Comparison of Methylation Procedures for Conjugated Linoleic Acid and Artifact Formation by Commercial (Trimethylsilyl)diazomethane

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Four different methods for methylating conjugated linoleic acid (CLA) were compared. The HCl/MeOH and BF_3/MeOH methods were tested under different time and temperature combinations. Increasing temperature and/or incubation time for either method decreased the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers, but *trans*-9,*trans*-11/*trans*-10,*trans*-12 isomers and artifacts (allylic methoxide) were increased. In addition, the triacylglyceride form of CLA was tested using the above methods and NaOMe at various temperatures for 20 min. The NaOMe did not generate methoxy artifacts. However, there were impurities in GC after methylation with NaOMe as well as with BF_3/MeOH. The (trimethylsilyl)diazomethane method, which is a mild and easy alternative, was tested. Free forms of fatty acids were easily, but not completely, methylated by this method. Also, the method generated artifacts (trimethylsilyl CLA esters) and impurities (trimethylsilyl) that would interfere with short-chain fatty-acid analysis by GC.

Keywords: *Conjugated linoleic acid; CLA; methylation; (trimethylsilyl)diazomethane.*

INTRODUCTION

Conjugated linoleic acid (CLA) refers to a group of geometric and positional isomers of conjugated linoleic acid. It was originally isolated from ground beef extract as an anticancer principal and showed a variety of biologically beneficial activities (*1*). The main isomer present in foods such as meat and dairy products is the *cis*-9,*trans*-11 isomer (*2*). This isomer can be formed during the biohydrogenation of linoleic acid to stearic acid by rumen bacteria (*3*). Alternatively, it can form in animals via Δ^9 desaturation of *trans*-11 octadecenoic acid (vaccenic acid) (*4*−*6*). The *trans*-10,*cis*-12 CLA isomer is also naturally present, but to a lesser extent (*7*). For experimental purposes, CLA is prepared by alkali isomerization from pure linoleic acid. This preparation contains about 95% CLA, mainly the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers (85−90%) along with other minor isomers (trans,trans or cis,cis)(*8*). However, depending on the source of linoleic acid (pure fatty acid or vegetable oil) and conditions of isomerization, the isomer distribution can vary significantly. In some studies, CLA preparations contain a more complex mixture of isomers, such as 8,10 and 11,13 isomers as well as 9,11 and 10,12 isomers (*9*). In addition, we previously reported that the *trans*-10,*cis*-12 CLA isomer is responsible for the reduction of body fat while enhancing lean body mass (*8*). Meanwhile, it was suggested that the *cis*-9,*trans*-11 CLA isomer is active in growth promotion (*10*) and anticancer effects (*11*). In this regard, it is important to avoid modifying the original isomer distribution when analyzing the composition of CLA used in any given experiment. Derivatizations of original compounds are often used to improve separation, and methylation is most commonly used for fatty acids. Acid-catalyzed methylations using HCl/MeOH and BF_3/MeOH are methods commonly used to methylate free fatty acids, phospholipids, or triacylglycerides, but usually require high temperatures to complete the reactions. Also, these methods have been reported to change the isomer distribution of CLA and generate allylic methoxide from CLA (*12*). Base-catalyzed methods, for example those using NaOMe or tetramethylguanidine, are mild ways to methylate the triacylglyceride form but not free fatty acids. Diazomethane can easily convert free fatty acids to methyl esters without changing the isomer distribution of the fatty acid at room temperature or lower with a short incubation time. However, the reagent preparation and toxicity of the original materials are a cause for concern. Alternatively, (trimethylsilyl)diazomethane ((TMS)diazomethane) is commercially available as a convenient source of diazomethane. In this report, we will compare temperature and time effects on acid- and base-catalyzed methods, focusing on isomer distribution of CLA. In addition, artifact formation from commercial (TMS)diazomethane will be discussed.

