Journal of Animal and Feed Sciences, 11,2002, 517–526

A highly efficient method for derivatization of fatty acids for high performance liquid chromatography*

M. Czauderna, J. Kowalczyk, K.M. Niedźwiedzka and I. Wąsowska

The Kielanowski Institute of Animal Physiology and Nutrition,
Polish Academy of Sciences
05-110 Jabłonna, Poland

(Received 16 May 2002; accepted 2 August 2002)

ABSTRACT

A new derivatization procedure for fatty acids followed by HPLC analysis with UV detection is described. Originally, derivatization was carried out with acetone solutions of 2,4'-dibromoacetophenone and triethylamine at 50°C for 2h. To prevent oxidation and isomerization, unsaturated fatty acids and conjugated dienes in particular were derivatized at a lower temperature (40°C) for 30 min using a concentrated solution of 2,4'-dibromoacetophenone in acetone and triethylamine. The new derivatization method decreases the risk of degradation and isomerization of unsaturated fatty acids. Due to the use of higher concentrations of substrates in reaction mixtures, significantly enhanced sensitivity of fatty acid analysis is observed. In contrast to the original derivatization method, the new one can be successfully used for both large and trace concentrations of fatty acids in various types of biological materials.

KEY WORDS: fatty acids, derivatization, determination

INTRODUCTION

The fatty acid profile in the animal body depends on the fat composition of feed mixtures. Various additions to these mixtures possess different abilities of modifying fatty acid proportions in animal tissues (Bas and Morand-Fehr, 2000; Bessa et al., 2000; Chilliard et al., 2000; Mir et al., 2000). Moreover, interest in meat and milk fats has reappeared because of many studies aimed at elevating essential unsaturated fatty acid (FA) contents and accurately quantifying trace amounts of *trans* FAs and low levels of conjugated linoleic acid isomers (Demeyer et al., 1999; Parodi, 1999; Wood et al., 1999). Furthermore, not only is the range of FA

* Supported by State Committee for Scientific Research, Grant No 3 P06Z 034 22

carbon chain length from C_4 to C_{26}, including branched-chain FAs (Kramer et al., 1997), but, in milk samples in particular, there are low quantities of various geometric and positional isomers of mono-, di-, and tri-unsaturated FAs. Therefore, investigations on FA metabolism require an accurate, sensitive, precise, and reliable method for FA determination. Various HPLC methods, capillary electrophoresis or gas chromatographic (GC) techniques have been used for analysis of FA profiles. Unfortunately, difficulties may occur in quantitative formation of esters from such a complex mixture of FAs in rumen fluid, intestinal digesta, milk, or meat samples. Indeed, sodium methoxide-catalyzed methylations have been used, however, free FAs, sphingolipids, and glycosphingolipids are not methylated under these conditions (Kramer et al., 1997). On the other hand, acid-catalyzed methylations convert all lipid and FAs, but during derivatization conjugated dienes are isomerized. The main advantage of HPLC over GC methods is the possibility of collecting fractions for further analysis and lower temperatures during the analysis, which reduce the risk of izomerization of double bonds. Unfortunately, fatty acid methyl esters, like free fatty acids separated by HPLC, have high molar absorption at low UV wavelengths (<205 nm). Therefore, for FA derivatization it is better to convert FAs with reagents possessing high molar absorptivity at longer UV wavelengths. Thus, the present study was undertaken to develop a method for preparation of FA derivatives possessing high molar absorptivity at longer UV wavelengths (>240 nm). To ensure stability of the formed product we used 2,4'-dibromoacetophenone as the derivative agent. The high molar absorption (with the maximum at ~256 nm), low temperature of the derivatization procedure make this FA aromatic-derivative suitable for reversed-phase HPLC with UV detection.

The aim of the presented study was to obtain better sensitivity of assayed FAs after their derivatization with 2,4'-dibromoacetophenone in the presence of triethylamine. The proposed method should provide a better method for determining trace amounts of essential FAs in prticular.

