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Comparison of Different Methylation Methods for the Analysis of Conjugated Linoleic Acid Isomers by Silver Ion HPLC in Beef Lipids

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Four different methods for the methylation of conjugated linoleic acid isomers (CLA) in ruminant lipids were compared by silver ion (Ag^+) HPLC. The combination of base-catalyzed methods followed by an acid-catalyzed method with BF_3/MeOH was tested under different temperatures (room temperature and at 60 °C), along with based-catalyzed methylation with $NaOCH_3$ and methylation with BF_3/MeOH after saponification with NaOH. The comparison among these four methods was done on muscle and adipose tissue samples from bulls. The repeatability θ of the combined base- and acid-catalyzed methylation ($NaOCH_3$/BF_3) at ambient temperature for 20 min and at 60 °C for 10 min was most suitable for the quantitative Ag^+-HPLC analysis of CLA isomers. At 60 °C the combined methods supplied the highest concentrations of most CLA isomers. The base-catalyzed methylation and the saponification followed by BF_3/MeOH methylation for 5 min generated significantly lower concentrations for most CLA isomers compared to the combined methods.

KEYWORDS: Conjugated linoleic acid isomers; methylation procedures; comparison; repeatability; beef

INTRODUCTION

There is increasing interest in conjugated linoleic acid (CLA) because of its potential therapeutic actions (see reviews in refs *1* and *2*). Consumers wish to know the total quantitative fatty acid composition including CLA of milk, meat, and processed products. Recently it has been shown that meat and meat products contribute about 20−25% of the total human CLA intake (*3*).

Investigation of the concentration of the CLA isomers in biological samples such as milk, muscle, or adipose tissues requires lipid extraction followed by preparation of methyl ester. Methyl esters are usually prepared according to a base or acid catalysis procedure. There is no single method that is optimal for all kinds of samples (see review in ref *4*). Methylation using sodium methoxide is preferred for the CLA isomer analysis (*5*, *6*). The problems with acid-catalyzed methods are the isomerization of CLA*cis*/*trans* isomers into *trans*/*trans* isomers and the production of allylic methoxy artifacts (*5*, *7*, *8*). The disadvantage of BF_3/methanol is the limited shelf life of the reagent and the production of artifacts at higher reaction temperatures and long reaction times (*8*). No single derivatization procedure exists that simultaneously methylates all different lipid fractions including triacylglycerols, free fatty acids, phospholipids, plasmalogens, sphingolipids and glycosphingolipids, and very short chain fatty acids (*6*). There have been a number of attempts to establish methylation procedures applicable for CLA in different tissues (*4*−*11*). Base-catalyzed methylation has the advantage of not isomerizing CLA, but free fatty acids, amides, and alk-1-enyl ethers are not methylated under these conditions (*6*). Beef muscle phospholipids contain about 52% alk-1-enyl ethers in plasmalogens (*12*). Approximately 0.1% of the lipid content of muscle consists of free fatty acids (*13*). There is a need to investigate exactly the concentration of CLA isomers in all different lipid fractions including free fatty acids, *N*-acyl lipids, and plasmalogens of ruminant meat and meat products because of their different functions and effects on metabolism (*1*, *2*, *4*, *6*). To measure the concentration of CLA isomers in ruminant meat and meat products, the reproducibility and the recovery using two combined methylation methods ($NaOCH_3$/BF_3/MeOH) at a room temperature of 20−21 °C (RT) and at 60 °C were compared with the base-catalyzed $NaOCH_3$ procedure and with saponification followed by BF_3/MeOH methylation. Because a sufficient separation of all CLA isomers present in biological tissues is not possible by gas chromatography (*6*), the study exploited Ag^+-HPLC for the determination of 10 individual CLA isomers in beef (240 analyses in total) and a quantitative reference material spiked with CLA*cis*-9,*trans*-11.

