$. Values are means ± SD for three measurements. –○– 9*c*,11*t* and 9*t*,11*c*; –●– 10*c*,12*t* and 10*t*,12*c*; –□– 9*c*,11*c* and 10*c*,12*c*; –■– 9*t*,11*t* and 10*t*,12*t*; –△– unknown peaks. See Figure 2 for other abbreviations.

ployed in the $BF_3$ method, the sum of *c,t* and *t,c* isomers was above 80%, while the *t,t* isomers and unknown peaks were below 5%, respectively (Fig. 5A). In the $H_2SO_4$ method with the highest level of DMSO or DMF, essentially no unknown peaks were detected (Fig. 5B).

*Influence of reaction solvent on CLA isomerization.* So far, we used methanol exclusively to prepare methyl esters of CLA. We also examined ethanol as a solvent to prepare CLA esters. When CLA ethyl esters were prepared by the $H_2SO_4$ method, the sum of *c,t* and *t,c* isomers was above 80%, whereas the concentration was 70% in the case of methyl esters after 120 min (Fig. 6). The *t,t* isomers and unknown peaks were below 5%, respectively.

In GC analysis, fatty acids are usually converted to stable volatile derivatives. Preparation of fatty acid esters is a simple procedure, and several procedures have been established (20–22). Preparation of fatty acid methyl esters by $BF_3$ is one of the most frequently adopted methods, but some artifacts are often produced during the esterification reaction (16,17,23,24).

For CLA, it was reported that some isomerization of *t,c* and *c,t* isomers to *t,t* isomers occurs, and methoxy artifacts are produced during methylation with $BF_3$ (15,25). This was confirmed in the present study. In this context, it was reported that a similar change was induced by iodine and light exposure (26). Thus, it is plausible that the change may be due to the presence of halogen atoms in $BF_3$.

However, the change occurred not only in the $BF_3$ method but also in the $H_2SO_4$ and HCl methods. These procedures were based on the method of Fischer-Speier (27), namely, esterification of acids by refluxing with excess alcohol in the presence of acid catalysts. The initial phase of the reaction is protonation of alcohol. There is an electron interaction between two double bonds of CLA because they are conjugated. Thus, there will be an interaction between a conjugated double bond and a protonated alcohol, which would not occur with nonconjugated double bonds.

2,2-Dimethoxypropane (2,2-DMP) is used to facilitate the transesterification of triglycerides (28). Though 2,2-DMP itself is condensed to produce yellow polymer, and this by-

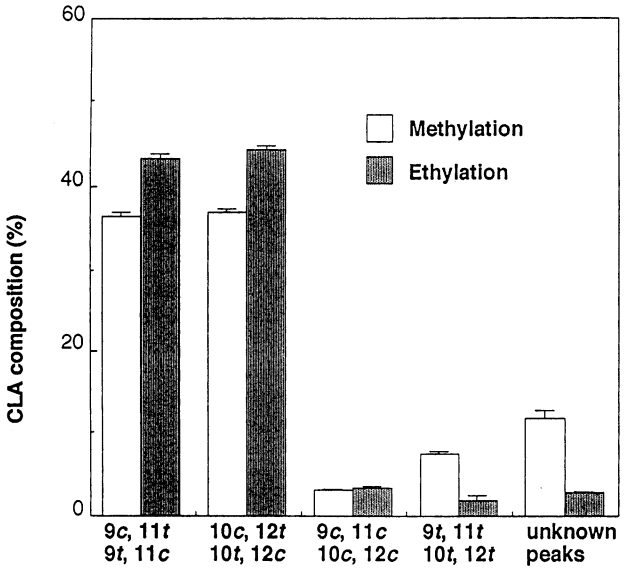


**FIG. 6.** Effect of reaction solvent on CLA composition during esterification catalyzed by $H_2SO_4$. Values are means ± SD for three measurements. Reaction time was 120 min. See Figure 2 for abbreviation.

product is detected on the chromatogram, the production could be inhibited by DMSO (29). Therefore, we tested whether DMSO could inhibit isomerization of CLA. We found that both DMSO and DMF inhibit isomerization of CLA. They are both aprotic solvents, and they possess a characteristic structure in which the oxygen atom projects, so they can easily interact with protonated alcohols. If the protonated alcohol is reacted with conjugated double bonds, CLA could be converted to methoxy compounds (25). When the methoxy base is detached and the conjugated double bond is reformed, the *t,t* isomers would be produced because they are more stable than the *c,t* and *t,c* isomers. Allylic hydroxy oleates (AHO) are the oxidation products of oleic acid and exist in natural foodstuffs. AHO are converted to methoxy oleate and CLA during acid-catalyzed methylation (30). We believe that DMSO and DMF can also inhibit this new CLA synthesis because this reaction is caused by the addition of methanol to double bonds, as recognized in this present report. Addition of DMSO and DMF to the reaction mixture in the transesterification of CLA could be an effective procedure because no detectable conversion of CLA constituents occurred during the 120 min that was needed to terminate transesterification.

In conclusion, the isomerization of *c,t* and *t,c* isomers to *t,t* isomers and the formation of unknown substances during the esterification of CLA should be considered. To avoid this, it is necessary to inhibit the interaction between protonated alcohol with the conjugated double bonds of CLA. One recommended method is the methylation catalyzed by $H_2SO_4$ in the presence of a proper amount of DMSO or DMF.

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