MATERIAL AND METHODS

Reagents

All chemicals were of analytical grade and organic solvents were of HPLC grade. Acetone, dichloromethane, glacial acetic acid, methanol were purchased from POCh (Gliwice, Poland) while acetonitrile from J.T. Baker (Holland). Triethylamine and 2,4'-dibromoacetophenone were from Merck (Darmstadt, Germany). Palmitic, stearic, ricinoleic, ricinelaidic, linolenic, γ-linolenic, linolenelaidic, linoleic, linolelaidic, *cis*-vaccenic, oleic, petroselinic, elaidic, *trans*-7-octadecenoic and petroselaidic acids were purchased from Sigma (St. Louis, MO, USA). A mixture of

conjugated linoleic acid isomers (CLA) was also from Sigma and represents a mixture of position and geometric isomers of linoleic acid, i.e.: *cis,trans/trans,cis*-9,11 (combined ~41.2%); *trans,cis*-10,12 (~44.1%); *cis,cis*-9,11 (~1.1%); *cis,cis*-10,12 (~9.4 %); *trans,trans*-9,11 and -10,12 (combined ~1.3%)). Nonanoic (an internal standard), caprylic, capric, lauric, myristic and behenic acids were from Fluka. All other chemicals were obtained from POCh (Gliwice, Poland). Water used for the preparation of mobile phases and chemical reagents was prepared using an Elix™ water purification system (Millipore, Canada). The mobile phases were filtered through a 0.45 μm membrane filter (Millipore) and then de-gassed for 2-3 min in vacuum with utrasonication prior to use.

Chromatographic equipment

The instrument used consisted of an alliance separation module (model 2690, Waters) and a Waters 996 photodiode array detector (DAD). Development of the gradient systems, collections and data integration were performed using a Pentium III computer (Compaq) and Millennium 2001 software (version 2.15). All derivatized fatty acids were detected at 256 and 234 nm. UV spectra of the eluate with a spectral resolution of 1.2 nm were obtained every second and were electronically stored on a computer hard disk. Separations of derivatized fatty acids were performed on two Nova Pak C_{18} columns (4 μm, 250 x 4.6 mm I.D., Waters) in conjunction with a Waters guard column of 10 x 6 mm I.D. containing reversed-phase C_{18} (30-40 μm) pellicular packing material.

Analytical solvents and gradient elution systems

Two HPLC grade solvents were used in this study. Solvent A was acetonitrile, while solvent B was water. For analysis of all derivatized fatty acids in standards and biological samples, a binary gradient elution program was used (Table 1).

TABLE 1

Binary gradient elution system used for separation of derivatized fatty acids

Time min	Flow rate ml/min	Composition, %		Curve[b]
		Solvent A	Solvent B	
5.0	2.4	68.0	32.0	11
23.0	2.4	76.5	23.5	6
34.5	2.7	85.0	15.0	6
80.9	2.7	85.0	15.0	11
81.0	3.0	100.0	0	6
100.0[a]	3.0	100.0	0	11

[a] – after 100 min the columns were re-equilibrated for 15 min in 68% solvent A and 32% solvent B at a flow rate 2.4 ml/min

[b] – Millennium[32] Chromatography Manager, 1998. Waters Corporation, Milford, MA 01757

All separations were performed at a column temperature of 37°C. Injection volumes were 5-20 µl. The maximum pressure of HPLC systems was 38.5 MPa. The ambient temperature was 20-23°C. Fatty acid derivative peaks were identified by the retention time of processed standards injected separately and by adding standard solutions to biological samples. The concentrations of fatty acids in biological samples were calculated using fatty acid standards and an internal standard (nonanoic acid) as a measure of the extraction and derivatization reaction yield. All samples and standards were protected from the light when the reaction components were carried through the hydrolysis and derivatization procedures described below.

The limit of detection (LOD) was calculated as a signal-to-noise ratio of 3, while the limit of quantification (LOQ) was defined as 10 times the noise level (Gratzfeld-Husgen and Schuster, 1996; Meyer 1999).

Preparation and hydrolysis of samples

Milk, meat, duodenal digesta and subcutaneous fat samples were collected from sheep. All samples were frozen, lyophilized and the obtained residues were stored in sealed tubes at -20°C until analyzed. Milk (0.7-78 mg), meat (4-65 mg), fat (2-7 mg) and duodenal digesta (10-110 mg) samples were hydrolyzed with 3.5-4.0 ml of 2 M NaOH at 80-85°C for 30-35 min in sealed tubes. The resulting solutions should be protected from the light and stored in sealed tubes under argon. After cooling the hydrolysates were acidified with 4 M HCl to pH ~ 2 and then free fatty acids were extracted four times with 3.5 ml of dichloromethane. The lower organic layer was dried with Na_2SO_4 (50 - 100 mg) and then the organic solvent was removed under a gentle stream of argon (Heinig et al., 1998). The residue was used for derivatization as below.