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METHODS

General. Before derivatization, the intramuscular fat and subcutaneous fat were extracted from bulls of a previous experiment (*14*) to produce lipid mixtures representing all of the tissue lipids containing CLA isomers. In total, the lipids of 10 muscle and 10 subcutaneous adipose tissue samples from bulls fed on pasture and slaughtered at 630 kg of live weight were included in this comparison. The frozen muscle and fatty tissue samples were partially thawed at 4 °C. After blending and mincing of the samples in a food processor (Multiboy, Ilmenau, Germany), lipids were extracted from 40 g of muscle or 10 g of subcutaneous fat with chloroform/methanol (2:1, v/v) according to the method of Folch et al. (*15*) by homogenization (Bühler, 3 × 10 s, 30000 revolutions per minute; Hechingen, Germany) at room temperature. All solvents contained 0.005% (w/v) of *tert*-butylhydroxytoluene to avoid oxidation of polyunsaturated fatty acids. The extraction mixture was stored at 5 °C for 18 h in the dark and subsequently washed with 0.02% aqueous $CaCl_2$ solution. The organic phase was dried with Na_2SO_4 and K_2CO_3 (10:1, w/w), the solvent was removed under vacuum with a rotary evaporator, and finally the mixture was dried under nitrogen at room temperature. The extracted lipids of muscle and adipose samples and the quantitative reference fat BCR-163 were weighed to determine the total lipid content. The lipid extracts were redissolved in toluene, and aliquots of 25 mg (in triplicates) in 300 μL of toluene were used for methyl ester preparation for each of the four methods. For the calculation of the recovery of the four methods the quantitative reference fat BCR-163 was used.

Reagents. Individual isomers of CLA methyl esters, C18:2*cis*-9,*trans*-11, C18:2*trans*-9,*trans*-11, C18:2*trans*-10,*cis*-12, C18:2*cis*-9,-*cis*-11, and the free CLA*cis*-11,*trans*-13 were purchased from Matreya (Pleasant Gap, PA). All solvents used were of HPLC grade from Lab-Scan (Dublin, Ireland). The methylation chemicals were purchased from Sigma (Deisenhofen, Germany). Because of the limited shelf life of the 14% BF_3/methanol solution, this reagent was purchased only in small amounts and stored in the refrigerator. For the investigations with a defined spiked concentration of CLA*cis*-9,*trans*-11 (Matreya) a quantitative reference fat BCR-163 (beef/pig fat blend reference material 163 of the European Community Bureau of Reference, Promochem, Wesel, Germany) was used.

1. Base-Catalyzed Method followed by Acid-Catalyzed Method at Room Temperature (Method 1). To prepare the fatty acid methyl esters (FAMEs) 2 mL of 0.5 M sodium methoxide was added, and the mixture was shaken in a water bath at room temperature for 20 min. Then, 1 mL of boron trifluoride in methanol (14%, w/v) was added to the solution and shaking continued for 20 min. Methyl esters of total fatty acids were extracted twice with 2 mL of *n*-hexane in the presence of 2 mL of saturated solution of $NaHCO_3$ and stirred with vortex. The upper phases containing FAMEs were pooled, and the solvent was evaporated under nitrogen. FAMEs were redissolved in 150 μL of *n*-hexane and stored at −20 °C until Ag^+-HPLC analysis ($NaOCH_3$/BF_3 RT).

2. Base-Catalyzed Method followed by Acid-Catalyzed Method at 60 °C (Method 2). To prepare the FAMEs 2 mL of 0.5 M sodium methoxide was added, and the mixture was shaken in a water bath at 60 °C for 10 min. Then, 1 mL of BF_3/MeOH (14%, w/v) was added to the solution and shaken in a water bath at 60 °C for 10 min. Methyl esters of total fatty acids were extracted twice with 2 mL of *n*-hexane in the presence of 2 mL of saturated solution of $NaHCO_3$ with vortex stirring. The upper phases containing FAMEs were pooled, and the solvent was evaporated under nitrogen. FAMEs were redissolved in 150 μL of *n*-hexane and stored at −20 °C until Ag^+-HPLC analysis ($NaOCH_3$/BF_3 60 °C).

3. Base-Catalyzed Method (Method 3). To prepare the FAMEs 2 mL of 0.5 M sodium methoxide was added, and the mixture was shaken in a water bath for 10 min at 50 °C ($NaOCH_3$, 10 min, 50 °C). Glacial acetic acid (100 μL) was added, followed by 5 mL of water. The methyl esters were extracted in *n*-hexane (2 × 5 mL), and the solvent was evaporated. FAMEs were then redissolved in 150 μL of *n*-hexane and stored at −20 °C until Ag^+-HPLC analysis ($NaOCH_3$).

