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Distributions of Conjugated Linoleic Acid (CLA) Isomers in Tissue Lipid Classes of Pigs Fed a Commercial CLA Mixture Determined by Gas Chromatography and Silver Ion–High-Performance Liquid Chromatography

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ABSTRACT: Pigs were fed a commercial conjugated linoleic acid (CLA) mixture, prepared by alkali isomerization of sunflower oil, at 2% of the basal diet, from 61.5 to 106 kg live weight, and were compared to pigs fed the same basal diet with 2% added sunflower oil. The total lipids from liver, heart, inner back fat, and omental fat of pigs fed the CLA diet were analyzed for the incorporation of CLA isomers into all the tissue lipid classes. A total of 10 lipid classes were isolated by three-directional thin-layer chromatography and analyzed by gas chromatography (GC) on long capillary columns and by silver-ion high-performance liquid chromatography (Ag^+-HPLC); cholesterol was determined spectrophotometrically. Only trace amounts (<0.1%; by GC) of the 9,11-18:2 *cis/trans* and *trans,trans* isomers were observed in pigs fed the control diet. Ten and twelve CLA isomers in the diet and in pig tissue lipids were separated by GC and Ag^+- HPLC, respectively. The relative concentration of all the CLA isomers in the different lipid classes ranged from 1 to 6% of the total fatty acids. The four major *cis/trans* isomers (18.9% 11 *cis*,13 *trans*-18:2; 26.3% 10 *trans*,12 *cis*-18:2; 20.4% 9 *cis*,11 *trans*-18:2; and 16.1% 8 *trans*,10 *cis*-18:2) constituted 82% of the total CLA isomers in the dietary CLA mixture, and smaller amounts of the corresponding *cis,cis* (7.4%) and *trans,trans* (10.1%) isomers were present. The distribution of CLA isomers in inner back fat and in omental fat of the pigs was similar to that found in the diet. The liver triacylglycerols (TAG), free fatty acids (FFA), and cholesteryl esters showed a similar pattern to that found in the diet. The major liver phospholipids showed a marked increase of 9 *cis*,11 *trans*-18:2, ranging from 36 to 54%, compared to that present in the diet. However, liver diphosphatidylglycerol (DPG) showed a high incorporation of the 11 *cis*,13 *trans*-18:2 isomer (43%). All heart lipid classes, except TAG, showed a high content of 11 *cis*,13 *trans*-18:2, which was in marked contrast to results in the liver. The relative proportion of 11 *cis*,13 *trans*-18:2 ranged from 30% in the FFA to 77% in DPG. The second major isomer in all heart lipids was 9 *cis*,11 *trans*-18:2. In both liver and heart lipids the relative proportions of both 10 *trans*,12 *cis*-18:2 and 8 *trans*,10 *cis*-18:2 were significantly lower compared to that found in the diet. The FFA in liver and heart showed the highest content of *trans,trans* isomers (31 to 36%) among all the lipid classes. The preferential accumulation of the 11 *cis*,13 *trans*-18:2 into cardiac lipids, and in particular the major phospholipid in the inner mitochondrial membrane, DPG, in both heart and liver, appears unique and may be of concern. The levels of 11 *cis*,13 *trans*-18:2 naturally found in foods have not been established.
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Interest in conjugated linoleic acid (CLA) has increased in the past decade as a result of reports of several health benefits related to its consumption. CLA has been reported to protect against cancer (1–8) and atherosclerosis (9,10). In addition, CLA has been reported to decrease body fat while increasing muscle (11,12) and bone mass (13). However, commercial CLA preparations are mixtures of several positional and geometric isomers of conjugated octadecadienoic (18:2) acid. Not all the isomers in these mixtures have been resolved chromatographically, and the active isomer(s) has (have) not been identified. It has been assumed that 9 *cis*,11 *trans*-18:2 is the active isomer, because it is the major isomer present in milk, dairy products and meats (14–18). A recent epidemiological study in Finland, which appears to support this assumption,

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Abbreviations: CE, cholesteryl ester; *cis/trans*, refers to the group of CLA isomers, or a specific positional isomer, having either a *cis/trans* or a *trans,cis* configuration; CLA, conjugated linoleic acid; DMOX, 4,4-dimethyloxazoline; DPG, diphosphatidylglycerol (cardiolipin); FAME, fatty acid methyl esters; FFA, free fatty acids; GC, gas chromatography; GC-DD-FTIR, -direct deposition-Fourier transform infrared; GC-EIMS, -electron ionization mass spectrometry; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; TAG, triacylglycerol; TLC, thin-layer chromatography.

found a significant inverse gradient between milk intake and incidence of breast cancer in 4,697 initially cancer-free women over a 25-yr follow-up period (19).

A number of studies reported the presence of CLA isomers in human adipose tissue (20,21), bile (22), blood (15,23–25), and milk (15). CLA isomers were incorporated into both tissue total neutral lipids (5,7,26,27) and total phospholipids (1,2,5,7,26–28), as well as cancer tissue (2) of animals fed CLA diets. Only one recent study presented the content of CLA isomers in the different liver phospholipids of rats fed CLA (28). An accurate assessment of CLA content has so far been prevented, firstly, because acid-catalyzed methylation procedures, which may isomerize the conjugated *cis/trans* to *trans,trans* 18:2 isomers (29), were used in the analysis of CLA isomers (1,2,5,7,16,18,20,26,28); secondly, by the lack of chromatographic methods to separate all the individual CLA isomers. We have just reported a silver-ion high-performance liquid chromatography (Ag^+-HPLC) method which clearly separated for the first time four isomers—11 *cis*,13 *trans*-18:2, 8 *trans*,10 *cis*-18:2, 9 *cis*,11 *trans*-18:2, and 10 *trans*,12 *cis*-18:2—that were found in a commercial CLA preparation, plus the corresponding four *cis,cis* and four *trans,trans* isomers (30, and references cited therein).

In the present communication, we report for the first time the distribution of CLA isomers in the tissues of pigs fed a commercial CLA mixture at 2% of the diet. The CLA isomers were separated by both gas chromatography (GC) and Ag^+-HPLC as their fatty acid methyl esters (FAME). The identity of major CLA isomers was confirmed by GC-direct deposition (DD)-Fourier transform infrared (FTIR) spectroscopy and GC-electron ionization mass spectrometry (EIMS). Adipose lipids generally showed the same distribution of the isomers fed. Liver phospholipids showed an increase of 9 *cis*,11 *trans*-18:2 isomer, while in heart phospholipids an increase of 11 *cis*,13 *trans*-18:2 isomer was found. Diphosphatidylglycerol (DPG) in both liver and heart lipids showed a uniquely characteristic incorporation of 11 *cis*,13 *trans*-18:2.

