Investigation of Some Preparation Procedures of Fatty Acid Methyl Esters for Capillary Gas-Liquid Chromatographic Analysis of Conjugated Linoleic Acid in Meat

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Abstract The five most widely accepted procedures for preparing fatty acid methyl esters in food lipids were investigated for their suitability in capillary gas-liquid chromatographic analysis of *cis*-9, *trans*-11 conjugated linoleic acid (c-9, t-11 CLA) in meat. A modified procedure of fatty acid methyl esterification was developed and the method was applied to determine c-9, t-11 CLA content in some meats. This method involved hydrolysis of lipids with 0.5 M KOH in methanol at 100°C for 5 min, followed by esterification with aqueous HCl (35%)/methanol (1 : 1 v/v) at 100°C for 5 min. The resulting c-9, t-11 CLA methyl ester was separated by gas-liquid chromatograph equipped with a capillary column and determined using tricosanoic acid as an internal standard. Meat from ruminant animals considerably contained more c-9, t-11 CLA than those from non-ruminant animals in the following order : goat meat, beef, mutton, pork, and chicken. The amount in goat meat (6.35 mg/g lipid) was 10-fold greater than those in pork and chicken.

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Conjugated linoleic acids (CLAs) have recently been studied by a number of researchers as one of the bioactive substances in food. CLAs are positional and geometrical isomers of linoleic acid, and they have conjugated double bonds at positions 9 and 11 or 10 and 12. In these isomers, *cis*-9, *trans*-11 CLA (c-9, t-11 CLA) which is found in beef¹³⁾ has beneficial effects such as antimutagenic activity and serum cholesterol-lowering activity^{4, 5, 8, 10, 12)}. Hughes *et al.*⁷⁾ suggested that c-9, t-11 CLA is produced by linoleate Δ^{12} -*cis*, Δ^{11} -*trans* isomerase, which is an enzyme synthesized in a rumen bacteria. Chin *et al.*²⁾ and Ha *et al.*⁶⁾ has found that meat, milk, and related processed food from ruminant animals contain a larger amount of c-9, t-11 CLA as compared with other types of food from non-ruminant animals.

On the other hand, CLAs are easily isomerized and oxidized during the preparation of their methyl esters^{2, 14)}. According to the literatures of Chin *et al.*²⁾ and Shantha *et al.*¹⁴⁾, decomposition of c-9, t-11 CLA isomer during methylation was in the range of 3% to 57% for the AOCS official method which involves methanolic sodium/potassium hydroxide followed by boron trifluoride in methanol, 5% for methanolic hydrochloride and 28% for methanolic sulfuric acid. Because CLAs are minor ingredients in food, it is important to develop a highly reliable method for determining them, especially c-9, t-11 CLA. Thus, the authors attempted to investigate whether the most widely accepted methods for preparing fatty acid methyl esters from food lipids could be applicable to determine the c-9, t-11 CLA in foods.

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A precise and reproducible method for determining the CLA in foods was proposed and used to determine the c-9, t-11 CLA content in various meats.

Materials and Methods

Materials

Conjugated octadecadienoic acid (a mixture of *cis*- and *trans*-9,11- and -10,12-octadecadienoic acids, described as free CLAs standard in this paper), conjugated octadecadienoic acid methyl ester (a mixture of *cis*- and *trans*-9,11- and -10,12-octadecadienoic acid methyl esters, described as CLA methyl esters standard in this paper), and tricosanoic acid were purchased from Sigma Chemical Company (St. Louis, MO). A four percent HCl/methanol was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo), and 14% BF₃/methanol, 1,1,3,3,- tetramethylguanidine, aqueous hydrochloric acid (35%, super special grade) and other chemical materials were from Wako Pure Chemicals Industries Ltd. (Osaka).

Commercial domestic and imported beef (round), pork (ham), and chicken meat (thigh) were used as samples for the determination of c-9, t-11 CLA. Round meat from carcasses of goats and sheep raised with roughage on Sumiyoshi Ranch, Miyazaki University, were also used. All samples were stored at -80°C until use.

Lipid extraction

Total lipids were extracted from meat following the method of Folch *et al.*³⁾, and their lipid content was determined by the gravimetric technique.