4. Base Hydrolysis and Acid-Catalyzed Method (Method 4). To prepare the FAME via saponification 2 mL of 0.5 N methanolic NaOH was added, and the mixture was shaken for 5 min at 60 °C. This was followed by methylation with BF_3/MeOH (14%, w/v) at 60 °C for 5 min (sapon/BF_3, 5 min, 60 °C).

Silver Ion High-Performance Liquid Chromatography (Ag^+-HPLC). The fatty acid methyl esters were analyzed by Ag^+-HPLC with four ChromSpher 5 Lipids analytical silver-impregnated columns (each 4.6 mm i.d. × 250 mm stainless steel, 5 μm particle size, Varian Inc.). The columns were maintained at 20 °C in an oven and were equilibrated with the solvent for at least 30 min prior to use. The isocratic mobile phase was 0.1% acetonitrile in *n*-hexane with a flow rate of 1 mL/min and was prepared fresh every other day. The injection volume was 15 μL. All conjugated compounds were detected with a photodiode array detector (SPD-M10, Shimadzu, Düsseldorf, Germany) by recording a spectrum from 200 to 400 nm with an extracted wavelength of 233 nm. The HPLC (LC-10AD, Shimadzu, Kyoto, Japan) was equipped with the operating system Class VP, version 6.12. The quantification of CLA isomers was based on the measurement of integrated area under the 233 nm peaks attributed to conjugated dienes. Identification of the CLA isomers was made using the retention times of the standard compounds (methyl ester of C18:2*cis*-9,*trans*-11, C18:2*trans*-9,*trans*-11, C18:2*trans*-10,*cis*-12, and C18:2*cis*-9,*cis*-11). Other isomers were identified from the known elution order (*6*, *16*, *17*). Additionally, the identity of each isomer was established by the typical UV spectra of CLA isomers from the photodiode array detector in the range from 190 to 360 nm with a maximum at 231−233 nm. The external calibration plots of the standard solutions were adapted to the different concentration levels of the individual CLA isomers in the lipid extracts. The external calibration plot of area versus micrograms of CLA*cis*-9,*trans*-11 was prepared for a standard mixture in a range of 9.0−700 μg/mL. External calibration plots of area versus micrograms of CLA for *trans*-9,*trans*-11, *trans*-10,*cis*-12, and *cis*-9,*cis*-11 were made for standard mixtures in the range of 0.5−20 μg/mL. The calibration of CLA*trans*-9,*trans*-11 was used for the other CLA *trans*,*trans* isomers, the calibration of *trans*-10,*cis*-12 for the *cis*,*trans* and *trans*,*cis* isomers, and the calibration of *cis*-11,*trans*-13 for the *trans*/*cis*-11/13 isomer (*18*). The detection limit of CLA isomers was calculated as 5 times the signal/noise ratio.

Statistical Evaluation. For each method the content of CLA isomers was estimated by the least-squares mean method using the GLM procedures of the Statistical Analysis System (SAS Systems, Release 8.2, SAS Institute Inc., Cary, NC (SAS). **Table 1** contains the least-squares mean (LSM) and the standard error (SE) of the LSM. All statistical tests of LSMs were performed for a significance level $\alpha = 0.05$. To calculate the repeatability θ of each method we used procedure VARCOMP of SAS to estimate the variance components σ^2_{sample} and σ^2_{error} [$\theta = \sigma^2_{sample}/(\sigma^2_{sample} + \sigma^2_{error})$]. The repeatability θ was calculated for all methods and 10 CLA isomers (**Figure 1**). To calculate the recovery of each method we used a reference fat BCR-163 (m_{native}) spiked with a known mass of CLA*cis*-9,*trans*-11 (m_{spike}). The recovery was calculated by $R = (m_{total} - m_{native})/m_{spike}$, where m_{total} and m_{native} are the masses of CLA*cis*-9,*trans*-11 found in the spiked and nonspiked sample, respectively (**Table 2**).