MATERIALS AND METHODS

Animals and tissues. Pigs (Landrace boar by Landrace × Large White sow) were fed a basal diet (barley, wheat, soybean meal, and canola meal) supplemented with either 2% added sunflower oil or a CLA mixture (Natural Lipids Ltd., Hovdebygda, Norway), from 61.5 to 106 kg live weight, as described elsewhere (12). The CLA content of the commercial product was 55.4% of the total FAME, and the distribution of CLA isomers is included in Table 2 (see below). The total fat content of the diet, which included the 2% added test oil, was 3.2% on a dry matter basis. Eight pigs from each diet were selected for total lipid analysis. At time of slaughter, liver, heart, inner back fat, and omental fat were removed, and portions were immediately frozen and maintained at −70°C until analyzed.

Separation of lipid classes and preparation of FAME. Liver and heart tissues were pulverized at dry ice temperature (31), and the total lipids were extracted with chloroform/methanol (1:1, vol/vol). All the lipid classes were separated on thin-layer plates by three-directional thin-layer chromatography (TLC) using silica gel H plates (32). For each tissue, two TLC plates were used to separate 3-mg portions of total lipids each, to ensure sufficient material was available for analysis of the minor lipid classes. To each isolated lipid class (combined from the two TLC plates), a known amount of methyl heptadecanoate (17:0) was added as an internal standard to permit quantitation of the lipids. The lipid classes, in the presence of silica gel, were methylated using sodium methoxide (Supelco, Inc., Bellefonte, PA) for 15 min at 50°C to avoid isomerization of CLA isomers (29), except free fatty acids (FFA) and sphingomyelin (SM); FFA were methylated with diazomethane (33) and SM (because of *N*-acyl fatty acids) with 5% anhydrous HCl/methanol (w/w) for 1 h at 80°C (34). The resultant FAME were purified by TLC using hexane/diethyl ether/acetic acid (85:15:1, by vol) before analysis by GC and Ag^+-HPLC. Back fat and omental fat were methylated directly with sodium methoxide, and the resultant FAME were purified by TLC. Cholesterol was determined spectrophotometrically as described previously (35).

GC. The total FAME of each individual lipid class from the pig tissues investigated were analyzed by GC (model 5890; Hewlett-Packard, Palo Alto, CA), equipped with a flame-ionization detector. A CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness; Chrompack, Bridgewater, NJ) was used, and H_2 was the carrier gas at a split ratio of 1:15 (29). The column was operated at 150°C for 2 min, then temperature-programmed at 1°C/min to 200°C, followed by a second temperature program at 5°C/min to 215°C, and finally held for 20 min at 215°C; the total run time was 75 min.

HPLC. The total FAME mixtures were separated on a ChromSpher 5 Lipids (4.6 mm i.d. × 25 cm stainless steel; 5-μm particle size) silver-impregnated column (Chrompack), as described recently (30). The mobile phase was 0.1% acetonitrile in hexane, prepared fresh each day. The column was operated isocratically at room temperature for 1 h prior to the initial injection of the day. The flow rate of the mobile phase was 1.1 mL/min, and detection was by ultraviolet at 233 nm. If the HPLC column required regeneration, it was flushed with 1% acetonitrile for 4 h, followed by 1 h with 0.1% acetonitrile. The FAME of each lipid class from all eight pigs were combined for the HPLC analyses. Duplicate HPLC analyses were performed but they were identical, and therefore, no standard errors were calculated. The limited data derived in this study suggest that the response of the different CLA isomers measured at 233 nm was accurate relative to one another. There was generally good agreement in the relative distribution of CLA isomers between GC and Ag^+-HPLC results.

Derivatives of 4,4-dimethyloxazoline (DMOX). Total tissue FAME were hydrolyzed to their FFA using 1 N KOH in 95% ethanol (33). The FFA were placed into a 1-mL screw cap reaction tube with a Teflon liner, and a threefold excess

of 2-amino-2-methyl-1-propanol was added. After purging with argon, the reaction vial was heated for 0.5 h at 170°C. The DMOX derivative was then partitioned into petroleum ether as described previously (36). The reaction product was finally taken up in a minimal amount of isooctane for subsequent GC-EIMS and GC-DD-FTIR analyses.

GC-EIMS and GC-DD-FTIR. The equipment and operating conditions used for the GC-EIMS (37) and GC-DD-FTIR (30,38) are given elsewhere. For GC-EIMS, a 100-m CP-Sil 88 column was used, whereas a 50-m CP-Sil 88 column was used for the GC-DD-FTIR work.

RESULTS

Lipid class composition. The inclusion of CLA in the diet of pigs, fed from 61.5 to 106 kg live weight, did not significantly alter the liver and heart lipid class composition, except for cardiac triacylglycerol (TAG) (Table 1). Heart TAG in pigs fed CLA showed a decrease which was not significant because of the large animal-to-animal variation. Characteristic differences evident between liver and heart lipids were related to the content of phosphatidylcholine (PC), diphosphatidylglycerol (DPG), FFA, and cholesteryl ester (CE). PC, FFA, and CE were lower, and DPG was higher in heart compared to liver lipids (Table 1). There were no diet effects on the total free cholesterol and CE content in liver and heart lipids. The heart contained less free and esterified cholesterol compared to the liver (Table 1).