MATERIALS AND METHODS

Materials. CLA (CLA 90) was obtained from Natural Lipids Ltd AS, Hovdebygda, Norway. The composition was CLA isomers 91.2%, oleic acid 6.3%, palmitic acid 0.7%, and linoleic acid 0.7%. The CLA isomers were *cis*-9,*trans*-11 or *trans*-9,*cis*-11, 41.9%; *trans*-10,*cis*-12, 43.5%; *trans*-9,*trans*-11 and *trans*-10,*trans*-12, 1.98%; others 1.47% (analyzed by 4% HCl/methanol at room temperature for 30 min to minimize artifact formation). The triacylglyceride form of CLA was prepared as described previously (*13*−*15*). Methanolic HCl (HCl/MeOH) was purchased from Supelco (Bellefonte, PA) and sodium methoxide (NaOMe), boron trifluoride (BF_3/MeOH), and (TMS-

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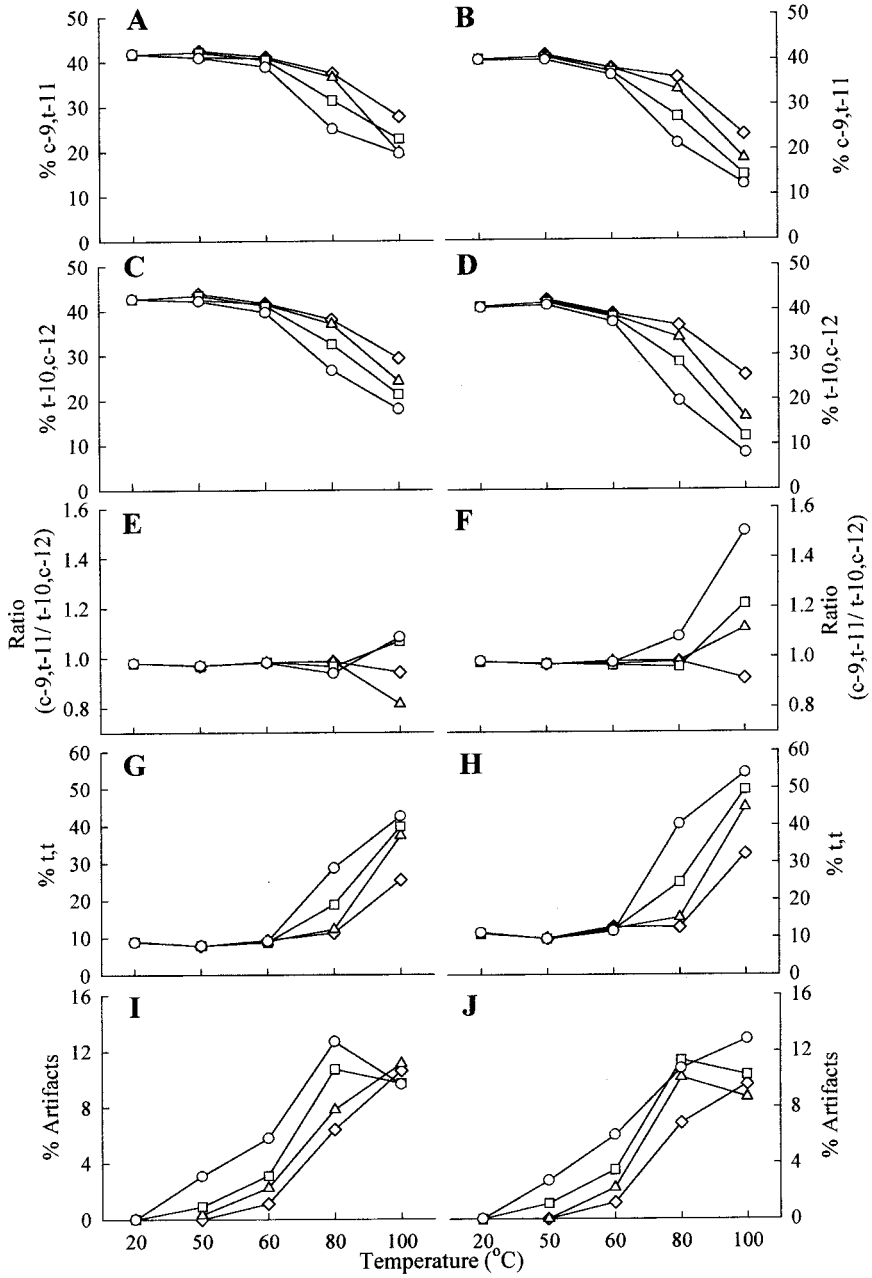

