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IMPROVED SAPONIFICATION THEN MILD BASE AND ACID-CATALYZED METHYLATION IS A USEFUL METHOD FOR QUANTIFYING FATTY ACIDS, WITH SPECIAL EMPHASIS ON CONJUGATED DIENES

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SUMMARY

The objective of this study was to evaluate mild lipid saponification then gentle base and acid-catalyzed methylation, in succession, at 80°C, 60°C, 40°C, or ambient temperature for quantification of fatty acids (FAs), with special emphasis on conjugated linoleic acid (CLA) isomers. Methylation at 80°C resulted in a substantial increase in the amount of *trans*,*trans* (*t*,*t*) CLA isomers and in loss of *cis*,*trans*/*trans*,*cis* CLA isomers, as a result of intra-isomerization and formation of artefacts. Methylation at 40°C, in contrast, seems to enable the most accurate quantification of CLA isomers, because it resulted in no noticeable intra-isomerization of conjugated dienes or formation of artefacts. Under these derivatization conditions, other FA methyl esters (FAMEs) also seem to be accurately quantified. The proposed procedure adequately prepares FAMEs from FA standards and from lipids in biological samples, because mild saponification and methylation at 40°C using typical basic and acidic catalysts and subsequent extraction with a smaller volume of heptane resulted in satisfactory accuracy of quantification, negligible changes in the composition of FAs, especially conjugated dienes, and a better yield of FAMEs. The proposed improved procedure, comprising mild lipid saponification and methylation at 40°C in solutions carefully flushed with argon (Ar) (derivatization–Ar), in the absence of 2,6-di-*tert*-butyl-*p*-cresol, then extraction with 4 mL heptane, seemed the most satisfactory method of preparing FAs, particularly CLA isomers, for chromatographic quantification by argentation–liquid chromatography, the method of choice for analysis of fatty acids containing conjugated double bonds.

INTRODUCTION

Recent efforts to increase the essential fatty acid content of foodstuffs has led to renewed interest in analytical methods for accurate quantification of mono and polyunsaturated fatty acids, particularly conjugated linoleic acid (CLA) isomers. No single chromatographic technique is capable of fractionating the approximately 400 fatty acids (FAs) present in biological materials, e.g. meat, fat, bovine milk, or rumen fluid lipids. The suitability of long capillary columns (100 m) for gas–liquid chromatography (GLC) has been improved by use of pre-fractionation by argentation–liquid chromatography (Ag^+–HPLC) [1,2]. Quantitative preparation of fatty acid methyl esters from the complex mixtures of FAs occurring in meat, internal organs, milk, or the fat derived from monogastric animals and ruminants is unfortunately, difficult. Sodium methoxide-catalyzed methylation has been used, but *N*-acyl lipids (i.e. glycosphingolipids or sphingolipids) and free FAs are not derivatized by this reaction [3]. Acid-catalyzed derivatization, in contrast, methylates all types of lipid. Unfortunately, numerous studies have shown that FAs with conjugated double bonds (CFAs; e.g. CLA isomers and their metabolites) are isomerized or intraisomerized during derivatization [3–5]. The presence of oxidizing or antioxidant species (for example tocopherols, pyrogallol, or 2,6-di-*tert*-butyl-*p*-cresol [2, 6]) results in significantly different profiles, particularly for unsaturated fatty acids, and formation of unidentified artefacts. Polyunsaturated fatty acids (PUFA) are especially sensitive to oxidation compared with monounsaturated (MUFA) or saturated fatty acids (SFA) in processed biological samples. Numerous studies have revealed that the profile of CFAs is markedly changed by use of elevated temperatures and also depends on the type of catalyst used to produce the methyl esters; for example, catalysis by *p*-toluenesulfinic acid and iodine resulted in migration of the double bonds and formation of eight geometric isomers of the 8,10, 9,11, 10,12, and 11,13-octadecadienoic acid methyl derivatives [1]. Boron trifluoride (BF_3) or trimethylsilyldiazomethane methylation, in contrast, result in extensive formation of *trans*,*trans* conjugated dienes and methoxy artefacts [3,5,7]. Liquid chromatographic analysis of CLA isomers, their metabolites, or other FAs containing conjugated double bonds has, therefore, usually been conducted on lipids mildly hydrolyzed to free fatty acids. Later, base and acid-catalyzed methylation was introduced for preparation of esters of all these FAs. The best compromise between assay accuracy and derivatization yield for FA assays, particularly for conjugated dienes, would, ne-

vertheless, be a procedure comprising mild saponification then gentle pre-column derivatization.