Derivatization procedures

New derivatization mode: To a residue in a reactivial, 0.5 ml of dibromacetophenone (48 g/L in acetone) and 60 µl of triethylamine were added together with vigorous mixing. The resulting solution was again mixed and reacted for 30 min at 40°C. All derivatized samples were protected from the light. It is recommended to store the reaction mixtures in sealed tubes under argon. The derivatized procedure for standards was the same as for biological materials. The resulting solutions were injected onto C_{18} columns (Nova Pak). All processed samples should be protected from light and stored at about -25°C until analysed.

Original derivatization mode (Heinig et al., 1998): To a residue in a reactivial, 1 ml of dibromacetophenone (12 g/L in acetone) and 1 ml of triethylamine (10 g/L in acetone) were added. The contents were mixed and reacted for 2 h at 50°C. The

derivatizing procedure was stopped by adding 100 µl of acetic acid (2 g/L in acetone). The resulting solutions were injected onto HPLC columns.

RESULTS AND DISCUSSION

The main analytical problem in the presented work was to obtain a better yield of the derivatization procedure in comparison with the original one. Moreover, the composition of the mixture of positional and geometric isomers of unsaturated FAs may depend on the reaction conditions applied for the formation of FA derivatives. Part of the polyunsaturated FAs, especially CLA isomers, may undergo isomerization or even degradation during derivatization carried out for a long time and at a high temperature. Therefore, it seems reasonable to examine in detail the derivatization of FAs carried out with a high concentration of 2,4'-dibromoacetophenone and triethylamine at a lower temperature (i.e. at 40°C) and for only 30 min. In the first set of experiments, various amounts of triethylamine (TEA) were added to hydrolysed milk samples (~15 mg) and 0.5 ml of 2,4'-dibromacetophenone (48 g/L in acetone). It is well documented that much better yields of the examined derivatization reaction are obtained in solutions containing a large excess of basic compounds (Czauderna et al., 2001). A systematic study of the effect of TEA concentration on the derivatization yield showed that the maximum yield of FA derivatives was obtained when 60 µl of TEA was utilized in the derivatization procedure (i.e. 0.5 ml of dibromacetophenone (48 g/L in acetone) and 60 µl of TEA). As expected, a larger excess of TEA in reaction mixtures does not elevate the yield of derivatized FAs. Subsequently, we studied the dependence of the mass of analysed biological samples on the yield of FA derivative formation using the new derivatization procedure. In these experiments it was demonstrated that the concentrations of formed FA derivatives were proportional to the amount of assayed biological samples within a very broad range. Indeed, the concentrations of formed FA derivatives in relation to the mass of assayed biological samples were linear functions within a broad range of examined samples (see sections: *Preparation and hydrolysis of samples* and *New derivatization mode*). This is a consequence of better UV detector response due to considerably higher concentrations of substrates in comparison with ones in the original derivatization procedure.

The evaluation of the new derivatization procedure was assessed by measuring concentrations of FA derivatives formed in milk, meat, duodenal digesta or subcutaneous fat samples as well as by the purity of analytical peaks corresponding to quantified FAs. On examining the yield of FA derivatives in milk on reversed-phase C_{18} HPLC columns (Czauderna et al., 2001) we found that our new derivatization mode significantly increased the concentrations of derivatized FAs (4-5 times) in comparison with the original derivatization procedure (Table 2). Further experiments

TABLE 2

Dependence of peak area (S_N) corresponding to derivatized FAs in milk samples (~6 mg) upon the derivatization procedure (i.e. the new (S_N^{new}) and original ($S_N^{original}$) derivatization process) and calculated limits of detection (LOD) and quantification (LOQ) derived from determination of FAs by the new derivatization method[a]