Preparation of fatty acid methyl esters (FAMES)

The following five methods were used for the preparation of FAMES of beef lipid and free CLAs standard: Method I¹⁾, lipids (30 mg) were saponified with 5 ml 0.5 M KOH/methanol at 100°C for 5 min, then esterified with 5 ml of 14% BF₃/methanol at 100°C for 5 min in a boiling water; Method II¹¹⁾, lipids (30 mg) were directly esterified with 1.5 ml of 8% BF₃/methanol in the presence of 1 ml benzene at 100°C on a heat block for 10, 15, 20, 25, 30 or 60 min, respectively; Method III⁵⁾, lipids (30 mg) were directly esterified with 6 ml of 4% HCl/methanol at 100°C on a heat block for 10, 15, 20, 30 or 60 min, respectively; Method IV^{14,15)}, lipids (30 mg) were directly es-

terified with 2 ml 1,1,3,3,-tetramethylguanidine/Methanol (1 : 4, v/v) at 100°C in a boiling water for 10 min; Method V⁹⁾, lipids (30 mg) were saponified with 1.0 ml 0.5 M KOH/methanol at 100°C, then esterified with 0.4 ml aqueous HCl/methanol at 100°C on a heat block. In Method V, saponification of lipids was preliminarily examined for 5 and 10 min, both the 5 and 10 min-reaction resulted in a sufficient hydrolysis, thus, we adopted the 5 min-reaction. The resulting fatty acids were esterified for 5, 10, 15, 20 or 30 min, respectively, with aqueous HCl in methanol solution having various HCl concentrations (aqueous HCl/methanol = 4 : 1, 4 : 2, 4 : 3 and 4 : 4, v/v). All operations were performed under nitrogen, and with minimum exposure to air and light.

Gas-liquid chromatography

FAMES were analyzed on a GC-17A gas-chromatograph (Shimadzu Seisaku-syo, Tokyo) equipped with a flame-ionization detector (FID) using a capillary column (SUPELCOWAXTM 10, 60 m × 0.32 mm, *i.d.*, 0.25 μm film-thickness, SUPELCO, Inc., PA). This analysis adopted a split injection (split ratio 1:100). The GC analysis was temperature-programmed, at 195°C held at 8 min, and raised from 195°C to 220°C at a rate of 2°C/min and then held at 220°C for 40 min. The injection port and detector temperatures were set at 240°C and 250°C, respectively. Helium was used as the carrier gas at a rate of 2 ml/min. The peaks of the FAMES were identified by comparing the retention times to reference standards, FAMES standard from fish oil and CLA methyl esters standard, and the area of the c-9, t-11 CLA peak was calculated as mg/g of lipid or mg/100 g of sample using tricosanoic acid as an internal standard.

Results and Discussion

Figure 1 shows the respective typical capillary gas-liquid chromatograms of beef lipids and CLA methyl esters standard. As shown in this figure, c-9, t-11 CLA isomer was separated from beef lipid, and was identified. Preliminarily, we prepared FAMES of beef lipid by using Method I, III, IV and V (described in Materials and Methods), and compared the composition of fatty acid. Composition of c-9, t-11 CLA was 0.31% for Method I, but the other three

Fig. 1. Identification of conjugated linoleic acid (CLA) isomers in beef on capillary gas-liquid chromatography. Each panel shows (A): methyl esterified beef lipid with Method I, (B): CLA methyl esters standard, and (C): (A) + (B).

methods showed values ranging from 0.33 to 0.36%. This result suggested that Method I may lead to isomerization of c-9, t-11 CLA.

In order to more precisely determine a small amount of c-9, t-11 CLA without its decomposition and isomerization, the authors continued to examine the four methods by which a higher yield of total fatty acids and a lower decomposition and isomerization of the c-9, t-11 CLA could be obtained. Methyl esterification with Methods II and V gave adequate yields of total fatty acids from beef lipids at 25 and 5

Fig. 2. Effect of esterifying time of various methods on the yield of total fatty acids from beef lipid. Each panel shows the yield of total fatty acids with a: Methods II, III and V b: Method V.

—▲— : Method II
—■— : Method III
—●— : Method V

Table 1. Fatty acid content of beef lipid esterified with Methods II and V (mg/g of lipid)

Fatty acids	Method			
	II		V	
14 : 0	19.79	(2.46)	18.27	(2.31)
15 : 0	3.88	(0.48)	3.65	(0.46)
16 : 0	201.04	(24.94)	194.44	(24.64)
16 : 1	25.38	(3.15)	23.90	(3.02)
17 : 0	8.15	(1.01)	8.03	(1.01)
18 : 0	163.43	(20.28)	164.07	(20.71)
18 : 1(ω 9)	328.41	(40.73)	324.18	(40.92)
18 : 1(ω 7)	26.73	(3.32)	26.14	(3.30)
18 : 2(ω 6)	13.93	(1.73)	14.14	(1.78)
18 : 3(ω 3)	6.40	(0.79)	6.51	(0.82)
c-9, t-11 CLA	2.44	(0.30)	2.87	(0.36)
20 : 1(ω 9)	1.27	(0.16)	1.31	(0.17)
20 : 4(ω 6)	1.78	(0.22)	1.59	(0.20)
20 : 5(ω 3)	0.86	(0.11)	0.81	(0.10)
22 : 5(ω 3)	2.54	(0.32)	2.39	(0.30)
Total	806.03	(100.00)	792.30	(100.00)

Each value in parentheses represents composition of fatty acids.

min, respectively, whereas the yield at 60 min for Method III was only about 75% as compared with the yield at 10 min for Method V (Fig. 2a). In addition, Method IV resulted in a remarkably lower yield of total fatty acids, which corresponded to only about 10% of the yield at 10 min for Method V (data not shown). For Method V, there were almost no differences in the yield of total fatty acids between 5 and 10 min of esterification. On the other hand, much higher yield of total fatty acids was estimated with esterification for less than 5 min because of lower

recovery of internal standard (Fig. 2b). In this method, esterification for 5 min was performed since a longer reaction time in transmethyl esterification could cause an isomerization of CLA isomers. The results obtained in this study showed that Methods II and V were superior to both Methods III and V in terms of the yield of total fatty acids.