For these reasons it seemed desirable to develop a new method of sample preparation for gas chromatography of fatty acids that would be based on gentle saponification and esterification by procedures which did not cause isomerization or intra-isomerization of conjugated dienes, or formation of artefacts. The main objective of our study was, therefore, to investigate the effect of temperature on the profile of mono and polyunsaturated fatty acids, particularly CLA isomers, subjected to methylation catalyzed by commonly used basic and acidic catalysts. Our second objective was to examine the profile of fatty acids, particularly CLA isomers, methylated under optimum temperature conditions in solutions carefully flushed with argon (Ar) (derivatization–Ar) and in solutions containing 2,6-di-*tert*-butyl-*p*-cresol (BHT) (derivatization–BHT) [2]. The modified and original procedures were compared using fatty acid standards and samples obtained from laboratory rats fed a diet enriched with a 2% mixture of CLA isomers.

EXPERIMENTAL

Chemicals and Materials

Heptane and acetonitrile were HPLC grade; other reagents were of analytical grade. A mixture of free CLA isomers (95–97%) was supplied by Larodan Fine Chemicals (Sweden). Acetonitrile (99.9%) and *n*-heptane (95%) were purchased from Lab-Scan (Eire) and other FA standards and 2,6-di-*tert*-butyl-*p*-cresol were from Sigma (USA).

Kidneys and livers from rats fed a diet containing 2% of a CLA isomer mixture were frozen, lyophilized, powdered, and the samples obtained were stored at −20°C until FA analysis [8]. All FAMEs were prepared by derivatization of lipids and fatty acid standards in 20-mL tubes equipped with Teflon-lined screw-caps. All methylated FA solutions were protected from light.

Saponification and Extraction of Fatty Acids

Lyophilized samples of kidney and liver (~50 mg) were mixed with 2 mL 2 M aqueous KOH, 2 mL 1 M methanolic KOH, and 50 μL internal standard solution (17 mg mL^{-1} nonadecanoic acid in chloroform). The resulting mixture was carefully flushed with a stream of argon (Ar) for 4–

5 min. Finally, the solution obtained, in a tightly closed tube, was vigorously mixed, heated at 92–95°C for 10 min, cooled for 10 min at room temperature, then sonicated for 10 min. The solution obtained (under Ar) was protected from light and stored overnight in a sealed vial at room temperature.

Water (3 mL) was added to the hydrolysate, in a vial. After vigorous mixing the solution obtained was acidified to pH 1–2 with 4 M HCl and free FAs were extracted with dichloromethane (DCM; 4 × 3 mL). The lower, DCM, layer was dried with ~0.1 g Na_2SO_4. To avoid any loss of free FAs, the extraction was repeated with heptane (4 × 3 mL), the upper heptane layers were combined with the DCM extract, and the organic solvents were removed under a stream of Ar. The residue, I, was stored at −20°C until base and acid-catalyzed methylation, or re-dissolved in 1 mL DCM and 20–30 μL of this solution was injected on to an ion-exchange column loaded with silver ions (analysis of non-methylated CFA(s) by Ag^+–HPLC).

Separation of Free CLA Isomers and their Metabolites by Ag^+–HPLC

Residue I, or 7.2 mg of CLA isomer standard, was re-dissolved in 1 mL DCM and 20–30 μL of the solution was injected on to an HPLC silver-ion column. Direct determination of non-methylated CLA isomers and their metabolites (i.e. all fatty acids containing conjugated double bonds) was performed by use of two analytical ion-exchange columns loaded with silver ions (250 mm × 4.6 mm ChromSpher 5-μm Lipids column; Chrompack, The Netherlands), connected in series, with photodiode-array detection (DAD) at 234 nm (Waters Model 996 photodiode-array detector). Elution was performed as described by Czauderna et al. [9].

