Acta Univ. Sapientiae, Alimentaria, **5** (2012) 52–62

Gas chromatographic analysis of conjugated linoleic acids

É. Varga-Visi[1]
email: vargane.eva@ke.hu

R. V. Salamon[2]
email: salamonrozalia@sapientia.siculorum.ro

K. Lóki[1]
email: loki.katalin@ke.hu

J. Csapó[1,2]
email: csapo.janos@ke.hu

[1]Kaposvár University, Faculty of Animal Science,
7400 Kaposvár, Guba S. u. 40., Hungary

[2]Sapientia Hungarian University of Transylvania,
Faculty of Technical and Social Sciences, Miercurea-Ciuc,
RO-530104, Piaţa Libertăţii nr. 1., Miercurea-Ciuc

Abstract. The aim was to determine the conjugated linoleic acids in beef with GC-FID. Perfect separation of all of the isomers cannot be achieved, though conjugated linoleic acid isomers proved to be separable from the other fatty acids that are present in beef in a significant amount related to conjugated linoleic acids. Lipid-extraction was carried out with n-hexane/i-propanol, giving 6.8 percent higher yield than Soxhlet extraction. The variance – due to different parts of the sample preparation process – was quantified. The accuracy of the whole process and the efficiency of triglyceride conversion/fatty acid methyl ester extraction were determined.

1 Introduction

The physiological effects of conjugated linoleic acid isomers (CLA) are in the scope of interest and as a consequence there is an increasing need for the quantification of CLA from food. At the beginning of this decade, it has been

Key words and phrases: conjugated linoleic acid, GC-FID, beef, fatty acid methyl ester.

shown that silver ion liquid chromatography (Ag-HPLC) possesses the best resolving power for the separation of CLA-isomers [1,2]. Now, when it is coming to an end, the question arises why Ag-HPLC does not become the dominant analytical technique for CLA analysis. Authors in the previous years still often used GC alone or Ag-HPLC together with GC [3-5]. The possible cause may be that in Ag-HPLC the potential source of errors could be not only the variations in factors that are common in LC, but other parameters (e.g., sample size, solvent composition and even storage times) could also hamper obtaining reproducible results. Moreover, the batch-to-batch variation in the silver loadings of the columns is also a problem [6]. On the contrary, the gas chromatographic determination of fatty acid composition is easier to implement and proper identifying tools are available. The serious disadvantage of this technique is the improper resolution of CLA-isomers. The best gas chromatographic separation of fatty acid methyl esters (FAME) has been achieved with the use of 100 m long, 100% cyanopropyl polysiloxane stationary phase columns [7-9], and recently fast gas chromatographic methods have been developed in order to achieve the same resolution in much less time [3,4].

Our analytical task was to achieve a reliable method for the determination of the CLA content of beef. Owing to the local conditions, only gas chromatographic analysis was considered, and we tried to carry out the best realizable performance. In one respect, our aim was to determine the possibility and the limits of CLA determination with GC-FID; partly, the reliability of the sample preparation steps was investigated. The main steps of the method were lipid-extraction, transesterification, extraction of fatty acid derivatives and gas chromatographic analysis. Lipid extraction was achieved using a mixture of hexane/isopropanol, because it has been shown to have more advantages over the extraction with chloroform/methanol, which are rapid phase separation, less proteolipid contamination and less toxic solvents [10]. CLA and the other fatty acids present in the glycerides were transesterified by an alkali-catalysed reaction with sodium methoxide in order to avoid changes in the ratio of the CLA-isomers [11].

2 Experimental

Meat samples

Beef samples were obtained from Priváthús Ltd., Kaposvár. Samples originated from four parts of the carcass (thick flank, fore rib, thick rib and neck) were collected five different times. The weight of the individual samples was approx. 100 g. Beef samples were mixed and stored in a freezer (-24 °C).

General scheme of sample preparation and analysis

Lipids were extracted with a mixture of n-hexane/i-propanol 3:2 (v/v)[10]. 80 mL n-hexane/i-propanol mixture (HIP) was added to approx. 10 g meat sample (max. 0.3 g fat); then a suspension was prepared with an Ultra-Turrax T25 basic type dispersion tool (IKA WERKE GMBH, Germany). Solid particles were removed with vacuum filtration. After filtration had been completed, the filter funnel was rinsed three times with 10 mL HIP. Liquid phase was clarified from the soluble non-lipid fraction: it was extracted with 60 mL 0.47 M sodium sulphate in water. The organic phase was separated, dried under water-free Na_2SO_4 and the solvent was then removed under vacuum in a rotary evaporator. The crude lipid extract was dissolved in 10 mL of n-hexane.