Figure 1. Comparison of acid-catalyzed methylation methods using CLA free forms: HCl/MeOH (left panel, A, C, E, G, and I) and BF_3/MeOH (right panel, B, D, F, H, and J). *Cis*-9,*trans*-11 isomer (A, B), *trans*-10,*cis*-12 isomer (C, D), ratio of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers (E, F), trans,trans isomers (G, H), and artifacts (I, J). Free form of CLA was methylated with either HCl/MeOH or BF_3/MeOH methods for 10 min (diamonds), 20 min (triangles), 30 min (squares), or 60 min (circles). Numbers are mean of duplicate determination.

)diazomethane were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Methylations. Free form and methyl esters of CLA were methylated with HCl/MeOH (4%) or BF_3/MeOH (12%) under the conditions indicated later in the figures, and changes in isomer distribution were observed. The triacylglyceride form of CLA was methylated with the above two methods as well as NaOMe at various temperatures (indicated later in Figure 3) for 20 min. For all three methods, the samples were dissolved in anhydrous methanol, and the reaction was started with the addition of methylation reagent. At the end of the incubation time, water was added, and methyl esters were extracted with hexane. Completion of methylation was checked with thin-layer chromatography (developing solvent hexane/diethyl ether/methanol/acetic acid, 90:20:5:2 by vol) and visualized with H_2SO_4. Percentages of CLA isomers were determined on the basis of their corresponding area of the gas chromatography (GC) chromatogram. For the (TMS)diazomethane method, free CLA (*12*) was dissolved in either methanol/benzene (2:1) or hexane, and (TMS)diazomethane was added for 30 min at room temperature. For specific conditions, see legends of figures. Impurities refers to the peaks that originated from reagents, and artifacts refers to new peaks generated from the sample by the procedure. GC was conducted with a Hewlett-Packard 5890 series II fitted with a flame ionization detector and 3396A integrator. A Supelcowax-10 fused-silica capillary column (60 m × 0.32 mm i.d., 0.25 μm film thickness) was used, and the oven temperature was programmed from 50 to 200 °C, increased 20 °C per min, held for 50 min, increased 10 °C per min to 230 °C, and held for 20 min. NMR was conducted using a Bruker WP200SY (200 MHz, Ettlingen, Germany). GC/MS was performed with a Hewlett-Packard 5890A GC with a HP5970 mass selective detector (MSD). (TMS)diazomethane artifact I (Figure 6), EIMS *m*/*z* (rel int.): 351 (28), 159 (21), 143 (23), 95 (33), 81 (53), 79 (45), 73 (100), 67 (91), 55 (70). (TMS)diazomethane