Preparation of Fatty Acid Methyl Esters (FAMEs) in the Presence of BHT (Derivatization–BHT)

NaOH in methanol (2 M, 2 mL) and a solution of BHT in methanol (20 mg mL^{-1}, 50 μL) were added to residue I, in a tube. The solution was flushed with a stream of Ar for 3 min then reacted for 1 h at 80°C, 60°C, or 40°C, or for 10–12 h at ambient temperature (22–24°C). After cooling (5–10°C) 2 mL 25% BF_3 in methanol was added, and the reaction mixture was flushed with a stream of Ar for 3 min and again heated for 1 h at 80°C, 60°C, or 40°C, or for 10–12 h at ambient temperature (22–24°C). Water (5 mL) was then added to the cooled reaction mixture and the FAMEs

were extracted with 5 mL heptane. The clear supernatant was transferred to a vial.

Preparation of Fatty Acid Methyl Esters (FAMEs) in Solutions Flushed with a Stream of Ar (Derivatization–Ar)

NaOH in methanol (2 M, 2 mL) was added to residue I, to the mixed CLA isomer standard (7.2 mg), or to standards of other fatty acids (0.63 mg). The resulting solution was carefully de-aerated by use of a stream of Ar for 4–5 min and then reacted for 1 h at 40°C. After cooling, 2 mL 25 % BF_3 in methanol was added to the reaction mixture. This was carefully flushed with a stream of Ar for 4–5 min and again heated for 1 h at 40°C. Water (5 mL) was then added to the cooled reaction mixture and the FAMEs were extracted with 5 mL *n*-heptane. The clear supernatant was transferred to a vial.

Fractionation of FAMEs by Gas Liquid-Chromatography (GLC) and by Ag^+–HPLC

Separation of all FAMEs was performed as described elsewhere [2,8] by use of an Agilent 6890N GC equipped with a CP7489 fused silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness; Varian, USA) and a flame-ionization detector (FID). Analysis of methylated CLA isomers and of their metabolites with conjugated double bonds was also performed with the method of choice for conjugated fatty acids, as described by Czauderna et al. [2]. Briefly, direct determination of methylated CFAs (i.e. CLA isomers and their metabolites) was performed with two analytical ion-exchange columns loaded with silver ions (250 mm × 4.6 mm i.d. ChromSpher 5 μm Lipids column; Chrompack, The Netherlands), connected in series, in conjunction with photodiode-array detection at 234 nm (Waters Model 996 photodiode-array detector). Elution was performed as described elsewhere by Czauderna et al. [2]. UV spectra of the eluate (spectral resolution 1.2 nm) were acquired every second and were electronically stored on a computer hard disk [2].

RESULTS AND DISCUSSION

Analysis of Free and Methylated Conjugated Dienes by Ag^+–HPLC

To investigate the tendency of conjugated fatty acid (CFA) isomers to intra-isomerise, we assumed that fractionation of non-methylated CFAs

by direct Ag^+–HPLC analysis [9] resulted in complete recovery of all isomers and gave a true picture of the CLA isomer composition. In this study, therefore, we first attempted to fractionate the free CLA isomers formed during mild saponification of lipids from the kidneys and livers of rats fed a CLA isomer-enriched diet. As expected (Table I), liquid chromatography using two Ag^+ columns, connected in series, with photodiode-array detection at 234 nm [2,9], enabled highly discerning separation of free and methylated *trans–trans* (*t*,*t*), *cis*,*trans*/*trans*,*cis* (*c*,*t*/*t*,*c*), and *cis*,*cis* (*c*,*c*) CLA isomers in the Larodan standard CLA isomer mixture, and in extracts of the kidneys and livers of rats fed a CLA isomer-enriched diet.

