Journal of Animal and Feed Sciences, 14, Suppl. 1, 2005, 563–566

Improving the analysis of fatty acids using a combination of gas chromatography and Ag^+ liquid chromatography[2]

M. Czauderna[1], J. Kowalczyk, K. Korniluk and I. Wąsowska

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

ABSTRACT

A method is proposed for routine determination of fatty acids (FAs), including conjugated fatty acids, in the liver of rats and sheep using gas-liquid chromatography (GLC) and Ag^+-HPLC. The proposed procedure includes new methods for saponification carried out at room temperature and extraction of free FAs. The obtained free FAs are methylated in an acid-base catalyzed procedure. Due to simultaneous fractionation of methylated FAs using GLC and Ag^+-HPLC, the accuracy and sensitivity of the procedure is improved (especially for conjugated linoleic acids).

KEY WORDS: fatty acids, conjugated dienes, gas-liquid, silver ion chromatography

INTRODUCTION

Characterization of the complex lipid fraction in food by its fatty acid me-thyl esters (FA-MEs) is a commonly accepted practice, particularly in the nutritional and biomedical fields. No single method is presently able to resolve all positional and geometric isomers of mono- and polyunsaturated fatty acids (MUFA, PUFA), conjugated linoleic acid isomers (CLA), saturated and branch-chain fatty acids (FAs). Capillary gas-liquid chromatography (GLC) is a widely used analytical technique, but liquid chromatographic methods are also receiving increased attention. The major objective of the current work was to develop a method for hydrolysis and derivatization of FAs. Attention has also been paid to improving the quantification of complex mixtures of FAs in the liver of rats and sheep. Therefore, long-capillary GLC and silver ion liquid chromatography (Ag^+-HPLC) were adapted to analyse complex mixtures of FAs, as well as CLA[2] isomers and other FAs containing conjugated double bonds (CFA).

[1] Corresponding author: e-mail: m.czauderna@ifzz.pan.pl

[2] the profile of CLA isomer mixture was presented previously (Czauderna et at., 2003)

MATERIAL AND METHODS

Two groups of 7 female Wistar rats each, 8 weeks of age were housed individually as described previously (Czauderna et al., 2004). Briefly, rats were fed a control diet or a diet enriched with 2% of a CLA isomer mixture and 2 ppm Se (as Na_2SeO_4). After 28 days the rats were killed and their livers were removed.

Sample preparation and saponification. Liver samples were frozen, lyophilized and the obtained residue was stored at -20°C until analysed. In vials, finely powdered liver samples (~50 mg) were treated with a mixture of 2 ml of 2M KOH in water, 2 ml 1M KOH in methanol, and 50 µl of BHT in methanol (20 mg/ml). The mixture was then vigorously mixed in a closed vial and heated under argon at 95°C for 10 min, cooled for 10 min at room temperature, and sonicated for 10 min. The solution was protected from light and stored in a sealed vial at 22-25°C overnight.

Extraction of free FAs. To the hydrolysate in the vial, 3 ml of water were added with vigorous mixing. Next, the solution was acidified with 4M HCl to pH 1-2 and free FAs were extracted four times with 3 ml of dichloromethane (DCM). The lower DCM layer was dried with 0.1g of Na_2SO_4. To avoid any loss of free FAs, extraction was repeated 4 times using 3 ml of n-hexane. The upper n-hexane layer was then combined with the DCM layer and the organic solvents were removed under a stream of argon (Ar).

Preparation of fatty acid methyl esters. To the residue, 2 ml of 2M NaOH in me-thanol and 50 µl of BHT in methanol (20 mg/ml) were added and mixed, flushed with Ar, and reacted for 1h at 80°C. After cooling, 2 ml of 25% BF_3 in methanol were added to the mixture, flushed with Ar, and again heated for 1h at 80°C. To the cooled reaction mixture, 5 ml of water were added and FA-MEs were extracted with 5 ml of n-hexane. The clear supernatant was transferred to a vial. FA-MEe were separated by GLC, FAs containing conjugated double bonds, by isocratic liquid chromatography (Ag^+-HPLC) with photodiode array detection (DAD) at 234 nm.

Analytical conditions. The analyses were performed on an Agilent 6890N GC equipped with CP7489 fused silica capillary column (100 m × 0.25 mm i.d. × 0.2 µm film thickness; Varian, USA) and FID. Split injection was performed using an Agilent 7683 autosampler; Ag^+-HPLC equipment as previously described (Czauderna et al., 2003) (mobile phase: n-hexane:acetonitrile -99.915:0.085, v/v; column temperature 31°C; flow-rate -1.35 ml/min).

RESULTS AND DISCUSSION

A chromatogram of FA-MEs standards is shown in Figure 1. Separation is satisfactory except for *c6*C18:1 and *c7*C18:1[2] and C20:0 and t9t12t18C18:3.

