Journal of Chromatography, 504 (1990) 202–206
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 226

Note

Argentation chromatography of fatty acid methyl esters using silver-loaded solid-phase extraction columns

FRANZ ULBERTH* and ELISABETH ACHS
Department of Dairy Research and Bacteriology, Agricultural University, Gregor Mendel Str. 33, A-1180 Vienna (Austria)
(First received August 24th, 1989; revised manuscript received December 19th, 1989)

Argentation chromatography, either on thin layers (TLC)[1] or on conventional columns[2], has proved to be of great value for the separation of unsaturated fatty acid methyl esters (FAMEs) according to the degree of unsaturation and geometry of the double bond(s)[3]. Recently, these established methods were adapted to high-performance liquid chromatographic (HPLC) techniques[4]. For this type of fractionation, custom-prepared silica gel impregnated with silver ions is used as the stationary phase by most workers[5,6]. One disadvantage of HPLC with silver-loaded silica phases is the relative short column life, due to the leaching of silver ions from the column by polar solvents[6]. In order to circumvent this problem, ion-exchange resins[7] and later benzenesulphonic acid, chemically bonded to silica gel[8–10], were successfully introduced as alternatives. With ion-exchange materials, the feasability of *in situ* impregnation of prepacked columns with silver ions by simply injecting an aequous silver solution into the column offers an additional benefit to the analyst[9–11].

As HPLC is expensive for sample pretreatment, it would be advantageous to separate FAMEs according to the number of double bonds by means of commercially available solid-phase extraction (SPE) tubes packed with cation-exchange material. This paper deals with the development of such a technique for the fractionation of milk fat FAMEs for subsequent gas–liquid chromatographic (GLC) analysis.

EXPERIMENTAL

Materials

Reagents and solvents were of analytical-reagent grade and used as received (E. Merck, Darmstadt, F.R.G.). The solid-phase extraction (SPE) columns, CHROMABOND SA (64 mm × 9 mm I.D., packed with 500 mg of benzenesulphonic acid, solvent volume 3 ml), were obtained from Macherey, Nagel & Co. (Düren, F.R.G.), Cat. No. 730 077. Reference FAMEs were purchased from E. Merck and Sigma (St. Louis, MO, U.S.A.).

0021-9673/90/$03.50

Argentation chromatography (Ag^+/SPE)

SPE columns were converted to the NH_4^+ form by flushing with 1% (w/v) ammonium acetate solution (10 ml), followed by distilled water (10 ml). A 2-ml volume of 1% (w/v) silver nitrate solution was allowed to drain by gravity flow through the column to impregnate the solid phase with silver ions. Excess silver ions were removed with methanol (10 ml), followed by 10 ml of dichloromethane (DCM) and *n*-hexane (10 ml) in order to re-equilibrate the column for apolar solvents. Typically, 1 ml of standard or sample solution was applied to the prepared column. After washing the column with 2 ml of *n*-hexane, FAMEs were eluted stepwise with various solvent mixtures at ambient temperature (20–25°C). The separated FAMEs were determined by drying the relevant fractions at 40°C in a stream of nitrogen and adding 1 ml of *n*-hexane containing methyl heptadecanoate as an internal standard for subsequent GLC analysis. Further details of the Ag^+/SPE procedure are outlined under Results and Discussion.

A standard FAME mixture (FAME standard) consisting of methyl esters of stearic acid (18:0), elaidic acid (18:1t), oleic acid (18:0c), linoleic acid (18:2), linolenic acid (18:3) and arachidonic acid (20:4) was prepared by dissolving approximately equal amounts (1 mg/ml) of each in *n*-hexane.

Milk fat was transesterified with methanolic potassium hydroxide as described by Christopherson and Glass[12], and finally diluted with *n*-hexane to give a FAME content of 2 mg/ml. Identification of major FAMEs in milk fat was performed by comparing the retention times obtained with those for a FAME standard resembling the approximate composition of milk fat (MF-FAME standard).

Gas–liquid chromatography

A Carlo Erba Mega 5300 high-resolution gas chromatograph, fitted with a dual injection system (on-column and split/splitless) and a flame ionization detector connected to a Spectra-Physics 4270 integrator, was used. The column installed was a 25 m × 0.32 mm I.D. fused-silica capillary column, coated with CP-Sil-88, $d_f = 0.2$ μm (Chrompack, Middelburg, The Netherlands). The carrier gas was hydrogen and the make-up gas was nitrogen. For split injection the column was operated isothermally at 150°C, whereas for on-column injection the temperature was programmed from 50°C (1 min isothermal) at 7°C/min to 220°C. Usually the FAME standard was split injected and the MF-FAME standard or milk fat FAMEs (MF-FAMEs) injected on-column.