It is clear from the data summarized in Table I that the *t*,*t* and *c*,*c* isomers were of minor abundance in the CLA isomer pool whereas *c*9*t*10CLA and *t*10*c*12CLA (i.e. *c*,*t*/*t*,*c* isomers) were the most abundant isomer group in all the samples assayed. The results presented in Table I also reveal that for the CLA isomer standard and the kidneys and livers of rats our mild saponification and base and acid-catalyzed methylation at 40°C resulted in no observable intra-isomerization of the original CLA isomers and/or formation of artefacts containing conjugated double bonds. Although methylation at 40°C resulted in a lower derivatization yield than methylation at 60°C and 80°C, the sum of CLA isomer peak areas was largest when derivatization was performed at 40°C. These results indicate, therefore, that methylation at 40°C resulted in a smaller loss of CLA isomers by conversion into non-CLA fatty acid methyl esters and/or unidentified artefacts. When base and acid-catalysed methylation of the Larodan standard CLA isomers was conducted at 40°C the percentage distribution of *t*,*t*, *c*,*t*/*t*,*c*, and *c*,*c* CLA isomers was, moreover, almost equal to their distribution when analyzed directly (as non-methylated CFA) by silver-ion liquid chromatography (Ag^+–HPLC) [9-11]. Larger amounts of methylated *c*9*t*11CLA were found when samples were analysed by GLC than when they were analysed by Ag^+–HPLC, because the resolution of capillary GLC [2] was poorer than that of Ag^+–HPLC performed with two silver-ion-loaded columns connected in series [2,8,9]. Indeed, the close proximity of numerous *c*,*t*/*t*,*c* CLA isomers [9] resulted in overlapping of the *c*9*t*11CLA peak with some *c*,*t*/*t*,*c* isomer peaks, and, therefore, in GLC chromatograms the *c*9*t*11CLA peak is not symmetrical. Our Ag^+–HPLC system, in contrast, resolved all the geometrical and positional isomers of CLA (*c*11*t*13/*t*11*c*13, *c*10*t*12/*t*10*c*12, *c*9*t*11/*t*9*c*11, and *c*8*t*10/*t*8*c*10) very efficiently [2,8,9]. The *c*9*t*11CLA peak is, therefore, symmetrical, because this isomer did not interfere with other *c*,*t*/*t*,*c* CLA isomers. Consequently,

the amount of *c*9*t*11CLA measured by use of Ag^+–HPLC is less than that measured by use of GLC.

Table I

CLA isomer composition (%) of the Larodan CLA isomer standard mixture[a] and of extracts of the kidneys[b] and livers of rats, by use of base and acid-catalyzed methylation at different temperatures. Separations were performed by gas–liquid chromatography and argentation–chromatography (Ag^+–HPLC)

Sample	Analytical method	Methylation temperature	Yield, from GLC assay[c] (%)	*t*,*t* CLA[d] (%)	*c*9*t*11 CLA (%)	*t*10*c*12 CLA (%)	Sum of *c*,*t*/*t*,*c* CLA isomers (%)	*c*,*c* CLA (%)	Sum of peak areas of CLA isomers
Kidney extract	HPLC[e]	Non-methylated FAs[e]	–	5.8	44.6	33.1	91.0	3.3	–
	GLC	80°C	82.5	44.5	31.9	22.0	53.9	1.6	–
		60°C	72.2	11.0	52.2	32.7	85.6	3.4	–
		40°C	62.1	7.0	56.3	31.5	89.9	3.1	–
		ambient, 23°C	27.6	6.4	55.8	32.1	90.5	3.1	–
Liver extract	HPLC[e]	Non-methylated FAs[e]	–	5.7	43.3	32.3	92.8	1.5	–
	GLC	80°C	92.3	34.3	38.7	26.2	64.7	1.0	–
		40°C	50.7	5.9	50.5	33.2	92.7	1.4	–
Larodan CLA isomer standard mixture	HPLC[e]	Non-methylated FAs[e]	–	19.0	36.6	37.1	77.6	3.4	–
	GLC	80°C	74.8	56.7	17.1	14.3	37.1	6.2	7457
		60°C	70.6	21.4	37.1	35.8	74.2	4.4	6790
		40°C	64.7	18.7	38.6	38.9	77.5	3.9	8605
				(18.8)[f]	(38.4)[f]	(37.7)[f]	(77.5)[f]	(3.8)[f]	
	Ag^+–HPLC analysis of methylated CLA isomers	80°C	–	64.2	14.7	11.5	32.6	3.2	38.6×10^6
		40°C	–	19.7	36.1	39.0	77.2	3.1	49.5×10^6
				(20.1)[f]	(35.4)[f]	(37.3)[f]	(76.7)[f]	(3.1)[f]	