RESULTS

Before the derivatization, the intramuscular, subcutaneous fat and the reference fat material were extracted with chloroform/methanol to produce lipid mixtures representing all of the tissue lipids. Conversion of these to methyl ester is the most usual preparation for the fatty acid analysis. In this study four different methylation methods were tested for the quantitative analyses of CLA isomers in both adipose tissues of bulls and a reference fat. Ag^+-HPLC was used for the separation and identification of the CLA isomers. In total, 240 Ag^+-HPLC analyses have been done for this comparison. The concentrations (milligrams per gram) of 10 different CLA isomers were determined. In accordance with previous findings (*16*, *18*) the cis/cis isomers were below the determination limit and were not included here. Furthermore, this Ag^+-HPLC method cannot distinguish the conjugated fatty acids with different carbon lengths. As a

Table 1. Effect of Methylation Method on CLA Isomer Concentrations (Micrograms per Gram of Fat) in Ruminant Lipid Extracts[a]

	LSM$_{SEM}$			
	$NaOCH_3/BF_3$ RT, method 1	$NaOCH_3/BF_3$ 10 min, 60 °C, method 2	$NaOCH_3$, method 3	sapon BF_3, 5 min, 60 °C, method 4
CLA*trans*-12,*trans*-14	$146.04_{12.70}$[a]	$153.54_{12.68}$[a]	$127.64_{12.71}$[b]	$125.24_{12.69}$[b]
CLA*trans*-11,*trans*-13	$192.74_{10.41}$[a]	$196.92_{10.37}$[a]	$164.02_{10.43}$[b]	$171.89_{10.39}$[b]
CLA*trans*-9,*trans*-11	$96.97_{8.09}$	$109.43_{8.05}$[a]	$86.82_{8.11}$[b]	$94.44_{8.07}$
CLA*trans*-8,*trans*-10	$25.30_{2.36}$[a]	$29.39_{2.34}$	$25.63_{2.38}$	$31.77_{2.35}$[b]
CLA*cis*-12,*trans*-14	$62.94_{5.91}$	$68.03_{5.89}$[a]	$55.70_{5.92}$[b]	$59.14_{5.90}$[b]
CLA*trans*/*cis*-11/13	$1730.05_{151.00}$[a]	$1736.38_{150.86}$[a]	$1495.74_{151.08}$[b]	$1328.42_{150.93}$[b]
CLA*trans*-10,*cis*-12	$109.16_{24.96}$	$104.09_{24.92}$	$100.18_{24.99}$	$93.10_{24.94}$
CLA*cis*-9,*trans*-11	$4923.40_{414.70}$[a]	$4980.76_{414.25}$[a]	$4347.16_{414.97}$[b]	$4647.71_{414.47}$
CLA*trans*-8,*cis*-10	$100.00_{11.01}$[a]	$103.76_{10.94}$[a]	$95.57_{11.06}$	$72.40_{10.97}$[b]
CLA*trans*-7,*cis*-9	$354.26_{33.17}$[a]	$353.80_{33.10}$[a]	$306.05_{33.21}$[b]	$371.28_{33.13}$[a]

[a,b] Different superscripts indicate significant difference between methods ($P = 0.05$). LSM, least-squares means of randomly selected 10 muscle and 10 subcutaneous fat samples in triplicates; SEM, standard error of mean.