Transesterification of glycerides was carried out with sodium methylate in methanol: 0.5 mL lipid extract in n-hexane and 0.5 ml sodium methoxide solution (0.5 M) was mixed and warmed at 50 °C for 30 min. When the reaction had been completed, 1 mL of distilled water was added and FAME was extracted four times with 1 mL hexane and diluted up to the final volume of 5 mL. All solvents and reagents were of analytical grade, the '37-component FAME mix' was obtained from Supelco, while 'conjugated linoleic acid mixture' was purchased from Sigma.

The separation of FAME was accomplished with a Chrompack CP 9000 gas chromatograph. The injection was manual, the split ratio was 16:1 and the injected amount was 6 μL at 270 °C. The column was a CP-Sil 88 (FAME) with a dimension of 100 m × 0.25 mm and the film thickness of the stationary phase was 0.2 μm. The final temperature programme: the temperature of the column was immediately increased from 130 °C to 225 °C at a rate of 2 °C min^{-1}. At 225 °C, isotherm conditions were applied for 20 min; the carrier gas was He (230 kPa, 16.1 cm/s). The temperature of FID was 270 °C. The initial temperature programme that was developed for other FAMEs was changed in order to optimize the resolution of CLA-isomers. The shifts in retention times were observed with the use of the '37-component FAME mix' and also with 'conjugated linoleic acid mixture'. The determination of the limit of detection (LoD) and limit of quantification (LoQ) was based on the slope of calibration curve and the noise of blank (n-hexane).

Checking the reliability of the sample preparation steps

Meat samples were extracted with HIP and the fat contents were determined. These values were compared with the crude fat contents obtained with the Soxhlet method, which is applied for the determination of the fat content of

meat in Hungary (MSZ ISO 1443:2002). In the case of fat determination with HIP-method, sample preparation was carried out as described above, until the evaporation of the solvent from the clarified HIP solution. The residuum was kept at 98 °C for 2 hours in a drying chamber, let to cool down in a desiccator, then the extract was weighed. The drying was repeated until the weight was constant.

In the case of the following two steps of sample preparation, the monitoring of transesterification and FAME extraction were not separated. The precision of transesterification and FAME extraction with hexane was determined with the parallel methylation of the same HIP-extract following extraction with hexane. The accuracy was determined with the transesterification of the known amount of glycerides. The ideal model solution would be an 'artificial beef fat' with similar fatty acid pattern as in beef, or at least CLA glycerol esters with a similar ratio as in beef fat. Due to purchasing difficulties, the efficiency of the above processes was determined with the use of myristic acid glycerol ester (MGE). The methyl ester of myristic acid (MME) appears at the beginning of the chromatogram and its peak area ratio within the sum of the FAMEs in beef is approx. 2%. During the determination of efficiency of transesterification and extraction, 0.150 g MGE was dissolved in 5 mL n-hexane and 0.5 mL from this solution was used for transesterification with 0.5 mL 0.5 M sodium methylate in methanol following the same method as in the case of the meat samples. The amount of the resulting MME derivatives was determined with external standard calibration.

Besides the examination of particular parts of the sample preparation, the reliability of the whole procedure was also evaluated. The MGE stock solution contained 199.2 mg MGE in 100 mL HIP and 2.5 mL of this solution was added to the initial HIP solution, then extracted, clarified, transesterified, and the resulting MME was extracted and measured. The recovery of MGE was determined in the form of MME.

Two-samples T-test was used for the comparison of means, the homogeneity of the variances were checked with F-test and the type of the T-test was chosen according to the results of the F-test. Due to the manual injection and the long cycle time (68 min), the number of measurement/day was restricted according to the up-to-date GC systems; consequently, the number of repetitions was limited.