[a] 8.39 mg free CLA isomer mixture was methylated with 2 mL 2 M NaOH in methanol then 2 mL 25% BF_3 in methanol

[b] Average composition (%) of CLA isomers in kidneys obtained by saponification and extraction; average of four results (saponification then methylation at 80, 40 or 23°C) or three results (saponification then methylation at 60°C) for four rats fed a diet enriched in 2% CLA isomer mixture [7]

[c] Result from GLC assay based on the internal standard (C19:0) (comprising yield from methylation and two extractions, after saponification and methylation)

[d] *t*,*t* and *c*,*c* geometric forms of CLA isomers – *trans*,*trans* and *cis*,*cis*, respectively

[e] Direct Ag^+–HPLC analysis of non-methylated CFAs (i.e. CLA isomers and other fatty acids containing conjugated double bonds) in kidneys (from four rats) and liver (from one rat) and in the mixture of free CLA isomers supplied by Larodan Fine Chemicals

[f] Results in parentheses: base and BF_3-catalyzed methylation was performed for 75 min at 40°C

Analysis of Fatty Acids (FAs) Using Base and Acid-Catalyzed Methylation at Different Temperatures

After several recent investigations it has been reported that polyunsaturated fatty acid composition, particularly conjugated linoleic acid isomer content, was greatly affected by the catalysts used for formation of methyl esters. Our studies (data not presented) and several others [2,3,7,8, 12,13] revealed that NaOH-catalyzed methylation for 1 h at 80°C resulted in the formation of, predominantly, *c*9*t*11CLA-MEs with only a small concentration of artefacts and/or isomerization products of the original *c*9*t*11CLA in analysis of processed milk samples whereas acid-catalyzed methylation using HCl–methanol or BF_3–methanol for 1 h at 80°C resulted in reduction in the amounts of *c*9*t*11CLA and *t*10*c*12CLA and formation of additional positional and geometric CLA isomers, especially *t*,*t* CLA isomers. Our results from combined methylation, summarized in Table I, are consistent with those from studies on single methylation cited above and reveal that combination of NaOH–methanol and BF_3–methanol-catalyzed methylation at 80°C also resulted in reduced amounts of *c*,*t*/*t*,*c* CLA isomers and significantly increased amounts of *t*,*t* CLA isomers as major intra-isomerization products of conjugated dienes. Formation of additional *t*,*t* CLA isomers and artefacts was, fortunately, less during base and acid-catalyzed methylation at lower temperatures. Formation of *t*,*t* CLA isomers and artefacts during base and acid-catalyzed methylation decreased as the temperature used for methylation was reduced. The profile of the Larodan CLA isomer standard methylated at 40°C for 1 h and that of non-methylated CLA isomers analyzed directly by Ag^+–HPLC were, consequently, similar [9]. As is apparent from the data in Table I, there were no significant differences between the composition of the Larodan CLA isomer standard and no increase in the derivatization yield when base and acid-catalyzed methylations at 40°C were also performed for longer (75 min rather than 1 h). Under these gentle basic and acidic methylation conditions, furthermore, the composition of methylated CLA isomers was equivalent to that for non-methylated CLA isomers analysed directly by Ag^+–HPLC [9]. The data presented here indicate, therefore, that combination of our mild saponification procedure with base and acid-catalyzed methylation at 40°C for 1 h seems to be the most suitable method for quantification of CFAs (i.e. CLA isomers and/or their metabolites) by GLC and Ag^+–HPLC [2].