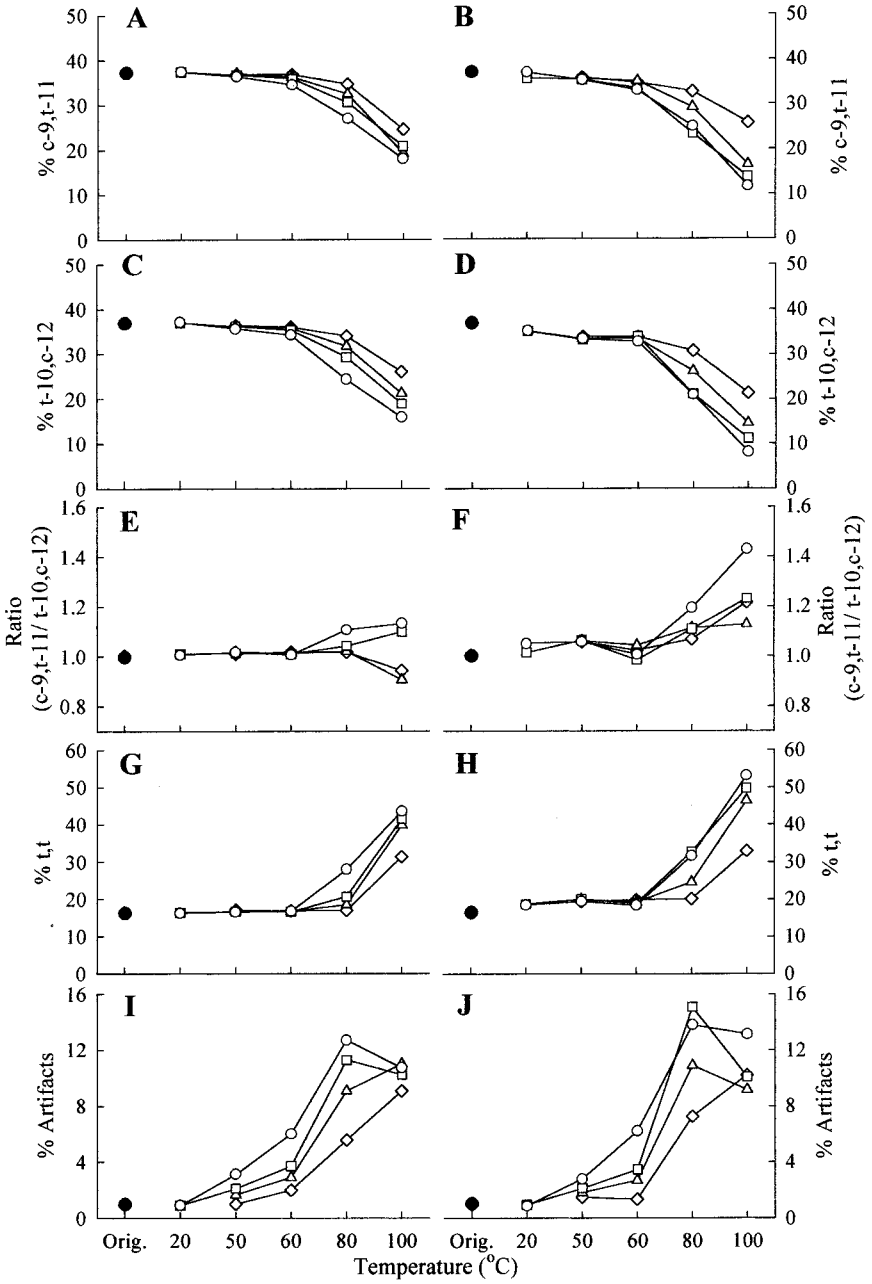

Figure 2. Comparison of acid-catalyzed methylation methods using CLA methyl ester forms: HCl/MeOH (left panel, A, C, E, G, and I) and BF_3/MeOH (right panel, B, D, F, H, and J). *Cis*-9,*trans*-11 isomer (A, B), *trans*-10,*cis*-12 isomer (C, D), ratio of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers (E, F), trans,trans isomers (G, H), and artifacts (I, J). Methyl esters of CLA were re-methylated with either HCl/MeOH or BF_3/MeOH methods for 10 min (diamonds), 20 min (triangles), 30 min (squares), or 60 min (circles). Original composition is indicated by filled circle in each figure. Numbers are mean of duplicate determination.

artifact II (Figure 6): 351 (23), 159 (23), 143 (19), 95 (34), 81 (56), 79 (42), 73 (100), 67 (95), 55 (71).

RESULTS AND DISCUSSION

Comparisons of Temperature and Incubation Time. Figure 1 shows the isomer distribution of CLA (free form) after methylation was performed under various incubation times and temperatures using acid-catalyzed methods (HCl/MeOH and BF_3/MeOH). Complete methylation was confirmed by TLC for all conditions tested in Figure 1. At a constant incubation time, *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers of CLA were decreased by increasing reaction temperature with both HCl/MeOH (Figure 1A, C) and BF_3/MeOH methods (Figure 1B, D). Both methods showed similar trends, but the BF_3/MeOH method had a slightly and consistently greater loss of these isomers compared to that of the HCl/MeOH method. The ratio of these two isomers (*cis*-9,*trans*-11/*trans*-10,*cis*-12) was changed when temperatures were 80 and 100 °C (Figure 1E, F). The BF_3/MeOH method increased this ratio considerably at 100 °C with increasing incubation time, which represented a greater loss of the *trans*-10,*cis*-12 isomer than of *cis*-9, *trans*-11 (Figure 1F).