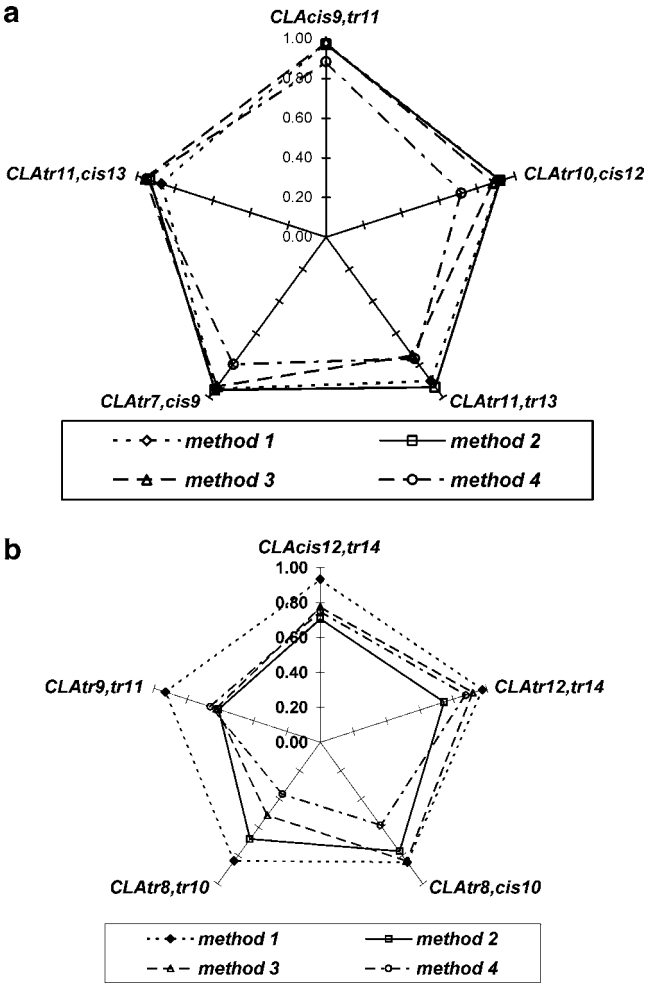

Figure 1. Repeatability θ for the (**a**) major and (**b**) minor CLA isomer concentrations (in total $n = 20$ fat samples in triplicates).

measure of the reproducibility of the four methods we used the coefficient of repeatability θ (see Statistical Evaluation). The value of the parameter $\theta > 0.8$ represents the high reliability of the method (**Figure 1**). For the most abundant isomers (*cis*-9,*trans*-11, *trans*-11,*trans*-13, *trans*/*cis*-11/13, and *trans*-7,*cis*-9) and *trans*-10,*cis*-12, methods 1 and 2 showed the highest values for the repeatability (all >0.87) and method 4 the lowest (0.72−0.95). The repeatability of method 3 was low (0.74) for only the CLA*trans*-11,*trans*-13 concentration. We found the most differences among the four methods for the other five minor CLA isomers (*trans*-12,*trans*-14, *cis*-12,*trans*-14, *trans*-8,*cis*-10, *trans*-8,*trans*-10, and *trans*-9,*trans*-11). For these CLA isomers the range of repeatability was 0.84−0.98 for method 1, 0.61−0.77 for method 2, 0.51−0.78 for method 3, and 0.37−0.88 for method 4. The variability of the three replicates for all CLA isomers was lowest for method 2 (data not shown).

Table 2. Effect of Methylation Method on CLA*cis*-9,*trans*-11 Concentration of the Reference Fat BCR-163 and Spiked with 100.05 μg of CLA*cis*-9,*trans*-11 [m_{spike}][a]

method	CLA*cis*-9,*trans*-11 of BCR-163 [m_{native}]	CLA*cis*-9,*trans*-11 + 100.05 μg spiked [m_{total}]	recovery[b]
1	32.57 (31.1–34.8)[c]	121.20 (115.2–128.8)	0.89
2	35.86 (35.8–35.95)	129.23 (126.5–131.8)	0.93
3	32.98 (31.6–34.6)	125.46 (120.7–132.0)	0.92
4	31.63 (30.1–33.0)	123.61 (121.2–126.7)	0.92

[a] Values are means of triplicates. [b] Recovery = $(m_{total} - m_{native})/m_{spike}$. [c] Minimum and maximum values of the triplicates are given in parentheses.

Overall, for CLA isomers the reproducibility was best for method 1 and the worst for method 4 (only for three CLA isomers was the repeatability >0.8).

Table 1 shows the quantitative isomer distribution for the four different methylation procedures. Method 2 supplied the highest concentration of all single isomers. The mean differences between methods 2 and 3 were significant for the following CLA isomers: *trans*-12,*trans*-14, *trans*-11,*trans*-13, *trans*-9,*trans*-11, *cis*-12,*trans*-14, *trans*/*cis*-11/13, *cis*-9,*trans*-11, and *trans*-7,*cis*-9. No significant differences were found between methods 1 and 2. For the main CLA isomer, CLA*cis*-9,*trans*-11, method 3 found the lowest concentration (**Table 1**).