3 Results

During the examination of the specificity of the analytical method, it was observed – as in the case of the other studies [9] – that with the use of the above means the separation of the CLA-isomers cannot be achieved perfectly, though the CLA-isomers proved to be separable from the other fatty acids that are present in the beef in significant quantities related to CLA. The most abundant isomer, c9,t11-CLA-ME, coelutes with t8,c10-CLA-ME, while the separation of c11,t13-CLA-ME and t10,c12-CLA-ME is adequate; then, the minor c,c- and t,t-isomers come after (Figure 1).

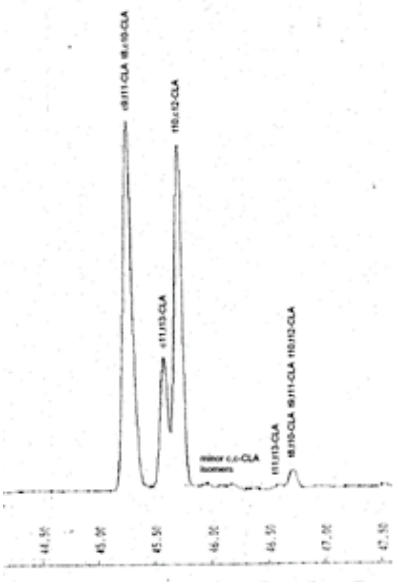

Figure 1: The partial separation of conjugated linoleic acid methyl esters with GC. Standard: 'Conjugated linoleic acids' from Sigma. Chrompack CP 9000 GC; 6 μL manual split (16:1) injection (270 °C); column: 100 m × 0.25 mm CP-Sil 88 (FAME) from 130 °C to 225 °C at a rate of 2 °C min^{-1}, at 225 °C isotherm for 20 min; carrier gas: He (230 kPa, 16.1 cm/s); FID (270 °C)

The resolution could be improved but the cycle time would be too long, more hours. In the chromatogram of the mixture of the two test solutions, it can be seen that heneicosanoic acid methyl ester (C21:0-ME) eluates between the first and the second CLA-ME peaks (Figure 2).

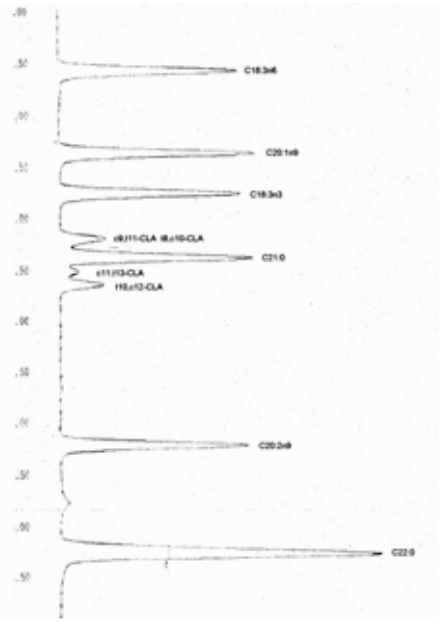

Figure 2: The separation of conjugated linoleic acid methyl esters from the other fatty acid methyl esters with GC. Standards: 'Conjugated linoleic acids', from Sigma and '37 component FAME mix' from Supelco'. GC conditions as Fig. 1

In the beef samples, only the signal of the first CLA peak (c9,t11-CLA-ME/t8,c10-CLA-ME) was big enough for quantification, although more minor isomers were present, but their signal was about or less than the limit of detection (Figure 3).

Table 1 presents the linearity of the examined CLA-isomers.

Table 1: Comparison of the amount of lipids extracted with hexane/isopropanol to the amount of lipids extracted with the Soxhlet method. Sample: fore rib.

Component	t_R	Concentration range (μg mL^{-1})						Slope [S]**	Intercept [I] **	r	LoD (μg mL^{-1})	LoQ (μg mL^{-1})
		No.1	No.2	No.3	No.4	No.5	No.6					
c9,t11-CLA-ME t8,c10-CLA-ME*	45.26	0.511	2.40	4.78	6.90	9.39	18.85	1.461	1.092	0.9992	0.193	0.643
c11,t13-CLA-ME	45.58	0.104	0.771	1.40	2.09	2.87	5.70	1.461	0.333	0.9992	0.193	0.643
t10,c12-CLA-ME	45.71	0.385	1.83	3.82	6.01	7.74	15.45	1.458	0.877	0.9991	0.193	0.645

*c9,t11-CLA and t8,c10-CLA were not separated.