Trans,trans isomers were also observed under the same conditions (Figure 1G, H, trans,trans is the combined term for *trans*-9,*trans*-11 and *trans*-10,*trans*-12 because both eluted at the same retention time in our GC). Up to 60 °C, levels of trans,trans isomers were similar at all time points. At 80 and 100 °C, extending

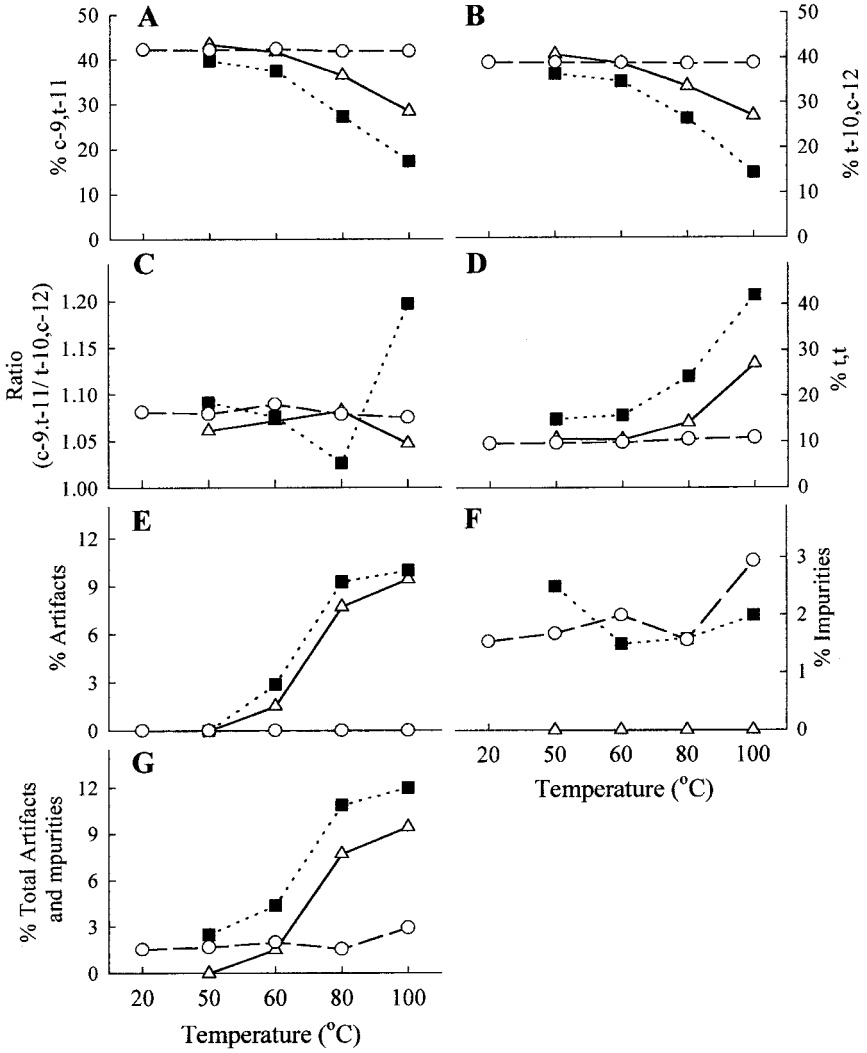

Figure 3. Comparison of acid-catalyzed and base-catalyzed methods. Triacylglyceride form of CLA was methylated with one of the following methods for 20 min: HCl/MeOH (triangles), BF_3/MeOH (filled squares), or NaOMe (open circles). *Cis*-9,*trans*-11 isomer (A), *trans*-10,*cis*-12 isomer (B), ratio of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers (C), trans,trans isomers (D), artifacts (E), impurities (F), and total artifacts and impurities (G). Numbers are mean of duplicate determination.

the incubation time considerably increased these isomers. At 100 °C for 60 min with BF_3/MeOH, these isomers were 54% of the overall CLA. The BF_3/MeOH method had more trans,trans isomer than the HCl/MeOH method under the same conditions.