Separation of CLA isomers by GC. The fatty acid composition of liver, heart, inner back fat, and omental fat lipids was determined by GC using a long capillary column (100 m) with a polar liquid phase (CP-Sil 88), as demonstrated previously for milk analysis (29). The CLA region of the GC chromatogram that is bracketed between linoleic (18:2n-6) and arachidonic (20:4n-6) acids is presented (Figs. 1 and 2) in order to show the position of CLA isomers in relation to neighboring FAME present in tissue lipids. The CLA diet (Fig. 1A) and selected tissue lipid classes of pigs fed CLA (Fig. 1B–D and Fig. 2A–D) are shown. On this GC column, all the CLA isomers eluted between linolenic acid (18:3n-3) and 20:2n-6, a relatively clear region in the GC chromatogram of tissue FAME, with two exceptions. A small (<0.1%) unidentified peak marked "x" (Figs. 1A and 2A) was observed in the GC chromatograms of all lipid classes of pigs fed the control diets, and was also found in the dietary commercial CLA oil. Therefore, minor peak "x" was presumably a combination of an unknown non-CLA and CLA isomer; peak "x" was not included in Table 2. In addition, 21:0, found only in SM, coeluted with the 8 *cis*,10 *cis*-18:2 peak. The GC column was used to separate the *cis/trans* CLA isomers into four peaks in the order: 9 *cis*,11 *trans*-18:2 plus 8 *trans*,10 *cis*-18:2 (tailing peak); a minor component tentatively identified as 9 *trans*,11 *cis*-18:2; 11 *cis*,13 *trans*-18:2; and 10 *trans*,12 *cis*-18:2. The three major *cis/trans* peaks were identified by GC-DD-FTIR and GC-EIMS as their DMOX derivatives. The minor *cis/trans* peak was identified as 9 *trans*,11 *cis*-18:2 based on comparison with the 9 *cis*,11 *trans*-18:2 commercial standard mixture from Matraya Inc. (Pleasant Gap, PA), which contained trace amounts the 9 *trans*,11 *cis*-18:2 isomer (21). The *cis,cis* CLA isomers also separated into four peaks (Figs. 1 and 2). The *cis,cis* configuration was established by GC-DD-FTIR, and the molecular weight was established by GC-EIMS as the DMOX derivative. The elution order of the positional *cis,cis* CLA isomers by GC (8,10; 9,11; 10,12; and 11,13) was determined by comparison with Ag^+-HPLC results (*vide infra*) and found to be the opposite of the latter. The *trans,trans* CLA isomers separated into two peaks: 11 *trans*,13 *trans*-18:2; and 8 *trans*,10 *trans*-18:2, 9 *trans*,11 *trans*-18:2 plus 10 *trans*,12 *trans*-18:2. The *trans,trans* configuration was confirmed by GC-DD-FTIR and the molecular weight and double-bond positions by GC-EIMS.

Separation of CLA isomers by Ag^+-HPLC. The Ag^+-HPLC method, just developed (30), was applied to the separation of FAME CLA isomers in the diet (Fig. 1A') and pig tissue lipid classes (Fig. 1B'–D', and Fig. 2A'–D'). Ultraviolet detection at 233 nm selectively identified FAME with a conjugated double-bond system. Ag^+-HPLC was used to separate the *cis/trans* CLA isomers into four major peaks in the order: 11 *cis*,13 *trans*-18:2, 10 *trans*,12 *cis*-18:2, 9 *cis*,11 *trans*-18:2, and 8 *trans*,10 *cis*-18:2; see Figures 1 and 2. The structures of all four *cis/trans* CLA isomers were identified by GC-DD-FTIR and GC-EIMS as their DMOX derivatives, and by comparison with known standards (30). The *trans,trans* CLA isomers eluted first from the silver ion column. The structures of four *trans,trans* isomers were established by comparison to known and/or standard mixtures of CLA isomers (30); see Figures 1 and 2. Based on the absorption at 233 nm, there were another four unidentified peaks of FAME in this region, including the shoulder on 8 *trans*,10 *trans*-18:2. Infrared and

TABLE 1
Composition (% of total lipids) of Liver and Heart Lipid Classes of Pigs Fed Control or CLA Diets as Determined by GC[a]

Lipid class	Liver			Heart		
	Control	CLA	SD[b]	Control	CLA	SD
PC	46.9[c]	50.5	5.3	39.8	40.5	4.5
PE	24.2	23.7	1.4	22.4	24.4	3.3
PS	4.4	4.4	0.5	3.9	3.7	0.7
PI	7.5	7.3	0.9	5.8	5.8	0.7
DPG	3.2	3.2	0.6	14.6	15.3	2.0
SM	2.9	2.7	1.0	2.4	3.0	0.7
TAG	4.7	4.9	1.2	10.4	6.6	6.0
FFA	3.4	2.0	1.0	0.8	0.6	0.3
CE	1.6	1.3	0.3	<0.1	<0.1	
Cholesterol[d]	3.60	3.70	1.05	2.20	2.02	0.58

[a]CLA, conjugated linoleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; DPG, diphosphatidylglycerol (cardiolipin); SM, sphingomyelin; TAG, triacylglycerol; FFA, free fatty acid; CE, cholesteryl ester; GC, gas chromatography.
[b]Pooled standard deviation (SD).
[c]Mean of six pigs/diet.
[d]Cholesterol expressed as μg/g wet weight.