Derivatized fatty acids	$S_N^{new}/S_N^{original}$	LOD (pg)	LOQ (pg)
Caprylic acid	4.66	$0.57 \cdot 10^{-4}$	$1.90 \cdot 10^{-4}$
Capric acid	4.52	0.07	0.23
Lauric acid	4.78	$0.10 \cdot 10^{-2}$	$0.33 \cdot 10^{-2}$
Linolenic acid	4.39	$0.40\ 10^{-2}$	$1.21\ 10^{-2}$
g-Linolenelaidic acid	4.61	0.04	0.13
Myristic acid	4.42	0.66	2.18
Linoleic acid	4.27	0.52	1.74
Linolelaidic acid	4.68	0.27	0.90
Palmitic acid	4.42	0.66	2.21
Oleic acid	4.53	0.28	0.94
Petroselinic acid	4.81	1.17	3.91
Elaidic acid	4.32	0.31	1.27
Petroselaidic acid	4.29	0.45	1.50
Stearic acid	4.14	0.44	1.46
Internal standard (Nonanoic acid)	4.67	–	–

[a] - the amount of 2,4'-dibromoacetophenone was established as the same in the both derivazation modes, while the volume of added triethyłamine was in agreement with given derivatization procedure

performed to compare the derivatization yield also showed that concentrations of the reaction products were always higher for saturated, mono- and polyunsaturated FAs in meat, duodenal digesta and fat samples. Based on HPLC analysis of FA derivatives in milk and meat samples using both derivatization procedures and FA standards processed by the original derivatization method, we calculated the limits of detection (LOD) and quantification (LOQ) for assayed FAs using the new derivatization procedure. As can be seen from experiments performed to compare both derivatization procedures (Table 2) and our earlier investigations (Czauderna and Kowalczyk, 2002) the proposed derivatization method offered considerably better LOD and LOQ in comparison with the original derivatization procedure (Czauderna and Kowalczyk, 2002). Considering the above results, it seems reasonable to suggest that the new derivatization procedure can be carried out for 30 min at 40°C. Further tests are obviously needed, especially for assessment of the purity of analytical peaks corresponding to derivatized FAs in various types of biological materials. Therefore, the accuracy of the new derivatization procedure was evaluated by determining relationships between the monitoring wavelength (λ_{nm}) and ratios (R^{nm}) of individual FAs in assayed biological materials (R^{nm}_{sample}) by the new

TABLE 3

Average±SD of R^{nm} values[a] obtained in the UV monitoring range for assayed FA derivatives in various types of biological samples

Fatty acids	Milk (241-269 nm)[b]	Meat	Duodenal digesta	Subcutaneous fat
Caprylic acid	1.05±0.17	1.06±0.07 (249-260 nm)	1.04±0.14 (241-269 nm)	0.98±0.10 (241-269 nm)
Capric acid	1.04±0.07	-[c]	1.01±0.10 (241-269 nm)	1.08±0.13 (249-266 nm)
Lauric acid	1.10±0.17	1.01±0.04 (246-260 nm)	0.97±0.05 (241-269 nm)	0.94±0.11 (246-266 nm)
Linolenic acid	0.95±0.11	0.98±0.16 (241-269 nm)	1.03±0.04 (230-280 nm)	0.97±0.23 (246-266 nm)
γ-Linolenic acid	1.02±0.27	1.08±0.13 (241-269 nm)	1.05±0.07 (241-269 nm)	0.94±0.14 (249-263 nm)
Linolenelaidic acid	-[c]	1.04±0.07 (230-275 nm)	1.00±0.12 (241-269 nm)	1.02±0.07 (249-269 nm)
Myristic acid	1.02±0.11	1.04±0.05 (241-266 nm)	0.95±0.14 (246-266 nm)	1.05±0.16 (249-263 nm)
Palmitic acid	0.99±0.12	0.98±0.01 (230-280 nm)	1.02±0.04 (230-280 nm)	1.01±0.02 (230-280 nm)
Oleic acid	0.95±0.12	1.00±0.03 (230-280 nm)	1.03±0.09 (241-269 nm)	1.01±0.02 (230-280 nm)
Petroselinic acid	1.02±0.27	0.98±0.04 (230-280 nm)	1.02±0.08 (241-269 nm)	0.97±0.08 (241-280 nm)
Elaidic acid	1.00±0.09	-[c]	-[c]	-[c]
Petroselaidic acid	1.08±0.11 (252-258 nm)	1.00±0.06 (235-269 nm)	1.03±0.08 (252-263 nm)	-[c]
Stearic acid	1.04±0.17	1.01±0.03 (241-269 nm)	1.02±0.07 (241-269 nm)	0.99±0.15 (241-269 nm)
Internal standard (C9:0)	0.95±0.21	1.04±0.06 (241-280 nm)	0.95±0.06 (241-269 nm)	0.99±0.01 (241-269 nm)