** $A = S \cdot c - I$ A = peak area (mV·s); S = slope; I = intercept; c = concentration (μg mL^{-1})

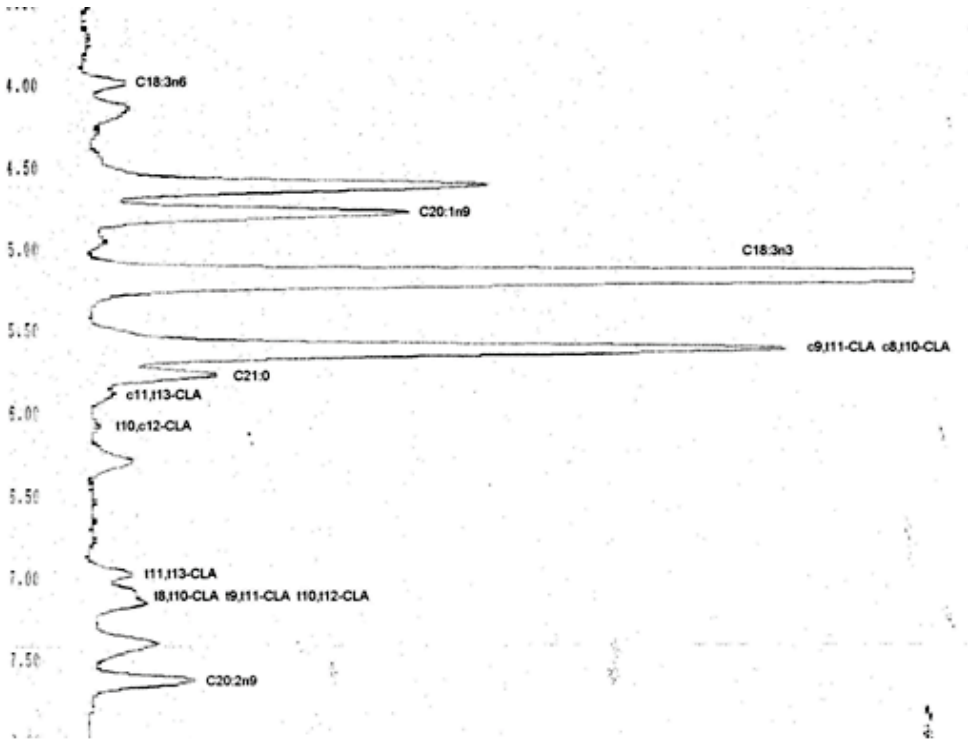

Figure 3: The part of the chromatogram of fatty acid methyl esters of beef, in which conjugated linoleic acid methyl esters are present. Sample: fore rib. GC conditions as Fig. 1

The slope of the equation describing the relationship between the concentrations of isomers and the peak area are similar to each other (1,458-1,461 $mV{\cdot}s{\cdot}mL{\cdot}\mu g^{-1}$); consequently, the LoD and LoQ values of the CLA-isomers are very close. Since slope values of the isomers were very alike, the amount of the two coeluting compounds in the first CLA-peak was evaluated together. The concentration of the most diluted (No.1.) standard solution was below the LoQ, thus standard solutions from No.2. to No.6. were used for calibration and result evaluation. The chromatogram of No.1. standard solution is shown in Figure 4.

The amount of residuum was significantly ($p \leq 0.05$) higher in the case of HIP-extraction than in that of the Soxhlet method (Table 2). With HIP, on average, we extracted 6.8 percent more material than with the other method.