It has been reported that acid-catalyzed methods generate allylic methoxides from CLA (*12*). There were no artifacts formed at room temperature or at 50 °C for 10 min in either method (Figure 1I, J). At temperatures of 50 °C (longer than 20 min) or higher, artifacts were generated that equaled up to 13% of the total. As both acid-catalyzed methods generated allylic methoxide artifacts and changed isomer distribution, these methods need to be carefully monitored for accurate analysis. HCl/MeOH (4%) at 50 °C for less than 20 min generated no or minimal artifacts, whereas higher temperature and/or longer incubation time generated considerable amounts of artifacts.

To determine whether any of the CLA isomers had been changed from the original composition, we prepared methyl esters of CLA and repeated similar experiments (Figure 2). The original CLA methyl esters contained 37.2% *cis*-9,*trans*-11 and 37.1% *trans*-10,*cis*-12 isomers, which was similar to the content after remethylation with HCl/MeOH at room temperature and at 50 °C up to 30 min (Figure 2A, C), but the BF_3/MeOH method slightly reduced these two isomers even at room temperature (Figure 2B, D). For both methods, incubation at 80 and 100 °C for longer than 30 min reduced the *cis*-9,*trans*-11 or *trans*-10,*cis*-12 isomers (Figure 2A–D). The ratio of these two isomers was similar up to 60 °C. But at 80 and 100 °C, the BF_3/MeOH method tended to increase the ratio, which indicated greater loss of the *trans*-10,*cis*-12 isomer than of the *cis*-9,*trans*-11 (Figure 2F). On the contrary, the HCl/MeOH method tended to decrease the ratio of these two isomers compared to the original composition, indicating the *cis*-9,*trans*-11 isomer was lost more than the *trans*-10,*cis*-12 isomer with this method.

Trans,trans isomers were increased by both methods (Figure 2G, H). In fact, these isomers account for up to 53% of CLA when methylated at 100 °C for 60 min (Figure 2H). The allylic methoxide amount (artifacts) was not different from the original at room temperature and at 50 °C for up to 20 min with the HCl/MeOH method (Figure 2I) but started to increase from 30 min for both methods. At 60 and 80 °C the artifacts increased with increasing temperature and/or incubation time, as was observed with the free form. At 100 °C, these artifacts were less than those observed at 80 °C under

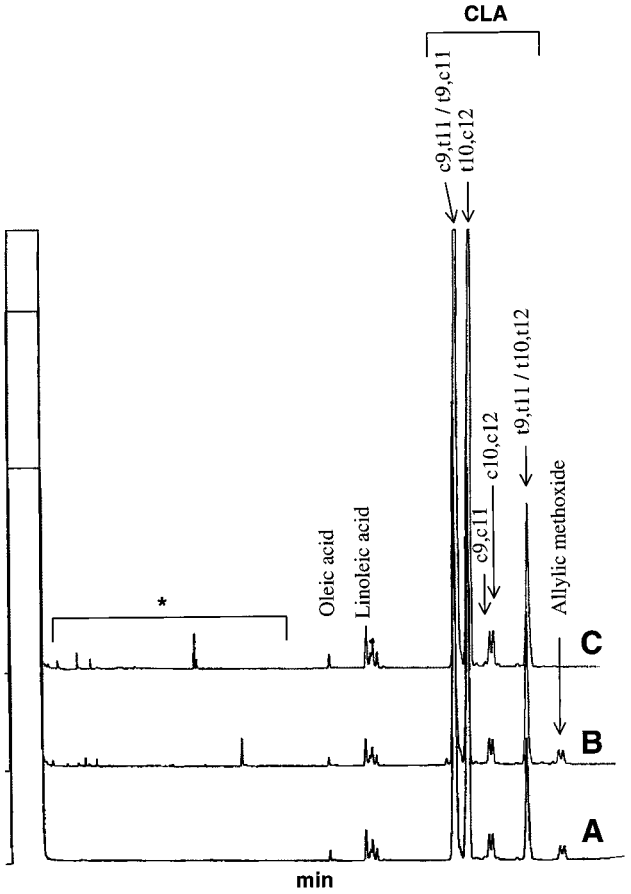

Figure 4. Gas chromatograms of CLA methyl esters after methylation with HCl/MeOH (A), BF_3/MeOH (B), or NaOMe (C); *, indicates impurities.

the same conditions, possibly because artifacts other than allylic methoxides were generated. Impurities originating from the BF_3/MeOH reagent will be discussed further in the next section.