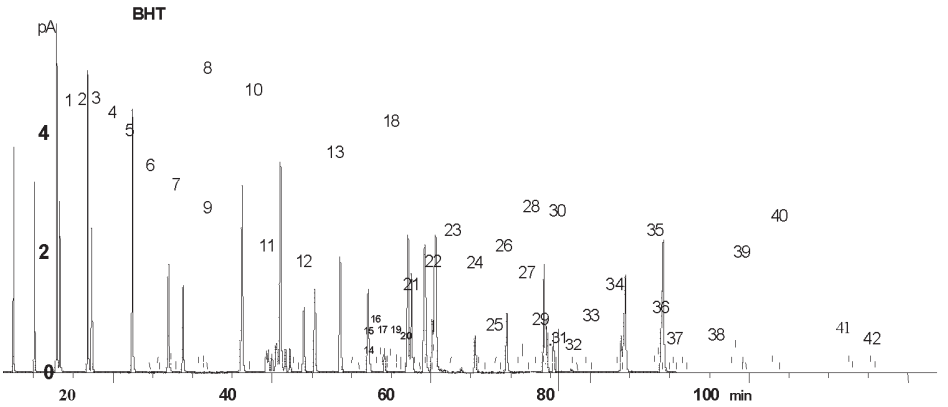

Figure 1. A chromatogram of FA-MEs[3]. The column was operated at 70°C for 4 min, then temperature programmed to rise by 12°C/min to 150°C, held for 6 min, then by 8°C/min to 168°C, held for 27 min, next by 0.75°C/min to 190°C, held for 10 min, then by 1.8°C/min to 210°C, held for 15 min, next by 6°C/min to 234°C, held for 4 min, then by 6°C/min to 236°C, held for 24 min. Peaks: 1 - C8:0; 2- C9:0; 3- C10:0; 4- C11:0; 5- C12:0; 6 - C13:0; 7 - C14:0; 8 -C14:1; 9 - C15:0; 10- C16:0; 11 –*c9*C16:1; 12 - C17:0; 13 - C18:0; 14 - *t6*C18:1; 15 - *t9*C18:1; 16 - *t11*C18:1; 17- *c6*C18:1 + *c7*C18:1; 18 - *c9*C18:1; 19 - *c11*C18:1; 20 - *c12*C18:1; 21 - C19:0; 22 - *t9t12*C18:2; 23 - *c9c12*C18:2; 24 - C20:0 + *t9t12t15*C18:3; 25 - *c6c9c12*C18:3; 26- *c11*C20:1; 27- c*9c12c15*C18:3; 28 - *c9t11*CLA; 29 - *c11t13/t11c13*CLA + *c8t10/t8c10*CLA; 30 - *t10c12*CLA + C21:0; 31 - *cc*CLA; 32- *tt*CLA; 33 -*c11c14*C20:2; 34 - C22:0; 35 - *c13*C22:1; 36 - *c11c14c17*C20:3; 37 - *c5c8c11c14*C20:4; 38 - *c5c8c11c14c17*C20:5; 39 - C24:0; 40 - *c15*C24:1; 41-*c7c10c13c16c19*C22:5; 42 -*c4c7c10c13c16c19*C22:6

[3] abbreviations for *cis* and *trans*: *c* and *t*, respectively

Using the temperature program from 70 to 236°C permitted separation of short-chain to very-long-chain saturated FAs (C24:0), as well as MUFA, PUFA and CLA isomers. Fatty acids, as FA-MEs, were also identified in the liver of rats fed a control diet and a diet enriched in Se and CLA. No peaks corresponding to endogenous liver species co-eluted with FA-MEs peaks of calibration standards (Figure 1). Detailed analysis of the mixture profile of methylated CLA and CFA was performed using Ag^+-HPLC. UV absorption spectra of CLA isomers in a standard and in rat livers bear close resemblance. The obtained results indicate that all integrated CLA isomer peaks in the biological sample are "pure" (~100%) in the applied UV range (from 205 to 280 nm) and free from the presence of unidentified species and noise fluctuations.

CONCLUSIONS

The presented procedure provides a universal method for gentle saponification using a mixture of KOH in water and methanol for ~12 h. As the saponification is

carried out at room temperature, the method eliminates the risk of isomerization (e.g., *t9*C18:1 into *c6*C18:1) and PUFA degradation. Addition of an extra 3 ml of water to hydrolysates and then the use of DCM and n-hexane for extraction of free FAs improved the formation yield of FA-MEs. A long capillary GLA column and the proposed column temperature program seem to be the best compromise for satisfactory fractionation of physiologically important FAs and duration of GCL analysis. Excellent characterization of conjugated FAs was obtained using Ag^+-HPLC since DAD distinguishes only FAs containing conjugated double bonds (like CLA or CFA), while other FAs are undetectable.

REFERENCES

Czauderna M., Kowalczyk J., Niedźwiedzka K.M., Wąsowska I., Pastuszewska B., Bulska E., Ruszczyńska A., 2004. Liver and body mass gain, content of CLA isomers and other fatty acids in the liver of rats fed CLA isomers and selenium. J. Anim. Feed. Sci. 13, 353-369

Czauderna M., Kowalczyk J., Wąsowska I., Niedźwiedzka K.M., 2003. Determination of conjugated linoleic acid isomers by liquid chromatography and photodiode array detection. J. Anim. Feed Sci. 12, 269-382

STRESZCZENIE

Udoskonalenie metody oznaczania kwasów tłuszczowych wykorzystując jednocześnie chromatografię gazową i Ag^+ chromatografię cieczową

Opisano czułą i selektywną procedurę oznaczania kwasów tłuszczowych (FA) w wątrobie owiec i szczurów. Opracowano nową metodę hydrolizy w temperaturze pokojowej i metodę ekstrakcji wolnych FA z hydrolizatu; metylowe pochodne FA uzyskano stosując zasadowo-kwasowo katalizowaną reakcję upochodnienia. Dzięki jednoczesnej analizie pochodnych FA metodą chromato-grafii gazowej i Ag^+-HPLC uzyskano poprawę dokładności i selektywności oznaczania FA.