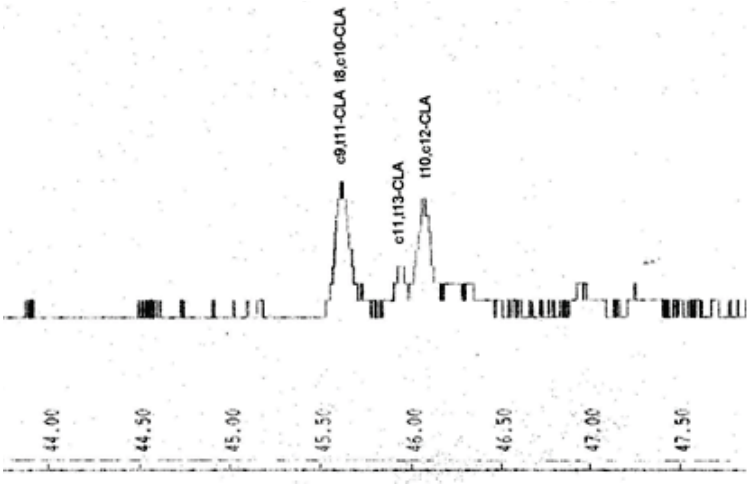

Figure 4: The chromatogram of standard solution No. 1 with concentrations close to the limit of detection. Concentration of conjugated linoleic acid methyl esters are shown in Table 1. GC conditions as Fig. 1

While HIP resolves some parts of carbohydrates and proteolipides, washing with sodium sulphate solution was reported to eliminate most of these compounds, as it was indicated with negative iodine test, and ninhydrine positive substances were also not detected [10]. In our application, the aim of the extraction was not the exact determination of crude fat content, but the extraction of all of the lipid classes containing fatty acids in ester bond; therefore, the possible presence of some other substances does not mean a problem.

Table 2: Comparison of the amount of lipids extracted with hexane/isopropanol to the amount of lipids extracted with the Soxhlet method. Sample: fore rib.

Method	Fat content (g fat/100 g sample)								
	No.1.	No.2	No.3	No.4	No.5	No.6	Mean	SD	RSD (%)
HIP-extraction	3.85	4.13	4.05	4.04	4.28	4.22	4.09	0.151	3.7
Soxhlet method	3.78	3.95	4.00	4.00	3.41	3.72	3.81	0.228	6.0

The precision of transesterification/FAME extraction can be seen in Table 3. The variation coefficient or relative standard deviation (RSD) is smaller for MME than in the case of c9,t11-/t8,c10-CLA-ME. The first component is

present in higher quantities in the fat of the beef. The same tendency can be noticed in the precision data obtained from the whole sample preparation process (Table 3).

Table 3: The precision of transesterification/FAME extraction and that of the total sample preparation and analysis for myristic acid methyl ester (MME) and conjugated linoleic acid methyl esters (CLA-ME). Sample: thick flank.

Component (mg/g sample)	Transesterification/FAME extraction (n=6)			Total sample preparation and analysis (n=3)		
	Mean	SD	RSD (%)	Mean	SD	RSD (%)
MME	0.300	0.0066	2.2	0.284	0.027	9
c9,t11-CLA-ME t8,c10-CLA-ME*	0.074	0.0028	3.8	0.070	0.0073	10

*c9,t11-CLA and t8,c10-CLA were not separated.

The sum of the error variances (s^2) of the individual steps gives the variance of the complete process:

$$s(\text{total})^2 = s(\text{sampling})^2 + s(\text{lipid extraction})^2 + s(\text{transesterification/FAME extraction})^2 + s\ (\text{analysis})^2$$

The above relationship can be described with the square of estimated relative standard deviation. For the extraction of lipids, $RSD^2 = 3.7^2$, but this value estimates the variation in total lipid content, which is not by necessity the same for the examined fatty acids.

In the case of the examination of transesterification/FAME extraction, the effect of GC-analysis on variance was not separated from the effect of sample preparation; thus, for the determination of MME, the next value can be substituted:

$$\text{RSD (transesterification/FAME extraction)}^2 + \text{RSD (analysis)}^2 = 2.2^2$$

For the complete process of MME analysis:

$$9^2 = \text{RSD (sampling)}^2 + \text{RSD (lipid extraction)}^2 + 2.2^2$$

Thus:

$$[\text{RSD (sampling)}^2 + \text{RSD (lipid extraction)}^2]^{1/2} = 8.7\%$$

For the determination of c9,t11-/t8,c10-CLA-ME:

$$\text{RSD (transesterification/FAME extraction)}^2 + \text{RSD (analysis)}^2 = 3.8^2$$

The equation describing the variance of the complete process in this case is:

$$10^2 = \text{RSD (sampling)}^2 + \text{RSD (lipid extraction)}^2 + 3.8^2$$

That is:

$[\text{RSD (sampling)}^2 + \text{RSD (lipid extraction)}^2]^{1/2} = 9.2\%$

Assuming that the variance in the concentration of the analytes – due to lipid extraction – is the same as the variance in lipid content, RSD(sampling) is 7.8% for MME and 8.5% for the examined CLA isomers. It would seem that sampling is the bottle-neck of the process because it is responsible for three-quarters of the total variation. The homogeneity and/or the size of the sample should be increased.