[a] – values (R^{nm}) of ratio R^{nm}_{sample} and $R^{nm}_{standard}$: $R^{nm} = R^{nm}_{sample}/R^{nm}_{standard}$. Absorption maximum in the UV range of 230 to 280 nm: at $\lambda_{maximum}$ = 256 nm. Values ($R^{nm}_{standard}$) of ratio of fatty acid peak area in a standard monitored at $\lambda_{maximum}$ (i.e. $S^{maximum}_{standard}$) and other examined wavelength (i.e. $S^{nm}_{standard}$): $R^{nm}_{standard} = S^{nm}_{standard}/S^{maximum}_{standard}$. Values ($R^{nm}_{sample}$) of ratio of fatty acids peak area in biological samples monitored at $\lambda_{maximum}$ (i.e. $S^{maximum}_{sample}$) and other examined wavelength (i.e. S^{nm}_{sample}): $R^{nm}_{sample} = S^{nm}_{sample}/S^{maximum}_{sample}$

[b] – the examined the UV range

[c] – non-integrated peaks (i.e., below LOQ)

derivatization procedure and the original one ($R^{nm}_{standard}$) (i.e. $R^{nm} = R^{nm}_{sample}/R^{nm}_{standard}$ for details see Czauderna and Kowalczyk (2001). Summaries of results obtained from monitoring at various wavelengths are given in Table 3. Fortunately, the ratios (R^{nm}) of saturated, mono- and polyunsaturated FAs for milk, meat, duodenal digesta and subcutaneous fat were nearly 1 in a broad UV range including the maximum of absorbance of FA derivatives (i.e. 256 nm). In wavelengths shorter or longer than 256 nm, background fluctuation or the close presence of interfering endogenous species impeded the accurate integration of some small FA peaks, especially when the original derivatization procedure was applied. Consequently, in this instance, the ratios (R^{nm}) were calculated for the narrower UV range, i.e. in the proximity of the maximum of FA derivative absorbance (Table 3). Comparison of the obtained results indicated that monitoring at 256 nm should be used for quantification of the assayed FAs because all of the analytical peaks corresponding to analysed compounds are pure and free from unidentified species. Subsequent experiments demonstrated that our new derivatization procedure in conjunction with HPLC analysis and UV detection at 234 and/or 256 nm (Czauderna and Kowalczyk, 2001) enabled the sensitive quantification of trace amounts of CLA (i.e. estimated LOD and LOQ values were 0.14 and 0.47 pg, respectively). Based on UV detection, the new derivatization method was found to produce a greater concentration of derivatized CLA standards in comparison with the original derivatization procedure. Fortunately, summaries of results of purity investigations revealed that peaks corresponding to derivatized CLA isomers are pure in a broad UV range from 210 to 275 nm. Indeed, for the main peaks corresponding to *cis-9 trans-11, trans-9 cis-11, trans-10 cis-12 and cis-10 cis-12* CLA isomers the average±SD of R^{nm} value was 1.02±0.06.

Exhaustive investigations of the proposed derivative method have demonstrated that no essential changes in the concentrations of FA derivatives in assayed biological samples were found when HPLC analysis was performed after 24 h of storage at -18°C, while only a slight decrease of FA derivative concentrations (~2-3 %) was observed in samples stored at 40°C for 2 h.

CONCLUSIONS

The proposed derivatization method provides a universal and simple procedure for derivatization of saturated and unsaturated FAs in various types of biological materials followed by liquid chromatographic analysis with highly efficient and selective UV detection. As FA derivatizations are carried out at a lower temperature (40°C) and for only 30 min, the new derivatization method considerably diminishes the risk of isomerisation and degradation of unsaturated FAs (especially conjugated dienes) in comparison with the original derivatization procedure (Heinig et al., 1998). Moreover, due to the use of higher concentrations of sub-

strates in reaction mixtures, a substantial increase of sensitivity of FA analysis without elevating the formation yield of interfering species was found. In contrast to the original derivatization procedure, our new method can be successfully used for quantification of both trace and large amounts of FAs in various types of biological samples.