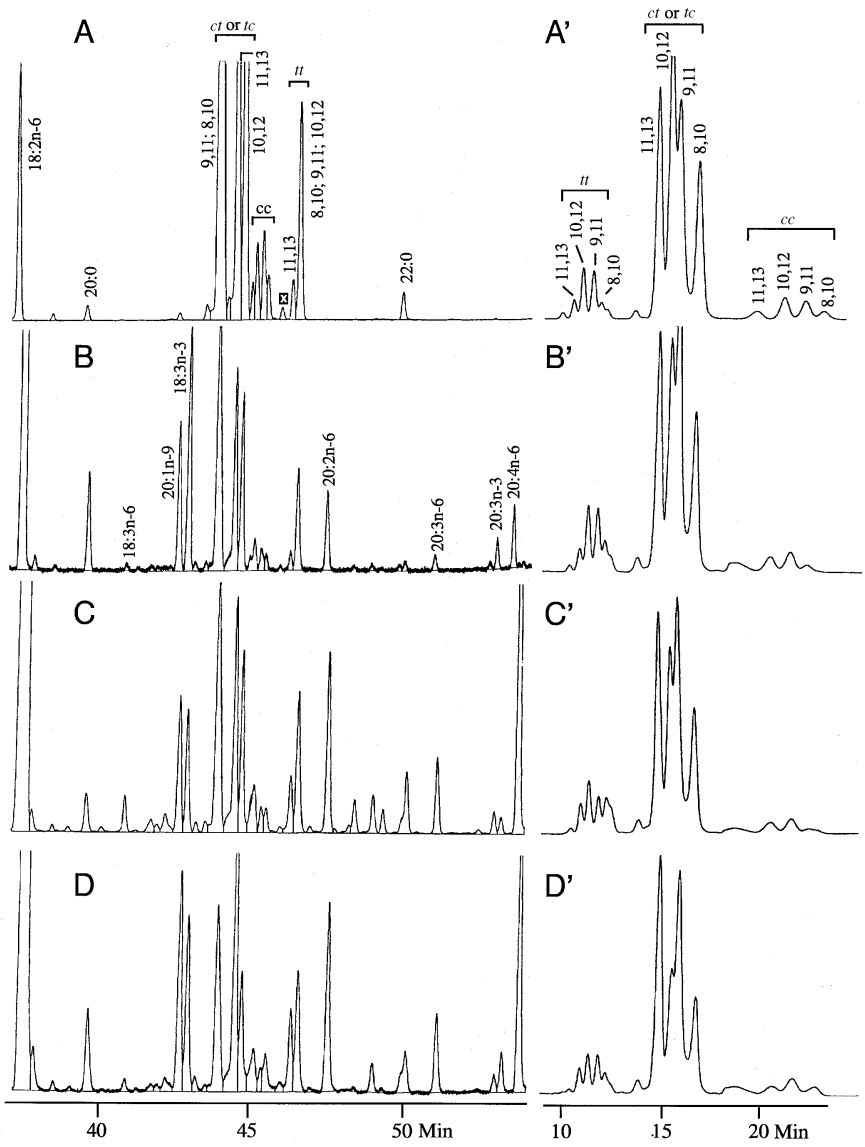

FIG. 1. Partial profiles obtained by gas chromatography (GC) (A to D) and silver-ion high-performance liquid chromatography (Ag^+-HPLC) (A' to D') of the conjugated linoleic acid (CLA) mixture fed to pigs (A,A'), and of CLA isomers found in selected tissues of pigs fed the CLA diet: omental fat (B,B'), liver triacylglycerols (TAG) (C,C'), and heart TAG (D,D'). The GC region selected was between linoleic (18:2n-6) and arachidonic (20:4n-6) acids. All known fatty acids and CLA isomers were labeled; "x" is an unknown in the GC chromatogram which was also found in the chromatograms of both the dietary CLA mixture and the tissues of pigs fed the control and CLA diets. The letters *c* and *t* refer to the *cis* and *trans* CLA isomers. The elution order of the cis,cis CLA isomers is: 8,10-, 9,11-, 10,12-, and 11,13-18:2.

mass spectral confirmation of these isomers is in progress. In the *cis,cis* region four peaks were separated which eluted in the same order as those of the *trans,trans* and *cis/trans* CLA isomers (30) (Figs. 1 and 2).

There may be slight differences in the distribution of CLA isomers between the GC and the Ag^+-HPLC chromatograms in Figures 1 and 2. There were two reasons for this difference. (i) One of eight available GC chromatograms was selected for display (Figs. 1 and 2) and may not reflect the average distribution, whereas for the Ag^+-HPLC, only a single chromatogram was available after the FAME for the same lipid class from all eight pigs on the CLA diet were combined. (ii) Other still unidentified minor components or metabolites of these complex mixtures may also be eluting in the CLA region of the Ag^+-HPLC chromatogram.

CLA content in pig tissues fed the control diet. Pigs fed the control diet showed relatively small peaks (<0.1%) corresponding in retention time to 9 *cis*,11 *trans*-18:2, 9 *trans*,11 *trans*-18:2, and unknown "x" on the GC chromatogram (chromatogram not shown). CLA isomers in tissues from control

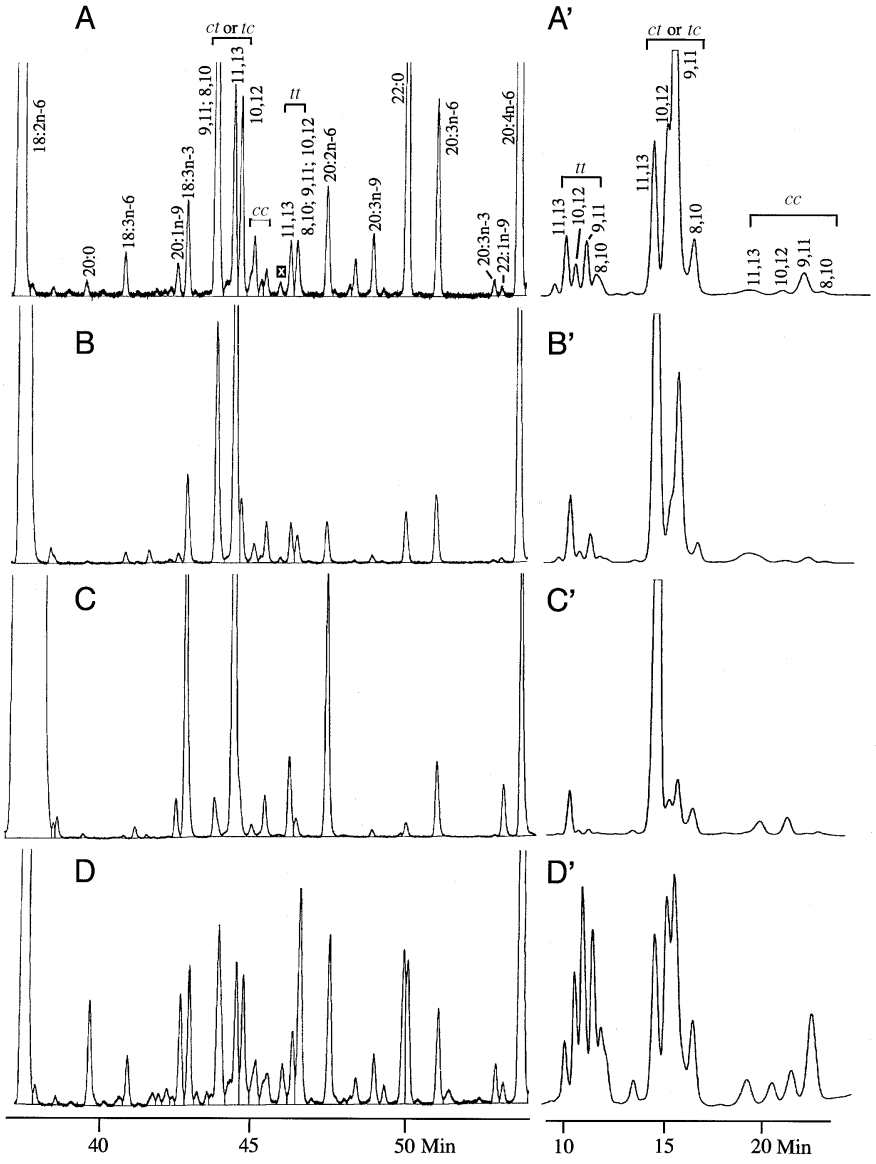

FIG. 2. Partial GC (A to D) and Ag^+-HPLC (A' to D') chromatograms of selected liver and heart lipid classes of pigs fed the CLA diet: liver phosphatidylcholine (PC) (A,A'), heart PC (B,B'), heart diphosphatidylglycerol (DPG) (C,C'), and liver free fatty acids (FFA) (D,D'). For other abbreviations see Figure 1.

animals were only confirmed by their relative retention time on GC. The concentrations of the CLA isomers in the lipids of pigs fed the control diet were too low under our experimental conditions to be detected by ultraviolet at 233 nm after Ag^+-HPLC separation.