The results in Table II show that mild saponification followed by methylation at 60°C, 40°C, or ambient temperature are appropriate analytical procedures for GLC analysis of saturated, monounsaturated, and non-

CLA polyunsaturated fatty acids in kidney and liver tissue (data for liver are not presented), because the relative amounts of these FAs did not differ among methylations at all the temperatures examined and the control derivatization performed at the temperature typically used (80°C). As expected, the most notable differences between results from derivatization at different temperatures were for CLA isomers only (Table II).

Table II

Comparison of fatty acid methylation performed at different temperatures (T). The fatty acid composition of the kidneys of rats was determined by gas–liquid chromatography (GLC)

Fatty acid[a]	T = 80°C	T = 60°C	T = 40°C	Ambient temperature
	(yield, 80.3%)[b]	(yield, 75.8%)[b]	(yield, 59.1%)[b]	(yield, 25.8%)[b]
C16:0	0.742	0.735	0.754	0.737
*c*9C16:1	20.74	20.05	20.90	19.94
C18:0	1.001	0.996	1.013	1.001
*c*9C18:1	1.502	1.426	1.501	1.445
*c*11C18:1	8.28	7.68	7.95	5.93
*c*9*c*12C18:2	0.984	0.966	0.984	0.970
*c*9*c*12*c*15C18:3	7.49	7.27	7.33	7.18
*c*9*t*11CLA	16.8	7.45	6.97	6.88
*t*10*c*12CLA	23.79	12.66	11.72	11.73
t,*t*CLA	10.13	39.08	45.88	33.94
*c*5*c*8*c*11*c*14C20:4	1.165	1.117	1.138	1.137
*c*5*c*8*c*11*c*14*c*17C20:5	9.90	11.79	12.62	12.66
*c*7*c*10*c*13*c*16*c*19C22:5	21.08	19.90	20.01	20.19
*c*4*c*7*c*10*c*13*c*16*c*19C22:5	10.80	9.99	9.82	10.09

[a] Fatty acids (FAs) represented as the ratio of the fatty acid peak area to that of the internal standard (C19:0) (i.e. $S_{FA}/S_{C19:0}$); *c* and *t* denote *cis* and *trans*, respectively

[b] Yield of FAMEs based on the internal standard (C19:0) [2]

Mild saponification of lipids and methylation at 40°C is, therefore, likely to lead to most accurate quantification of conjugated fatty acids (CFAs) in the presence of other FAs. Methylation at 40°C did, however, lead to a lower derivatization yield compared with methylation at higher temperatures (Table II). This disadvantage of base and acid-catalyzed methylation at 40°C was overcome by using a smaller volume of heptane for extraction of the methylated fatty acids (FAMEs). Yields of methylated fatty acids extracted with 4 and 5 mL heptane are compared in Table III. Higher

concentrations of all the FA standards assayed were obtained when extraction was performed with 4 mL heptane rather than 5 mL. As is apparent from the results in Table III, moreover, the extraction yield was the same for saturated and all mono and polyunsaturated fatty acids. Mild saponification and methylation at 40°C in combination with extraction with 4 mL heptane can be regarded as the most suitable method of sample preparation for determination of FAs as methyl esters.

Table III

Dependence of the peak areas (S_n) of the fatty acids assayed on the volume of heptane used for extraction of FAMEs (base and acid-catalyzed methylation were performed at 40°C)

Fatty acid	5 mL heptane	4 mL heptane	S_n (4 mL)/S_n (5 mL)
*c*9C18:1	263	359	1.363
C19:0	683	948	1.388
*c*9*c*12C18:2	175	241	1.376
*c*9*t*11CLA	5351	7144	1.335
*t*10*c*12CLA	5563	7173	1.290
t,*t*CLA	2563	3465	1.349
Sum of CLA isomers	13754	18471	1.343
*c*5*c*8*c*11*c*14C20:4	863	1164	1.349
*c*5*c*8*c*11*c*14*c*17C20:5	76	103	1.346

The Effect of BHT on the Profile of Methylated Fatty Acid Standards

Formation of unidentified artefacts during base and acid-catalyzed methylation resulted in inaccurate FA determination. The presence of antioxidants (e.g. tocopherols, pyrogallol, or BHT [2,6]) or elimination of oxidation-promoting species significantly improved the accuracy of the chromatographic quantification of FAs, particularly for CLA isomers and other long-chain PUFA. The CLA isomer mixture, other unsaturated fatty acid standards, and rat liver samples were used to evaluate the effect on the profile of the FAMEs of using Ar to remove air from solutions of the methylated FAs (derivatization–Ar). The profiles obtained were compared with those obtained after FA methylation in the presence of BHT (derivatization–BHT) (Table IV).