REFERENCES

Bas P., Morand-Fehr P., 2000. Effect of nutritional factors on fatty acid composition of lamb fat deposits. Livest. Prod. Sci. 64, 61-79

Bessa R.J.B., Santos-Silva J., Ribeiro J.M.R., Portugal A.V., 2000. Reticulo-rumen biohydrogenation and the enrichment of ruminant edible products with linoleic acid conjugated isomers. Livest. Prod. Sci. 63, 201-211

Chilliard Y., Ferlay A., Mansbridge R.M., 2000. Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated, *trans* and conjugated fatty acids. Ann. Zootech. 49, 181-205

Czauderna M., Kowalczyk J., 2001. Separation of some mono-, di- and tri-unsaturated fatty acids containing 18 carbon atoms by high-performance liquid chromatography and photodiode array detection. J. Chromatogr. B 760, 165-178

Czauderna M., Kowalczyk J., Chojecki G., 2001. An improved method for derivatization of fatty acids for liquid chromatography. J. Anim. Feed Sci. 10, Suppl. 2, 369-375

Czauderna M., Kowalczyk J., Potkański A., Szumacher-Strabel M., Chojecki G., 2001. Quantification of conjugated linoleic acid and other essential fatty acids in ovine meat, milk, fat, and intestinal digesta. J. Anim. Feed Sci. 10, Suppl. 2, 385-392

Demeyer D., Doreau M., 1999. Targets and means for altering meat and milk lipids. Proc. Nutr. Soc. 58, 593-607

Gratzfeld-Husgen A., Schuster R., 1996. HPLC for Food Analysis. Hewllett-Packard (Germany), p. 117

Heinig K., Hissner F., Martin S., Vogt C., 1998. Separation of saturated and unsaturated fatty acids by capillary electrophoresis and HPLC. Amer. Lab., May, 24-29

Kramer J.K.G., Fellner V., Dugan M.E.R., Sauer F.D., Mossoba M.M., Yurawecz M.P., 1997. Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total *trans* fatty acids. Lipids 32, 1219-1228

Meyer V.R., 1999. Practical High-Performance Liquid Chromatography. John Wiley and Sons, Chichester (UK), p. 78

Mir Z., Rushfeldt M.L., Mir P.S., Paterson L.J., Weselake R.J., 2000. Effect of dietary supplementation with either conjugated linoleic acid (CLA) or linoleic acid oil on the CLA content of lamb tissues. Small Ruminant Res. 36, 25-31

Parodi P.W., 1999. Conjugated linoleic acid and other anticarcinogenic agents of bovine milk fat. J. Dairy Sci. 82, 1339-1349

Wood J.D., Enser M., Fisher A.V., Nute G.R., Richardson R.I., Sheard P.R., 1999. Manipulating meat quality and composition. Proc. Nutr. Soc. 58, 363-370

STRESZCZENIE

Wysokowydajna metoda derywatyzacji kwasów tłuszczowych stosowana w wysokosprawnej chromatografii cieczowej

W pracy przedstawiono wysokowydajną metodę derywatyzacji kwasów tłuszczowych, którą można wykorzystać w wysokosprawnej chromatografii cieczowej. Reakcję derywatyzacji zaleca się prowadzić w obecności znacznego nadmiaru stężonego roztworu 2,4'-dibromoacetofenonu oraz trietyloaminy podawanej bezpośrednio do układu reakcyjnego. W trakcie wprowadzania trietyloaminy należy intensywnie mieszać mieszaninę reakcyjną. Reakcję derywatyzacji należy prowadzić w temperaturze 40°C przez 30 min. Z uwagi na łagodne warunki, w jakich prowadzona jest reakcja upochodnienia kwasów tłuszczowych, proponowana nowa metoda minimalizuje niebezpieczeństwo izomeryzacji oraz degradacji nienasyconych kwasów tłuszczowych. Prezentowana metoda pozwala na uzyskanie lepszej czułości oznaczania kwasów tłuszczowych w porównaniu z wcześniej stosowaną metodą ich derywatyzacji.