CLA isomeric distribution in the diet and adipose tissue. The CLA preparation, included at 2% by weight in the diet, contained 81.7% *cis/trans* CLA isomers, based on Ag^+-HPLC analyses. The reported values are expressed as percentages of total CLA content. The relative concentrations of the four *cis/trans* CLA isomers were found by Ag^+-HPLC to be: 18.9% 11 *cis*,13 *trans*-18:2; 26.3% 10 *trans*,12 *cis*-18:2; 20.4% 9 *cis*,11 *trans*-18:2; and 16.1% 8 *trans*,10 *cis*-18:2. The GC results of the diet are included in Table 2. The total *trans,trans* and *cis,cis* CLA contents were 10.1 and 7.4%, respectively. The inner back fat and omental fat (Figs. 1B and 1B') showed a similar distribution of CLA isomers as that found in the dietary CLA oil.

Total CLA content in tissue lipid classes. The total CLA content in all the tissue lipid classes is shown in Table 2 (last column). The total CLA content was highest in liver TAG (6%) and lowest in liver SM (1%). Back fat showed a higher accumulation of total CLA than omental fat (Table 2). The major phospholipids, PC and PE (phosphatidylethanolamine), incorporated between 2.8 and 5.8% of total CLA. The remaining phospholipids incorporated between 1.5 and 4% of total CLA. DPG, which is a major phospholipid in inner mitochondrial membranes of liver and heart, accumulated about

TABLE 2
Relative Composition (%) Determined by GC of CLA Isomers[a] in Diet, Liver, and Heart Lipid Classes, and Inner Back Fat and Omental Fat of Pigs Fed a CLA Diet

	CLA isomers										
Tissues Diet Classes[b]	9*c*,11*t* 8*t*,10*c*	9*t*,11*c*	11*c*,13*t*	10*t*,12*c*	8*c*,10*c*	9*c*,11*c*	10*c*,12*c*	11*c*,13*c*	11*t*,13*t*	10*t*,12*t* 9*t*,11*t* 8*t*,10*t*	Total CLA (%)[c]
Diet	35.3	0.8	19.9	24.9	1.2	2.7	3.5	1.6	1.5	8.7	32.9
Liver											
TAG	33.2 ± 1.2[d]	1.6 ± 0.3	21.6 ± 1.4	16.0 ± 1.2	2.5 ± 0.6	3.8 ± 0.7	2.1 ± 0.2	2.1 ± 0.1	4.4 ± 0.3	11.7 ± 1.1	6.0 ± 0.4
PC	48.7 ± 1.4	1.6 ± 0.4	16.1 ± 1.0	15.2 ± 1.0	1.4 ± 0.2	4.3 ± 0.5	0.9 ± 0.2	1.9 ± 0.2	4.1 ± 0.5	4.8 ± 0.4	3.2 ± 0.2
PE	53.9 ± 2.7	1.4 ± 0.4	15.5 ± 1.2	9.4 ± 0.7	1.7 ± 0.2	1.8 ± 0.3	1.0 ± 0.3	1.8 ± 0.3	5.4 ± 0.8	6.4 ± 0.5	2.8 ± 0.2
PS	39.9 ± 3.0	1.1 ± 0.6	11.9 ± 1.0	9.7 ± 1.3	1.3 ± 0.9	3.4 ± 1.4	0.6 ± 0.2	1.7 ± 0.7	11.4 ± 2.5	10.2 ± 0.8	1.6 ± 0.2
PI	38.3 ± 4.2	4.0 ± 1.2	12.1 ± 1.1	8.2 ± 0.9	3.4 ± 1.0	5.1 ± 1.1	1.3 ± 0.3	2.6 ± 0.4	6.2 ± 1.0	15.0 ± 1.9	1.9 ± 0.2
DPG	17.9 ± 1.2	1.2 ± 0.9	36.9 ± 5.8	8.1 ± 2.3	1.7 ± 1.1	3.9 ± 1.1	0.9 ± 0.7	3.5 ± 1.1	6.2 ± 1.3	11.5 ± 3.4	1.5 ± 0.2
SM	25.3 ± 2.9	5.0 ± 0.8	10.4 ± 1.1	10.4 ± 1.1	—[e]	1.2 ± 0.6	1.7 ± 0.4	3.6 ± 0.7	9.8 ± 0.2	31.1 ± 3.4	1.0 ± 0.1
FFA	19.0 ± 2.7	3.4 ± 0.7	11.1 ± 1.6	14.5 ± 1.1	2.7 ± 0.7	4.3 ± 0.4	2.3 ± 0.5	3.8 ± 0.5	7.7 ± 0.8	23.1 ± 2.5	2.9 ± 0.2
CE	31.1 ± 2.2	3.3 ± 1.4	15.4 ± 1.1	13.7 ± 1.5	1.8 ± 0.9	3.8 ± 1.8	1.4 ± 0.7	2.0 ± 0.9	6.7 ± 2.4	17.9 ± 3.2	3.5 ± 0.4
Heart											
TAG	26.5 ± 3.5	2.1 ± 0.7	25.7 ± 4.3	12.1 ± 0.6	1.5 ± 0.4	4.8 ± 0.5	2.1 ± 0.1	4.0 ± 0.5	7.0 ± 1.0	12.6 ± 1.8	3.6 ± 0.4
PC	25.1 ± 2.1	0.7 ± 0.2	55.5 ± 4.3	6.2 ± 0.3	0.4 ± 0.2	1.4 ± 0.3	0.7 ± 0.1	3.7 ± 0.5	3.3 ± 0.9	2.5 ± 1.1	5.8 ± 0.4
PE	29.7 ± 2.4	0.8 ± 0.3	42.2 ± 3.8	6.8 ± 0.8	1.0 ± 0.3	1.3 ± 0.3	0.8 ± 0.2	3.0 ± 1.1	6.9 ± 1.1	5.0 ± 1.0	2.8 ± 0.2
PS	24.1 ± 4.5	0.7 ± 0.3	34.3 ± 3.1	6.5 ± 1.3	2.4 ± 1.3	3.5 ± 0.7	0.5 ± 0.2	4.6 ± 0.7	10.2 ± 0.9	8.5 ± 1.0	4.1 ± 0.5
PI	18.7 ± 2.4	1.6 ± 0.5	40.0 ± 3.6	8.3 ± 2.4	2.3 ± 0.4	3.1 ± 0.4	1.1 ± 0.3	4.7 ± 0.5	9.5 ± 2.7	9.3 ± 1.4	3.6 ± 0.3
DPG	7.2 ± 1.4	1.1 ± 0.5	65.1 ± 4.8	4.5 ± 1.0	0.1 ± 0.1	2.0 ± 0.4	0.3 ± 0.02	4.8 ± 0.4	9.6 ± 1.1	4.4 ± 2.2	1.9 ± 0.2
SM	14.8 ± 5.5	<0.05	38.3 ± 9.3	6.1 ± 2.1	—[e]	1.7 ± 0.6	<0.05	4.0 ± 1.1	15.8 ± 5.7	19.3 ± 8.0	1.8 ± 0.5
FFA	10.9 ± 1.4	2.0 ± 1.2	25.1 ± 3.0	10.7 ± 1.1	0.6 ± 0.3	4.9 ± 0.4	0.9 ± 0.5	6.8 ± 0.7	13.4 ± 1.4	17.8 ± 3.6	3.2 ± 0.2
Back fat	38.7 ± 5.2	1.9 ± 0.3	20.5 ± 3.4	17.1 ± 3.2	1.2 ± 0.3	3.1 ± 0.5	2.5 ± 0.5	1.7 ± 0.3	2.0 ± 0.4	10.7 ± 1.5	4.7 ± 0.4
Omental fat	36.1 ± 1.4	1.8 ± 0.7	20.7 ± 3.6	17.9 ± 3.2	1.1 ± 0.6	3.5 ± 0.4	2.8 ± 0.4	1.8 ± 0.4	2.1 ± 0.3	11.6 ± 1.8	2.9 ± 0.5