Table 4 presents the recovery of transesterification/FAME extraction expressed as the conversion efficiency of MGE and the extraction efficacy of the resulting MME. On average, 91% of the glycerides were converted into FAME and extracted. The accuracy of the complete process is also shown in Table 4.

Table 4: The accuracy of transesterification/FAME extraction and that of the total sample preparation and analysis. (1) Myristic acid glycerol ester was transesterified and the formed myristic acid methyl ester (MME) was extracted and measured. (2) Myristic acid glycerol ester was extracted with HIP, transesterified and myristic acid methyl ester was extracted and measured.

Transesterification/FAME extraction (n=6) (1)				Total sample preparation and analysis (n=3) (2)			
MME (mg)	Mean	SD	RSD (%)	MME (mg)	Mean	SD	RSD (%)
Nominal *	15.54	–	–	Nominal **	4.93	-	-
Measured	14.17	0.85	6.0	Measured	3.53	0.09	2.6
Recovery %	91	5.4	6.0	Recovery %	72	1.9	2.6

*Nominal: the amount of MME that would form if the efficiency of transesterification and extraction were 100%

**Nominal: the amount of MME that would form if the efficiency of lipid-extraction, transesterification and extraction were 100%

The efficiency of lipid extraction can be estimated based on the following equations:

$$\text{Nominal MME (mg)} \cdot E \cdot A = \text{measured MME (mg)}$$

$$\text{Nominal MME (mg)} \cdot V = \text{measured MME (mg)},$$

where:

E = efficiency of lipid extraction

A = efficiency of transesterification/FAME extraction = 0.91

V = efficiency of the complete process = 0.72

Thus

$$E = V \cdot A^{-1} = 0.72 \cdot 0.91^{-1} = 0.79$$

It seems that the most important part of the losses could be assigned to the incomplete extraction of MGE.

Acknowledgement

This work was accomplished with the financial support of OTKA T049405 (National Foundation for the Subsidy of Research, Hungary). The project was partly financed by the Sapientia Hungarian University of Transylvania, Institute of Research Programs. (Contract no.: 59/9/08.11.2012)

References

[1] B. Nikolova-Damyanova, S. Momchilova, W. W. J. Christie, *High Resol Chromatogr*, 23 (2000) 348–352.

[2] M. P. Yurawecz, K. M. Morehouse, *Eur J Lipid Sci Technol*, 103 (2001) 594–632.

[3] C. Moltó-Puigmartí, A. I. Castellote, M. C. López-Sabater, *Anal Chim Acta*, 602 (2007) 122–130.

[4] I. Bondia-Pons, C. Moltó-Puigmartí, A. I. Castellote, M. C. López-Sabater, *J Chromatogr A*, 1157 (2007) 422–429.

[5] P. Luna, M. Juárez, M. A. de la Fuente, *J Chromatogr A*, 1204 (2008) 110–113.

[6] M. Collomb, A. Schmid, R. Sieber, D. Wechsler, E. L. Ryhänen, *Int Dairy J*, 16 (2006) 1347–1361.

[7] N. Sehat, J. K. G. Kramer, M. M. Mossoba, M. P. Yurawecz, J. A. G Roach, K. Eulich, K. M. Morehouse, Y. Ku, *Lipids*, 33 (1998) 963–971.

[8] J. K. G. Kramer, J. Zhou, *Eur J Lipid Sci Technol*, 103 (2001) 594–632.

[9] J. K. G. Kramer, C. Cruz-Hernandez, Y. Zhou, *Eur J Lipid Sci Technol*, 103 (2001) 594–632.

[10] A. Hara, N. S. Radin, *Anal Biochem*, 90 (1978) 420–426.

[11] Y. Park, K. J. Albright, Z. Y. Cai, M. W. Pariza, *J Agric Food Chem*, 49 (2001) 1158–1164.