To avoid compromising accuracy in the quantification of unsaturated fatty acids, particularly CLA isomers, all methylation was performed at the optimum derivatization temperature, 40°C. Detailed analysis of CLA

Table IV

Comparison of fatty acid methylation performed in the presence of 2,6-di-*tert*-butyl-*p*-cresol (BHT) (derivatization–BHT) and in the absence of BHT (derivatization–Ar)

Processed fatty acid standards[a] (average peak area ± *SD*)					
		Derivatization–BHT		Derivatization–Ar	
		Fully processed[b]	Base–acid methylation[c]	Fully processed[b]	Base–acid methylation[c]
t,*t*CLA	Ag^+–HPLC	$45 \times 10^5 \pm 3 \times 10^5$	$62 \times 10^5 \pm 5 \times 10^5$	$46 \times 10^5 \pm 1 \times 10^5$	$63 \times 10^5 \pm 1 \times 10^5$
	GLC	971 ± 30	1371 ± 1	946 ± 3	1369 ± 1
*c*9*t*11CLA	Ag^+–HPLC	$80 \times 10^5 \pm 5 \times 10^5$	$113 \times 10^5 \pm 4 \times 10^5$	$82 \times 10^5 \pm 2 \times 10^5$	$113 \times 10^5 \pm 1 \times 10^5$
	GLC	1968 ± 50	2716 ± 78	1949 ± 2	2802 ± 5
*t*10*c*12CLA	Ag^+–HPLC	$85 \times 10^5 \pm 5 \times 10^5$	$20 \times 10^5 \pm 5 \times 10^5$	$86 \times 10^5 \pm 3 \times 10^5$	$19 \times 10^5 \pm 1 \times 10^5$
	GLC	1894 ± 43	2723 ± 44	1871 ± 3	2721 ± 13
Sum of all *c*,*t*/*t*,*c* CLA	Ag^+–HPLC	$176 \times 10^5 \pm 10 \times 10^5$	$246 \times 10^5 \pm 1 \times 10^5$	$179 \times 10^5 \pm 6 \times 10^5$	$244 \times 10^5 \pm 1 \times 10^5$
	GLC	3952 ± 101	5544 ± 26	3915 ± 1	5676 ± 56
c,*c* CLA	Ag^+–HPLC	$10 \times 10^5 \pm 1 \times 10^5$	$13 \times 10^5 \pm 3 \times 10^5$	$10 \times 10^5 \pm 1 \times 10^5$	$11 \times 10^5 \pm 1 \times 10^5$
	GLC	208 ± 10	290 ± 3	200 ± 1	288 ± 1
*c*9C18:1	GLC	617 ± 5	1019 ± 1	614 ± 1	1021 ± 1
*c*9*c*12C18:2	GLC	568 ± 4	949 ± 1	564 ± 3	952 ± 1
*c*9*c*12*c*15C18:3	GLC	460 ± 4	777 ± 1	458 ± 4	781 ± 1
*c*6*c*9*c*12C18:3	GLC	547 ± 4	918 ± 1	544 ± 5	927 ± 1