Saponification followed by BF_3 methylation (method 4) gave higher CLA*trans*-7,*cis*-9 concentration compared with method 3. All of the other nine isomers were not significantly different. Method 4 always gave significantly lower concentrations for five CLA isomers (*trans*-12,*trans*-14, *trans*-11,*trans*-13, *trans*/*cis*-11/13, and *trans*-8,*cis*-10, except *trans*-7,*cis*-9) compared to methods 1 and 2. **Figure 2** shows one representative chromatogram of the separation of the CLA isomers in subcutaneous fat.

One commonly used method for the estimation of the recovery is to apply the analytical methods to two identical proportions of a test material, one of which is spiked with a known mass of the analyte (m_{spike}). A quantitative reference fat (BCR-163) used as a standard was spiked with a defined concentration of CLA*cis*-9,*trans*-11 (**Table 2**). After spiking

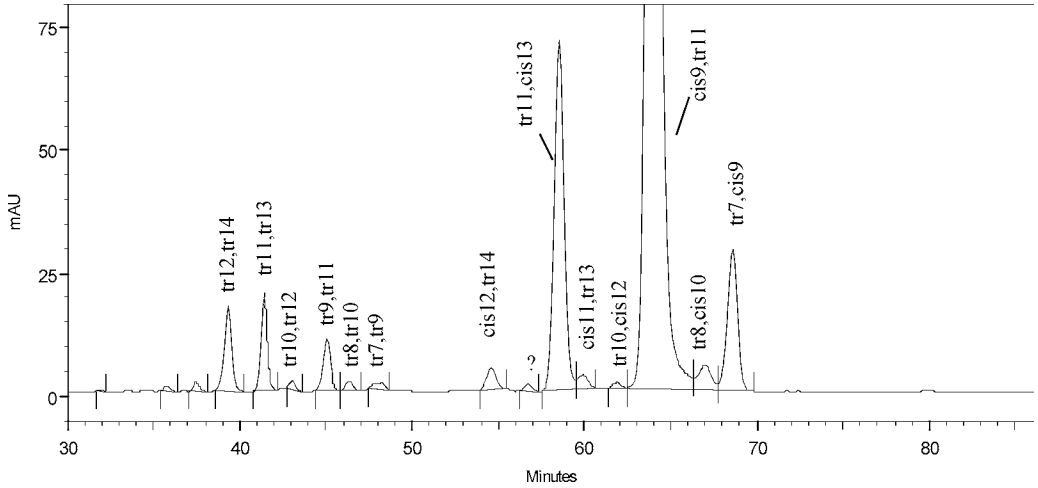

Figure 2. Partial Ag^+-HPLC chromatograms (four columns in row) of lipid extract of subcutaneous fat at 233 nm using method 2 ($NaOCH_3/BF_3$, 10 min, 60 °C).

with 100.05 mg of CLA*cis*-9,*trans*-11, method 2 showed the best recovery. In both reference fat samples (nonspiked and spiked) method 2 supplied also the highest CLA*cis*-9,*trans*-11 content and the lowest variations. The lowest concentration was detected with method 1.

DISCUSSION

The interest in CLA isomers started with the discovery of their positive effects on cancer, cardiovascular disease, diabetes, body composition, immune system, and bone health (*3*, *19*). Ruminant meats and milk and their products are the main sources of CLA in the human diet. The most abundant CLA isomer in dairy and beef fats is *cis*-9,*trans*-11, with smaller concentrations of *trans*-*7*,*cis*-9 and *trans*-11,*cis*-13 (*18*, *20*−*22*). The relative and the absolute content of the single isomers can be changed in the intramuscular, intermuscular, and subcutaneous ruminant fat by feeding (*21*). For calculation of the daily consumption of the CLA isomers, the analysis of the absolute content, not just the relative proportion, is needed. Therefore, complete and accurate analysis of the CLA isomer distribution in ruminant tissues is important. The findings that Ag^+-HPLC columns can separate geometric as well as positional CLA isomers (*23*, *24*) opened up a powerful alternative to GC analysis. The problem in CLA isomer investigation is the quantitative preparation of the methyl ester. The most used method is the base-catalyzed methylation with sodium methoxide. The disadvantage of this method is that free fatty acids, *N*-acyl lipids (i.e., sphingolipids and glycosphingolipids), and alk-1-enyl ethers are not methylated (*6*, *7*). The quantitative methylation of CLA in various lipid matrices of ruminant tissues and the separation and quantification of all isomers are important, on the one hand, but it is also necessary to avoid additional isomerization and the generation of allylic methoxide from CLA during derivatization (*7*, *8*). This is the first comparison of four different methods to evaluate the quantitative distribution of CLA isomers in beef by HPLC.