RESULTS AND DISCUSSION

Method development

In principle, the procedure for preconditioning the CHROMABOND SA column with silver ions resembles the method described by Christie[9] for the preparation of a silver-loaded HPLC column. However, the solvent volumes used were adapted according to the reduced column size (500 mg of sorbent in the SPE column). For proper column impregnation, the silver solution must be allowed to pass through the SPE column by gravity flow, whereas the other solvents could be forced through the sorbent bed at a flow-rate of *ca.* 1 ml/min.

In a first attempt to separate the FAME standard, various solvent mixtures with

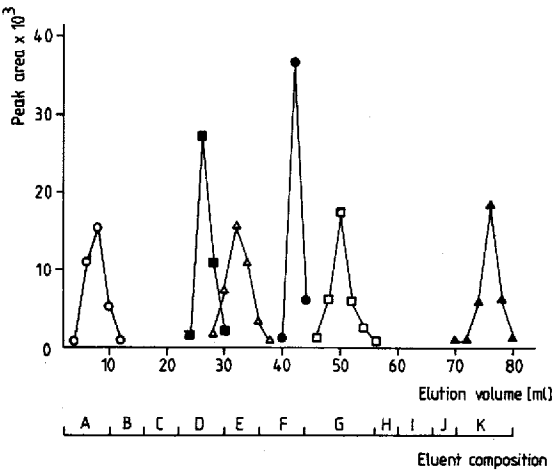

Fig. 1. Separation of FAMEs according to the geometry and number of double bonds. The Ag^+/SPE column was eluted batchwise (2-ml portions) with the eluents listed below. The amount (peak area) and identity of FAMEs (○ = 18:0; ■ = 18:1-*trans*; △ = 18:1-*cis*; ● = 18:2; □ = 18:3; ▲ = 20:4) present in each fraction was determined by GLC. Eluent composition (v/v): (A) 1% DCM in *n*-hexane; (B) 5% DCM in *n*-hexane; (C) 10% DCM in *n*-hexane; (D) 50% DCM in *n*-hexane; (E) 100% DCM; (F) 3% methanol in *n*-hexane; (G) 10% methanol in *n*-hexane; (H) 50% methanol in *n*-hexane; (I) 100% methanol; (J) 1% acetonitrile in methanol; (K) 10% acetonitrile in methanol.

increasing polarity were applied batchwise in 2-ml portions and the column effluents were analysed by GLC. In Fig. 1 the detector response (peak area) of the separated FAMEs is plotted against the elution volume and the eluent composition. The major proportion of 18:0 was eluted with 10 ml of 1% DCM in *n*-hexane. Unsaturated FAMEs were retained on the column unless the DCM concentration reached 50%. With this DCM–*n*-hexane mixture, 18:1t eluted as a relative sharp peak, but was not completely separated from 18:1c, which was recovered from the column with pure DCM. In order to elute the more unsaturated FAMEs, the polarity of the eluent had to be further increased by adding methanol to DCM. As is evident from Fig. 1, the dienoic and trienoic FAMEs were well resolved with DCM containing 3% and 10% methanol, respectively. Even with pure methanol, 20:4 did not elute. Thus acetonitrile, which is able to displace polyolefins from silver-loaded resin columns[13], was incorporated in the solvent. A concentration of 10% acetonitrile in methanol was necessary to speed up the elution of 20:4 and to recover this polyunsaturated FAME entirely. With a silver-loaded Nucleosil 5SA HPLC column and a linear gradient from methanol to methanol–acetonitrile (9:1), an excellent separation of bovine testis FAMEs according to the number of double bonds was obtained by Christie[9]. Although the SPE sorbent bed consisted of the same type of packing material, eluents with a lower polarity had to be used for the fractionation of the FAMEs in this study. Starting the procedure with methanol resulted in almost complete elution of the FAMEs without separation taking place.

Another series of experiments was carried out to optimize the elution strength and the amount of solvents needed to separate the FAME standard into pure components. Fractions of high purity and also excellent recoveries of the standard

compounds (usually $\geq 95\%$) were obtained with the selected solvent mixtures. Stearic acid was eluted with 5 ml of 10% DCM in *n*-hexane, 18:1t with 5 ml of 40% DCM in *n*-hexane, 18:1c with 5 ml of 0.5% methanol in DCM, 18:2 with 5 ml of 3% methanol in DCM, 18:3 with 5 ml of 1% acetonitrile in methanol and 20:4 with 10% acetonitrile in methanol. Only the geometric 18:1 isomers were not as well resolved as the remaining FAMEs; 2.3% of the applied 18:1t coeluted with the 18:1c fraction and 8.5% of the 18:1c with the 18:1t fraction. Complete resolution of a standard mixture containing geometric and positional 18:1 isomers was achieved by the aforementioned HPLC system, employing dichloromethane–1,2-dichloroethane (1:1) instead of the methanol–acetonitrile gradient as the elution solvent[10]. However, the separation efficiency of an analytical HPLC column is obviously not comparable to that of an SPE column commonly intended for sample pretreatment.