Comparisons of Acid-Catalyzed and Base-Catalyzed Methods. To compare acid-catalyzed and base-catalyzed methods, the triacylglyceride form of CLA was prepared. An incubation time of 20 min was used for all temperatures tested. The NaOMe method completely converted the triacylglyceride form to methyl esters of CLA at all temperatures tested (checked with TLC). The HCl/MeOH or BF_3/MeOH methods did not completely methylate at 50 °C, but above 60 °C methylation was complete. Figure 3A, B illustrates that HCl/MeOH or BF_3/MeOH decreased *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers, with BF_3/MeOH causing a larger decrease as discussed earlier. However, the NaOMe method did not change the amount of these isomers. The ratio of these two isomers (Figure 3C) was not changed by NaOMe but fluctuated with HCl/MeOH or BF_3/MeOH, trends which were consistent with Figures 1E, 1F, 2E, and 2F. Trans,trans isomers were increased by HCl/MeOH and to a greater extent with BF_3/MeOH, whereas NaOMe did not change these isomers (Figure 3D). Allylic methoxides, which were generated with acid-catalyzed methods (Figures 1I, 1J, 2I, 2J), were not observed with the NaOMe method (Figure 3E). However, peaks other than original compounds or allylic methoxides were observed with the BF_3/MeOH or NaOMe methods, but not with the HCl/MeOH method. Chromatograms are shown in Figure 4. All of the unknown peaks (* in Figure 4) are impurities from methylation reagents, as indicated in Figure 3F. HCl/MeOH (Figure 4A) did not have any impurities but BF_3/MeOH (Figure 4B) and NaOMe (Figure 4C) contained about 1.5−3%. Combined with allylic methoxide, total artifacts plus impurities are shown in Figure 3G. When the HCl/MeOH method was used at 50 °C for 20 min no artifacts or other impurities were present, although the methylation was not complete.

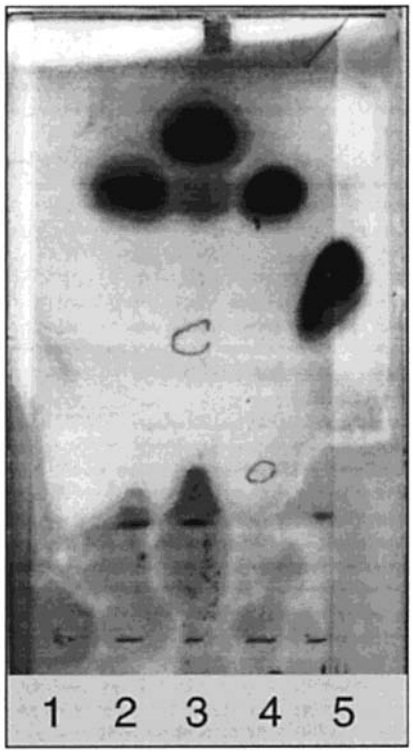

Figure 5. Artifact formation by (trimethylsilyl)diazomethane method. Thin-layer chromatogram shows (1) glycerol, (2) methyl esters from hydrolyzed corn oil after original method, (3) methyl esters and byproduct (on top) with the same sample after modified method, (4) CLA methyl esters standard, and (5) triacylglyceride of CLA standard.