In our study we compared two combined base- and acid-catalyzed methylation methods (methods 1 and 2) and an acid-catalyzed method (method 4) with the most commonly used base-catalyzed method for CLA analysis (method 3). Higher concentrations in all single isomers (including trans/trans and cis/trans) by combined base- and acid-catalyzed methylation at room temperature (method 1) and at 60 °C (method 2) were detected compared to the base-catalyzed (method 3). This indicates complete methylation of all lipid fractions including plasmalogens and free fatty acids. Using a quantitative reference standard fat and spiking the main isomer CLA*cis*-9,*trans*-11 generated also the highest CLA concentration and the best recovery rate for method 2 compared to the other three methods. Our findings (repeatability and quantification) led to the recommendation that both combined methods (1 and 2) can be used for the methylation of ruminant fat for the determination of CLA isomers by Ag^+-HPLC. Ostrowska et al. (*25*) reported a loss in cis/trans isomers, a reduced total conjugated diene content, and a significant elevated level of trans/trans isomers by HCl (1 h at 80 °C) and BF_3 methylations (2 min at 100 °C). The quantitative calculation of 10 different CLA isomers by HPLC showed no loss of cis/trans isomer content for the combined methods 1 and 2 compared to base-catalyzed method 3. The ratio between the sum of all cis/trans isomers and the sum of all trans/trans isomers was identical for all four methods. Igarashi et al. (*10*) recommended the BF_3/MeOH method (30 min at room temperature) for the methylation of free fatty acids and CLA to prevent artificial isomerization and the production of undesired byproducts. Investigations with a free CLA preparation (50 mg) by GC showed a complete methylation at 55 °C for 5 min with BF_3/MeOH and no increase of the trans/trans CLA concentration (*26*). Also, Park et al. (*8*) did not find an increase in trans/trans percentage until 60 °C with BF_3/MeOH independent of the incubation time (10, 20, 30, or 60 min). The proportion of artifacts also seems to be unaffected up to a reaction temperature of 60 °C and at all different reaction durations. However, the production of artifacts and trans/trans isomers increased considerably with higher temperatures (80 or 100 °C) and higher incubation times. Park et al. (*8*) measured up to 13% of total artifacts and impurities at a reaction temperature of 80 °C and 20−60 min of reaction time with BF_3/MeOH. For utilization of method 2 it is important to be aware of the reaction conditions and the stability of the reagent BF_3/MeOH.

To summarize our results (repeatability and recovery rate), for the quantitative determination of all CLA isomers in beef (intramuscular fat, subcutaneous fat) by Ag^+-HPLC a combination of the base-catalyzed (sodium methoxide) followed by an acid-catalyzed methylation (14% of BF_3/MeOH) at 60 °C for 10 min can be used. This method showed for most abundant CLA isomers a satisfying repeatability, highest recovery with low variations, and the highest concentration of all isomers. The utilization of this method for other ruminant tissues and meat

products and the use of other acid-catalyzed methylation procedures (HCl, H_2SO_4) for the quantitative Ag^+-HPLC analysis of all CLA isomers in different ruminant lipids have to be clarified by further investigations.

ABBREVIATIONS USED

CLA, conjugated linoleic acid; Ag^+-HPLC, silver ion high-pressure liquid chromatography; w, weight; $NaOCH_3$, sodium methoxide; v, volume; LSM, least-squares mean; SEM, standard error of LSM; ME, methyl ester, FAME, fatty acid methyl ester.

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