[a]The identification of the major CLA isomers was confirmed by spectroscopic analyses and comparison with Ag^+-HPLC. The peak between 11*c*,13*c*-18:2 and 11*t*,13*t*-18:2, labeled "x" in Figures 1A and 2A, was not included because it was also present in controls.
[b]For abbreviations see Table 1.
[c]Percentage of total CLA isomers in the total fatty acid methyl ester composition of each lipid class.
[d]Values are means ± SD (n = 8).
[e]The concentration of 8*c*,10*c*-18:2 could not be quantitated in the SM fraction using this GC column because this CLA isomer coeluted with 21:0 present in SM.

2% of total CLA. The highest relative content of *trans,trans* CLA isomers was found in the FFA fraction (Table 2); see liver FFA chromatogram (Figs. 2D and 2D').

Relative CLA isomeric distribution in liver lipids. The distribution and standard deviation (SD) data for CLA isomers determined by GC are presented in Table 2. The relative composition of CLA isomers in liver lipids is summarized graphically in Figure 3 in order to compare it to that present in the dietary CLA oil used. The sum of 9 *cis*,11 *trans*-18:2 and 8 *trans*,10 *cis*-18:2 was increased in liver PC and PE and decreased in DPG, SM, and FFA. The neutral lipids (TAG and CE) and the minor phospholipids (phosphatidylserine and phosphatidylinositol) showed no selectivity in the incorporation of these two CLA isomers compared to the diet. No further conclusions could be made on these data because these two CLA isomers were not separated by GC. To evaluate the effects of each of these two individual isomers, see the Ag^+-HPLC results (Fig. 4). On the other hand, 11 *cis*,13 *trans*-18:2 accumulated in DPG, where its relative concentration increased to 40%, compared to about 20% in the CLA oil. The 10 *trans*,12 *cis*-18:2 isomer was relatively low in all liver lipids compared to its level in the diet. The content of the four

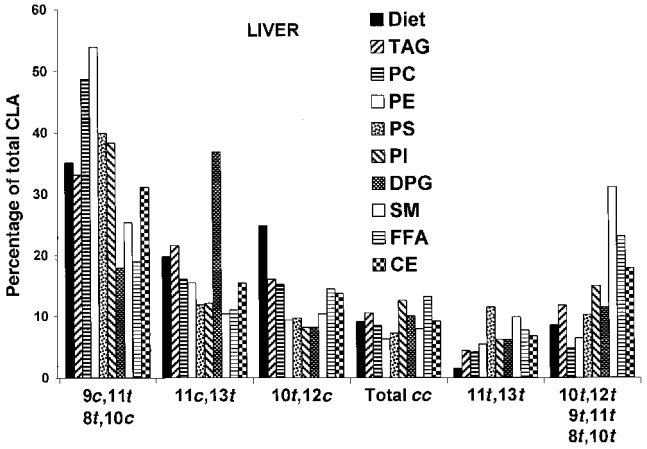

FIG. 3. Quantitative comparison of CLA isomeric distributions determined by GC for the different liver lipid classes that had been previously isolated by thin-layer chromatography (TLC). Relative amounts (*y* axis) are expressed as percentages of total CLA. The CLA isomers are shown (*x* axis) in the GC elution sequence. Total *cc* represents the total of four minor *cis,cis* CLA isomers. The distribution of the CLA isomers in the diet is included for comparison. PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; CE, cholesteryl ester. For other abbreviations see Figures 1 and 2.

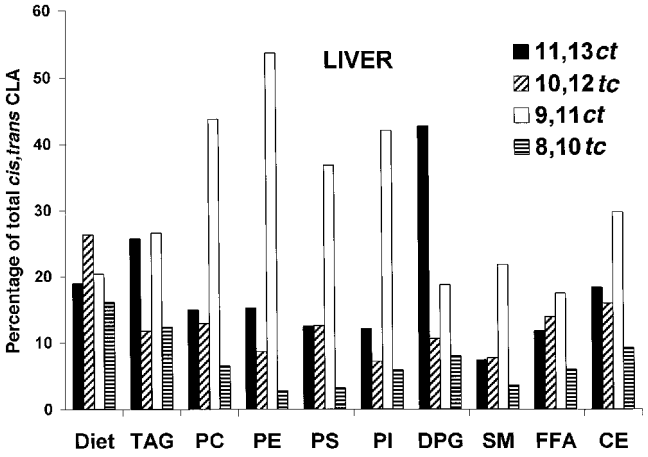

FIG. 4. Quantitative comparison of the four major *cis/trans* CLA isomers in the different liver lipid classes and the diet determined by Ag^+-HPLC. Relative amounts (*y* axis) are expressed as percentage of total *cis/trans* CLA isomers. The distribution of the *cis/trans* CLA isomers in the diet are included for comparison. For abbreviations see Figures 1–3.