Saponification and base and acid-catalyzed methylation of fatty acids in rat livers[d] (μg g^{-1} DM)			
		Base-catalyzed	Acid-catalyzed
C15:0	GLC	58 ± 5	57 ± 2
C16:0	GLC	4247 ± 59	4148 ± 102
*c*9C16:1	GLC	150 ± 6	142 ± 9
*t*11C18:1	GLC	18 ± 2	19 ± 2
*c*6C18:1	GLC	41 ± 4	40 ± 2
*c*9C18:1	GLC	920 ± 11	981 ± 93
*c*11C18:1	GLC	368 ± 6	369 ± 1
*c*9*c*12C18:2	GLC	3928 ± 53	3808 ± 44
C20:0	GLC	18 ± 1	18 ± 1
*c*9*c*12*c*15C18:3	GLC	610 ± 8	597 ± 6
*c*9*t*11CLA	GLC	621 ± 14	653 ± 39
*t*10*c*12CLA	GLC	443 ± 1	472 ± 46
c,*c*CLA	GLC	50 ± 9	64 ± 7
t,*t*CLA	GLC	194 ± 18	173 ± 1
*c*13C22:1	GLC	7 ± 1	5 ± 2
*c*11*c*14*c*17C20:3	GLC	50 ± 3	55 ± 6
*c*5*c*8*c*11*c*14C20:4	GLC	4229 ± 55	4109 ± 20
*c*5*c*8*c*11*c*14*c*17C20:5	GLC	1670 ± 5	1644 ± 18
*c*7*c*10*c*13*c*16*c*19C22:5	GLC	701 ± 20	686 ± 1
*c*4*c*7*c*10*c*13*c*16*c*19*c*C22:6	GLC	2889 ± 76	2845 ± 4

[a] Each result is the mean from methylation of two FA standard samples; *SD* denotes standard deviation and DM dry mass

[b] The procedure comprised saponification and base–acid catalyzed methylation at 40°C

[c] The procedure comprised solely base–acid catalyzed methylation at 40°C

[d] Each result is the mean FA concentration in the liver of rats fed a diet enriched with 2% CLA isomer mixture

isomers and other PUFA and MUFA (Table IV) revealed that derivatization–Ar resulted in almost the same results for FAMEs as derivatization–BHT. The results obtained from derivatization–Ar reveal this is a universal procedure for accurate quantification of FAs in biological samples. The proposed mild saponification and gentle base and acid-catalyzed methylation at 40°C, without use of BHT, (derivatization–Ar) caused no isomerization and produced no artefacts and can be recommended, particularly for analysis of conjugated FAs (e.g. CLA isomers, their catabolites, and higher metabolites), as FAMEs, using argentation–liquid chromatography (Ag^+–HPLC with DAD) then determination of other FAs by GLC–FID. The absence of BHT in processed FAMEs samples enabled accurate determination of all conjugated FAs by use of Ag^+–HPLC with UV detection at 232–234 nm without problems caused by overlapping of the peaks of assayed conjugated FAs with the broad peak of BHT, which contains an aromatic ring. In contrast, the peak of BHT added to processed samples (derivatization–BHT) overlapped some of the peaks of conjugated FAs (especially *c*,*c* and *c*,*t*/*t*,*c* CLA isomers and some CLA metabolites) when Ag^+–HPLC–DAD was used. The very large BHT peak also interfered in the region of the short-chain fatty acids (especially C14:0 and C14:1) in our GLC chromatograms (GLC–FID) [2,6].

CONCLUSION

Combination of mild saponification and gentle base and acid-catalyzed methylation at 40°C, without addition of BHT (derivatization–Ar), in combination with extraction with 4 mL heptane, is the best compromise between accuracy and the yield in methylation of FAs in the quantification of FAs, particularly conjugated dienes. The proposed procedure adequately prepared FAMEs from FA standards and lipids in biological samples, because the mild saponification and the basic and acidic catalysts typically used for methylation at 40°C without BHT (derivatization–Ar) resulted in negligible alteration of the FA composition, especially CLA isomers, and enabled simultaneous determination of the complex mixture of methylated conjugated FAs in the presence of other FAs by use of argentation–liquid chromatography (Ag^+–HPLC) and DAD. Argentation–liquid chromatography in conjunction with UV detection at 232–234 nm is the method of choice for analysis of fatty acids containing conjugated double bonds (e.g. CLA isomers and their catabolites and higher metabolites) [2,8,9]. Other methylated FAs, and the methylated conjugated FAs in samples processed by de-

rivatization–Ar, can subsequently be accurately quantified by use of high-resolution long-capillary column GLC–FID [2].

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