There were no significant differences in the composition of fatty acids of beef lipid between Methods II and V, and the yield of total fatty acid for Method V was approximately 98% that for Method II (Table

Fig. 3. Effect of esterifying procedures on the composition of CLA isomers. "others" represent total CLA peaks except c-9, t-11 ; t-10, c-12 ; t-9, t-11 and t-10, t-12 CLA isomers.

- : c-9, t-11 CLA isomer (1)
- ▨: t-10, c-12 CLA isomer (2)
- ▤: t-9, t-11 and t-10, t-12 CLA isomers (3)
- : others

1). However, when we used Methods II and V for methyl esterification of free CLAs standard, Method II resulted in a lower composition of c-9, t-11 CLA than that of Method V (Fig. 3). In this figure, "others" represented a sum of other CLA isomers except c-9, t-11 ; t-10, c-12 ; t-9, t-11 and t-10, t-12 CLA isomers, therefore, c-9, t-11 and t-10, c-12 CLA isomers were possibly isomerized to the other CLA isomers. This result suggested that the presence of BF_3 in reaction systems may easily induce the decomposition and isomerization of c-9, t-11 CLA

isomer.

The effects of varying concentrations of HCl in the methyl esterification reagent on composition of CLA isomers were examined. Decomposition and isomerization of c-9, t-11 and t-10, c-12 CLA isomers by using aqueous HCl/methanol solution (4 : 4, v/v) were lower than those by other higher HCl conditions (Fig. 4). These results indicated that decomposition and isomerization of c-9, t-11 CLA isomer were affected by aqueous HCl concentration in methanol solution, *i.e.*, more acidic condition would accelerate the

Fig. 4. Effect of HCl concentrations of aqueous HCl/methanol as esterifying reagent on the composition of CLA isomers.

- : c-9, t-11 CLA isomer
- ▨ : t-10, c-12 CLA isomer
- : t-9, t-11 and t-10, t-12 CLA isomers
- : others

Table 2. Comparison between the composition of CLA methyl esters standard and that prepared from free CLAs standard with Method V

Isomers	c-9, t-11	t-10, t-12	t-9, t-11 and t-10, t-12	Others
A	39.06 (0.26)	46.15 (0.27)	9.50 (0.05)	5.29 (0.48)
B	36.80 (0.47)	46.18 (0.16)	10.83 (2.00)	6.21 (0.67)

A : CLA methyl esters standard,

B : CLA methyl esters derived from free CLAs standard with Method V.

Each value in parentheses represents standard deviation (n=4).

Table 3. c-9, t-11 CLA content of various meats

Meat	c-9, t-11 CLA (mg/g of lipid)
Beef	3.21
Mutton	2.28
Goat meat	6.35
Pork	0.63
Chicken	0.56

decomposition and isomerization of c-9, t-11 and t-10, c-12 CLA. The composition of c-9, t-11 CLA isomer prepared from free CLA standard by Method V using aqueous HCl/methanol solution (4 : 4, v/v), however, decreased by 5.8% compared with that of CLA methyl esters standard (Table 2).

As a result of the investigation of some preparation procedures of fatty acid methyl esters, we could develop a modified method for determining c-9, t-11 CLA content in meat by dividing the value estimated using tricosanoic acid as an internal standard by 0.9261, which was a correction factor determined on the basis of the yield of total fatty acids and the decomposition ratio of c-9, t-11 CLA. In addition, this method can be used not only for determination of c-9, t-11 CLA but also for a routine analysis of fatty acids because it is easy to use and is less time consuming.

Table 3 shows the c-9, t-11 CLA content of some meat samples determined by the proposed method.

Chin *et al.*²⁾ found that CLA content of ruminant animal products was especially high in total CLAs and in c-9, t-11 CLA ratio of total CLAs. The c-9, t-11 CLA contents in their study were very similar to our values determined. Besides, in this study, meat from goats fed with roughage contained the highest amount of c-9, t-11 CLA. Stanton *et al.*¹⁶⁾ reported that some milk from low grass allowance (16 kg/cow × day) have lower CLA values than high grass allowance (20 and 24 kg/cow × day) after 19 week treatment. These results indicate that excessive feeding of roughage to ruminant animals and feeding system may lead to changes in the microflora of their rumen, which may alter the content of c-9, t-11 CLA in meat and milk products.

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