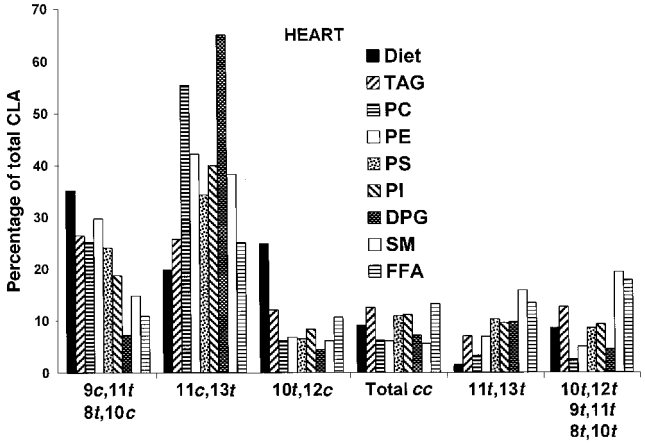

FIG. 5. Quantitative comparison of CLA isomeric distribution determined by GC for the different heart lipid classes that were previously isolated by TLC. For abbreviations see Figures 1–3.

cis,cis CLA isomers was low in liver lipids. Their total concentrations were combined for presentation in Figure 3. The *trans,trans* isomers appeared to accumulate in liver lipids: 11 *trans*,13 *trans*-18:2 into phosphatidylserine and SM, and the mixture of the three *trans,trans* CLA (10,12-, 9,11-, and 8,10-18:2) isomers into SM, FFA, and CE (Fig. 3). The increase of *trans,trans* CLA isomers in SM was due to the acid (HCl/methanol) methylation (29) required to hydrolyze the *N*-acyl fatty acids.

The distribution of the major *cis/trans* CLA isomers in all the liver lipid classes, identified by Ag^+-HPLC, is presented in Figure 4. The results indicate that 9 *cis*,11 *trans*-18:2 was the major isomer in all liver lipids, except DPG, which showed a marked increase in 11 *cis*,13 *trans*-18:2. Furthermore, most liver lipid classes showed markedly lower levels of 8 *trans*,10 *cis*-18:2, and in some lipid classes lower levels 10 *trans*, 12 *cis*-18:2 compared to the distribution found in the CLA diet.

Relative CLA isomeric distribution in heart lipids. Contrary to the liver lipids, the heart showed a high content of the 11 *cis*,13 *trans*-18:2 CLA isomer in all lipid classes, except TAG, as determined by both GC (Fig. 5) and Ag^+-HPLC (Fig. 6). The accumulation was highest in cardiac DPG, in which the relative concentration of the 11 *cis*,13 *trans*-18:2 isomer reached 77% of the total CLA (HPLC results). The second major isomer in heart lipids was 9 *cis*,11 *trans*-18:2. The heart lipids showed much lower levels of 8 *trans*,10 *cis*-18:2 and 10 *trans*,12 *cis*-18:2 compared to those found in the CLA diet (Fig. 6). The content of the *cis,cis* CLA isomers was not increased in the heart (Fig. 5), whereas the *trans,trans* CLA isomers appeared to accumulate relative to the CLA diet, especially in heart FFA (Fig. 5). Again the *trans,trans* content in the SM fractions should be viewed with caution because SM had to be methylated with HCl/methanol (29).

DISCUSSION

Improved separation of CLA isomers. By using optimal conditions, the long polar GC capillary column (100 m SP-Sil 88) separated 10 peaks attributed to CLA (Figs. 1 and 2). Some isomers coeluted, and their complete assignments were not possible, even using GC-DD-FTIR and GC-EIMS. However, identification of the CLA isomers became possible after the development of a new Ag^+-HPLC method (30). Ag^+-HPLC was used to separate *trans,trans*, *cis/trans*, and *cis,cis* CLA positional isomers found in the diet and pig tissues (Figs. 1 and 2). The CLA isomers were identified by comparison to known CLA mixtures and by GC-DD-FTIR and GC-EIMS as their DMOX derivatives (30). Some GC assignments were also confirmed by spiking the samples with known CLA standards. A systematic comparison of the relative intensities of the CLA isomers in the Ag^+-HPLC and GC chromatograms permitted the identification of the isomers resolved by GC. A good example was the one for heart lipids in which the

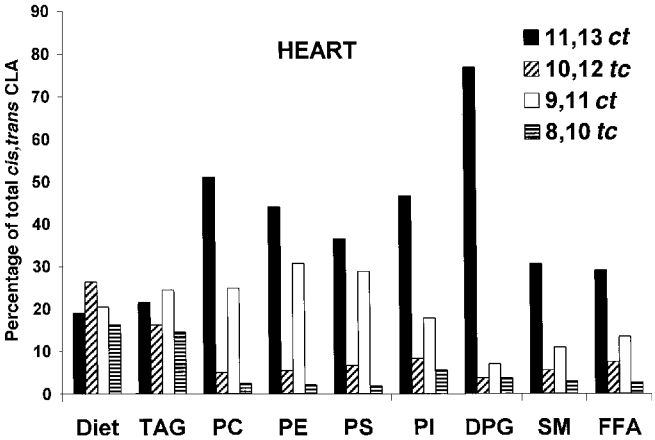

FIG. 6. Quantitative comparison of the four major *cis/trans* CLA isomers in the different heart lipid classes and the diet determined by Ag^+-HPLC. For abbreviations see Figures 1–3.

trans,trans, *cis/trans*, and *cis,cis* 11,13-18:2 were the major CLA isomers in both the Ag^+-HPLC (Fig. 2B') and GC (Fig. 2B) chromatograms.

Previous studies reporting the CLA content in animal tissues have not adequately resolved the CLA isomers, which is now possible using the Ag^+-HPLC method (30). The literature results were presented as 9 *cis*,11 *trans*-18:2 (1,2), total CLA (5,39), or total CLA in total neutral lipids and total phospholipids without (7,26) or with (27,28) some isomer identification. In one study the distribution of CLA isomers in the liver lipid classes was reported (28). Furthermore, in many studies, acid-catalyzed methylation procedures were used (1,2,5,7,13,16,18,20,26,28) to prepare FAME for GC analysis, which generally increased the content of *trans,trans* CLA isomers due to double-bond isomerization (29). That left much of the data on CLA incorporation difficult to interpret other than a general knowledge of total CLA levels. For example, Sugano *et al.* (28), who recently reported the CLA distribution in liver lipid classes of rats fed a 1% CLA preparation for 2 wk, methylated the isolated lipid classes with BF_3/methanol, which significantly increased the *trans,trans* CLA content in all lipid classes. Therefore, the reported CLA composition likely included artifacts: (i) the *trans,trans* isomers were reported as the major isomer in DPG (or cardiolipin), phosphatidylserine, phosphatidylinositol, and PE (28), and (ii) we suspect that one CLA isomer, 9 *cis*,11 *cis*-18:2, was mislabeled in liver DPG (Ref. 28, Fig. 2E). Based on our data (Fig. 2 C and C'), it is presumably 11 *cis*,13 *trans*-18:2. However, as noted earlier, the incorporation of total CLA isomers into hepatic phospholipids, which in their study ranged from 0.8 to 4.5% (28), was generally similar in magnitude to that found in pig liver phospholipids (1.0 to 3.2%, Table 2).