(Trimethylsilyl)diazomethane Method. Diazomethane is a good derivatization reagent for free fatty acid: minimum change to original compounds, mild conditions, and fast. However, there is concern about the toxicity of the compound used to prepare diazomethane. As a substitute, commercial (TMS)diazomethane was used. The methylation procedure with (TMS)diazomethane includes using benzene to dissolve fat, followed by extraction with hexane. Replacement of benzene with hexane was called the modified method. Figure 5 shows the TLC results of hydrolyzed corn oil derivatized by both the original and modified methods. The original method easily converted free fatty acids to methyl esters (Figure 5, lane 2). The modified method contained additional compound(s) (Figure 5, lane 3, upper spot, presumably artifacts), together with methyl esters. The TLC pattern of these artifacts was the same in hydrolyzed corn oil and CLA. Unreacted free forms are apparent in the middle of TLC with both methods, even if we increased the incubation time. The reaction with (TMS)diazomethane was very fast but was not complete. In addition, TLC of the original method contained small amounts of artifacts.

Figure 6 shows the GC chromatogram of CLA methyl esters prepared by the (TMS)diazomethane method, with both the original method (A) and the modified

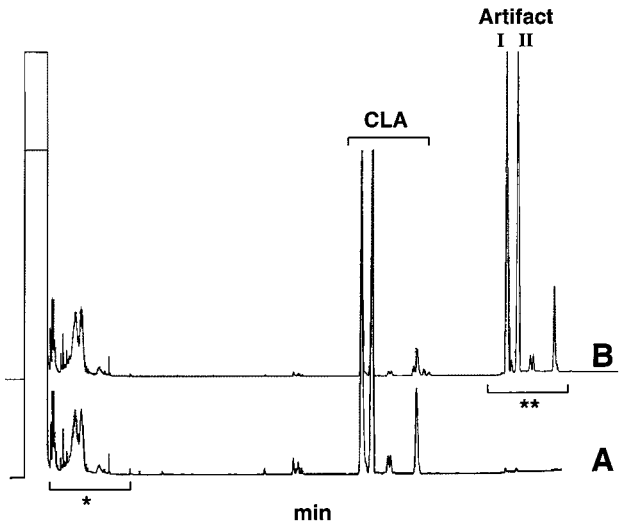

Figure 6. Gas chromatograms of CLA methyl esters prepared by (trimethylsilyl)diazomethane original method (A) and modified method (B). The * indicates impurities from (trimethylsilyl)diazomethane, and ** indicates artifacts generated from CLA after methylation with modified method of (trimethylsilyl)diazomethane. Please see Figure 4 for identification for CLA isomers.

method (B). GC indicated that the artifact fraction contained a pattern similar to that of CLA methyl esters at longer retention times (** in Figure 6). The chromatogram from the original method also contained small amounts of artifacts. In addition, we noticed a group of peaks due to impurities at shorter retention times (* in Figure 6), which we were not able to remove. These compounds were very volatile, and they easily contaminated other tubes even without direct contact. Based on the characteristics, we suggest that these are trimethylsilyl. They will interfere with short-chain fatty acids in GC.

To analyze unknowns, we collected artifact fractions originating from CLA using column chromatography, and we analyzed them further. GC/MS analysis indicated artifacts I and II both have molecular weights of 351. Trimethylsilyl bound to CLA would have a molecular weight of 366. Loss of a methyl group would give a molecular weight of 351. No change in the 5−6.5 ppm area in NMR analysis indicated that the double bonds are intact (data not shown). In addition, there were two TMS peaks (0 and −0.1 ppm), which indicated contamination with trimethylsilyl from the methylating reagent.

Of the methods tested, no single method will methylate CLA without any problems, either generating artifacts or changing isomer distribution, or both. When derivatization of CLA is necessary, we suggest using these methods with caution. Base-catalyzed methods, such as NaOMe, can be useful for completing derivatization easily. However, free forms of fatty acids need alterative methods, such as (TMS)diazomethane or acid-catalyzed methods. The (TMS)diazomethane method can generate artifacts (TMS esters) and contain contaminants that would interfere with other fatty acids in GC analysis. Both HCl/MeOH and BF_3/MeOH methods can change CLA isomer distribution, in a time- and/or temperature-dependent manner. When the HCl/MeOH (4%) method was used at a temperature less than 50 °C for less than 20 min, there were no impurities or artifacts generated.

ABBREVIATIONS USED

CLA, conjugated linoleic acid; TMS, trimethylsilyl.

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