Application of the Ag^+/SPE method to milk fat FAMEs

When the optimized Ag^+/SPE method was applied to the separation of an MF-FAME standard, results in agreement with the standard mixture used during method development were obtained, except for the impaired resolution of 18:1t and 18:1c. With this test mixture, about one third of the 18:1t coeluted with the *cis*-monoenoic fraction. Saturated FAMEs eluted with 5 ml of 10% DCM in *n*-hexane as one fraction, independent of the chain length. Short-chain FAMEs were not recovered completely, because of their partial evaporation during the nitrogen drying step involved in sample preparation. Modifications of the solvent systems applied did not lead to any significant improvement in the *cis*/*trans*-monoene separation. Thus

TABLE I

APPLICATION OF THE Ag^+/SPE PROCEDURE TO THE FRACTIONATION OF MILK FAT FAMEs

Eluent	*FAME*	*Recovery (%)*[a]	
		Milk fat I[b]	*Milk fat II*[c]
10% DCM in *n*-hexane (5 ml)	8:0	51.0 ± 4.0	35.9 ± 4.8
	10:0	82.3 ± 0.6	72.8 ± 1.9
	12:0	93.8 ± 1.2	92.9 ± 2.6
	14:0	97.0 ± 1.3	98.1 ± 0.3
	15:0	97.4 ± 1.1	98.0 ± 0.9
	16:0	99.1 ± 0.8	94.6 ± 3.7
	18:0	99.5 ± 0.9	97.9 ± 5.2
0.5% methanol in DCM (5 ml)	10:1	90.7 ± 1.8	96.3 ± 1.4
	14:1	94.9 ± 1.3	101.5 ± 0.8
	16:1	97.9 ± 1.1	98.0 ± 1.7
	18:1	101.5 ± 0.4	102.7 ± 0.7
	18:2conj[d]	100.5 ± 0.7	99.6 ± 0.9
3% methanol in DCM (5 ml)	18:2	95.1 ± 0.6	103.3 ± 0.6
1% acetonitrile in methanol	18:3	93.3 ± 0.7	99.1 ± 3.9

[a] Mean ± standard deviation of four determinations.
[b] Iodine value 31.4.
[c] Iodine value 40.0.
[d] 18:2 conj = 9-*cis*,11-*trans*-octadecadienoic FAME.

MF-FAMEs, serving as a real sample, were separated into saturated and mono-, di- and triunsaturated FAME fractions, according to the elution scheme given in Table I. As in conventional argentation chromatography, the conjugated 18:2 (*cis*-9,*trans*-11-octadecadienoic FAME) eluted with the monoenoic fraction. The recoveries of the separated FAMEs from two different milk fat samples (iodine values 31.4 and 40.0, respectively) were in accordance with the values obtained in the preliminary experiments.

CONCLUSION

A silver-loaded solid-phase extraction column (Ag^+/SPE) provides a simple and rapid means for the fractionation of FAMEs according to the degree of unsaturation for subsequent GLC analysis. Relevant fractions of high purity and good recoveries are obtained with the procedure described. Compared with the commonly used argentation TLC, the Ag^+/SPE method offers the opportunity to separate and recover FAMEs in a single step, requiring only small solvent volumes.

REFERENCES

1 L. J. Morris, *Lab. Pract.*, 13 (1964) 284.
2 B. de Vries, *Chem. Ind. (London)*, 16 (1962) 1049.
3 L. J. Morris, *J. Lipid Res.*, 7 (1966) 717.
4 W. W. Christie, *High-Performance Liquid Chromatography and Lipids*, Pergamon Press, Oxford, 1987, p. 137.
5 C. R. Scholfield, *J. Am. Oil Chem. Soc.*, 56 (1979) 510.
6 M. Ozcimder and W. E. Hammers, *J. Chromatogr.*, 187 (1980) 307.
7 E. A. Emken, J. C. Hartman and C. R. Turner, *J. Am. Oil Chem. Soc.*, 55 (1978) 561.
8 W. S. Powell, *Anal. Biochem.*, 115 (1981) 267.
9 W. W. Christie, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 148.
10 W. W. Christie and G. H. McG. Breckenridge, *J. Chromatogr.*, 469 (1989) 269.
11 W. W. Christie, *J. Chromatogr.*, 454 (1988) 273.
12 S. W. Christopherson and R. L. Glass, *J. Dairy Sci.*, 52 (1969) 1289.
13 W. J. DeJarlais, R. O. Adolf and E. A. Emken, *J. Am. Oil Chem. Soc.*, 60 (1983) 975.