Distributions of CLA isomers into pig tissue lipids. This is the first report showing the accurate separation of CLA isomers found in individual tissue lipid classes from test animals fed CLA. Work is still in progress on the identification of CLA metabolites in pig tissues, and hence was not included in this publication. The total CLA content in pig tissue lipids ranged from 1 to 6%, depending on the tissue and lipid class (Table 2). Generally, the CLA content was higher in the neutral lipids such as TAG, CE, and FFA (3–6%) than in phospholipids such as SM and DPG (1–2%). However, the changes in tissue CLA incorporation appeared to have had no effect on the quantitative lipid class composition in the liver and heart of pigs fed this CLA mixture, except possibly cardiac TAG (Table 1).

Our results indicate that the relative absorption of all the CLA isomers was similar, since there was generally no difference between the distribution of CLA isomers in the commercial CLA preparation fed to pigs and in inner back fat, omental fat, liver TAG, and heart TAG. Sugano *et al.* (28) measured the lymphatic recovery and CLA isomeric composition of the lymph fluid in the rat and found that total CLA was absorbed less (~55%) than linoleic acid (70–80%). However, the composition data included a high *trans,trans* content, presumably generated during methylation with BF_3/methanol. By ignoring their *trans,trans* content in the dietary emulsion and the lymph fluid, the relative distribution of the remaining CLA isomers was similar before and after absorption, which is consistent with our results. The feeding of CLA was reported to decrease subcutaneous fat and increase lean meat (11,12). However, this physiological change was not accompanied by any change in the distribution of CLA isomers in the inner back fat (subcutaneous fat).

Liver and heart each showed a uniquely characteristic distribution of CLA isomers even though both tissues received a generally similar CLA distribution, as evidenced by their respective TAG composition. The reason for the observed differences in relative accumulation of the different CLA isomers in the tissues lipid classes is not clear. The tissue fatty acid composition is a dynamic system constantly receiving, metabolizing, oxidizing, and incorporating dietary fatty acids. The accumulation of 11 *cis*,13 *trans*-18:2 could be due to slower metabolism of this isomer or preferential incorporation. On the other hand, the low content of both 10 *trans*,12 *cis*-18:2 and 8 *trans*,10 *cis*-18:2 could be due to rapid metabolism or selective discrimination. Metabolites of CLA isomers were shown to occur in animal tissues (40,41). In fact, Sébédio *et al.* (42) found that 10,12-18:2 was metabolized to 8,12,14-20:3 and 5,8,12,14-20:4 in essential fatty acid-deficient rats. This would indicate that 10 *trans*,12 *cis*-18:2 was metabolized.

The contents of FFA in both liver and heart lipids were less than 3 and 1%, respectively (Table 1). However, in the FFA fractions, the relative concentrations of the *trans,trans* CLA isomers were more than 30% of the total CLA isomers (Fig. 2D') compared to 10% in the diet. These results suggest that the *trans,trans* CLA isomers were metabolized more slowly than the corresponding *cis/trans* CLA isomers. This result is similar to that observed when rats were fed a diet rich in erucic acid, which accumulated in the cardiac FFA fraction (31). This was partially due to the slower rate of metabolism of erucic acid compared to other fatty acids (43).

Significance of the unique distribution of CLA isomers into pig tissue lipids. The information available to date does not permit drawing a definitive conclusion regarding the favorable or adverse biological activity of any of the CLA isomers. To our knowledge, there are no studies in which individual synthetic CLA isomers have been evaluated in biological systems. The only study in which a natural extract (from fried ground beef) was used exhibited anticarcinogenic properties (44).

If 9 *cis*,11 *trans*-18:2 is assumed to be the active CLA isomer, then the incorporation/accumulation of the 11 *cis*,13 *trans*-18:2 CLA isomer into heart phospholipids, and particularly DPG in both heart and liver, could be viewed with concern. DPG is found principally in the inner mitochondrial membrane and is intrinsically involved in many of the enzymes of bioenergetics of mitochondria; see reviews (45,46). The incorporation of 11 *cis*,13 *trans*-18:2 into mitochondrial DPG could adversely affect the activity of key enzymes in mitochondrial energetics, because DPG is firmly imbedded in

many of these enzymes (45). A marked decrease in the linoleic acid content of inner mitochondrial DPG could also affect enzyme activity. In fact, Sugano *et al.* (28) showed that feeding CLA to rats produced a significant decrease of linoleic acid from 69.6 to 54.7% in liver DPG. However, we did not find a change in the content of linoleic acid in pig liver (control 75% vs. CLA 73%) or heart (control 85% vs. CLA 86%) DPG fed the CLA diet (the complete fatty acid composition will be published elsewhere). We also did not find any gross pathological abnormalities in this pig study (12). It should be noted that specific mitochondrial functions have not yet been evaluated.

The large epidemiological study conducted in Finland (19) indirectly supports the conclusion that 9 *cis*,11 *trans*-18:2 is the active isomer, because this is the major CLA isomer in milk. We have reexamined milk and dairy products using the new Ag^+-HPLC method (30) and confirmed that 9 *cis*,11 *trans*-18:2 is the major CLA isomer in milk (Sehat, N., private communication). Based on this assumption, it might be prudent to revise commercial CLA preparations to exclude the 11 *cis*,13 *trans*-18:2 CLA isomer as a major component.

On the other hand, if we assume that 9 *cis*,11 *trans*-18:2 is not the only active isomer in CLA preparations, then the incorporation of 11 *cis*,13 *trans*-18:2 into cardiac and DPG lipids (this study), and/or the desaturation and elongation of 10 *trans*,12 *cis*-18:2 in essential fatty acid-deficient rats (42), could be viewed as beneficial. Certainly many of the feeding trials, which included several CLA isomers, have also shown anticarcinogenic (1–8) and other beneficial responses (9–13). Pariza *et al.* (47) reported that 9 *cis*,11 *trans*-18:2 was metabolized to 11 *cis*,13 *trans*-20:2, and the latter exhibited similar biological activity to that of CLA isomeric mixtures. This is most interesting, since the configuration of 11 *cis*,13 *trans*-20:2 from the carboxyl group to the conjugated system is identical to that of 11 *cis*,13 *trans*-18:2.

There is a definite need for further feeding studies using pure CLA isomers to clarify these issues.

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