Journal of Chromatography A, 659 (1994) 95–99
Elsevier Science B.V., Amsterdam

CHROM. 25 620

Separation of *cis* and *trans* unsaturated fatty acid methyl esters by silver ion high-performance liquid chromatography

Richard O. Adlof

US Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, 1815 N. University, Peoria, IL 61614 (USA)

(First received August 23rd, 1993; revised manuscript received October 4th, 1993)

ABSTRACT

A variety of isomeric fatty acid methyl esters were separated on a commercially available silver ion chromatography column utilizing a versatile, isocratic solvent system composed of acetonitrile in hexane and utilizing UV detection. Samples containing all possible *cis* and *trans* isomers derived from methyl oleate (*cis*-9-octadecenoate; 18:1), methyl linoleate (*cis*-9, *cis*-12-octadecadienoate; 18:2), methyl linolenate (*cis*-9, *cis*-12, *cis*-15-octadecatrienoate; 18:3) and methyl arachidonate (*cis*-5, *cis*-8, *cis*-11, *cis*-14-eicosatetraenoate; 20:4) were analyzed. The *cis* and *trans* isomers from methyl oleate, methyl linoleate and methyl linolenate were resolved, as were 15 of the 16 isomers from methyl arachidonate. The separation of the 20:4 isomers exceeded the capability of capillary gas and liquid chromatographic methodologies.

INTRODUCTION

While silver ion liquid chromatography has been utilized to separate fatty acid methyl esters (FAMEs) by number of double bonds and by the configuration (*cis*/*trans*) of the double bonds [1–3], lack of commercial HPLC silver ion columns has limited the impact of this technology. Christie and co-workers [4–7] utilized a commercially available Nucleosil 5SA HPLC column and, by adding silver ions, were able to fractionate a variety of fatty acid isomers.

A commercially available column containing silver ions has recently been developed by Chrompack (ChromSpher Lipids HPLC column). A solvent system (developed by Christie and co-workers [5,6]) composed of dichloromethane, dichloroethane and small amounts (0.01 to 0.025%) of acetonitrile (ACN) is used with both the Nucleosil and Chrompack HPLC columns. Because chlorinated solvents are opaque at the wavelengths (200–210 nm) used for FAME analyses, the use of UV detectors is precluded unless the phenacyl derivatives of the fatty acids are first prepared [6]. We developed a UV-compatible solvent system (acetonitrile in hexane) for the separation of polyunsaturated *cis*- and *trans*-FAMEs.

EXPERIMENTAL

Materials and reagents

Hexane (Allied Fisher Scientific, Orangeburg, NY, USA), isooctane, light petroleum (b.p. 35–60°C) and ACN (all E. Merck, Darmstadt, Germany) were, except for the isooctane, HPLC grade and used as received. The *cis* and *trans* isomer mixtures were prepared from the all-*cis* precursors by *p*-toluenesulfinic acid-catalyzed isomerization [8]. This procedure results in less than 0.5% double bond migration [8,9]. Methyl oleate (*cis*-9-18:1), linoleate (*cis*-9, *cis*-12-18:2), linolenate (*cis*-9, *cis*-12, *cis*-15-18:3) and arachidonate (*cis*-5, *cis*-8, *cis*-11, *cis*-14-20:4) were

SSDI 0021-9673(93)E1050-A

isomerized in this manner. The 50:50 safflower (SFO)–linseed oil (LSO) methyl ester (7.1% 16:0, 3.6% 18:0, 19.9% 18:1, 35.9% 18:2 and 33.1% 18:3) mixture was prepared from commercial samples. All samples were eluted with light petroleum through a silica gel Sep-Pak (Waters, Milford, MA, USA) and dissolved in isooctane.

High-performance liquid chromatography

The HPLC equipment consisted of a Spectra-Physics (Freemont, CA, USA) P2000 solvent-delivery system, a Rheodyne (Cotati, CA, USA) 7125 injector with a 20-μl injection loop and an ISCO (Lincoln, NE, USA) V^4 absorbance detector. The ChromSpher Lipids column (250 mm × 4.6 mm I.D. stainless steel; 5 μm) was purchased from Chrompack (Middelburg, Netherlands) and used as received. Solvent flow was standardized at 1.0 ml/min and run temperatures at 22–23°C. A small cooling fan was used to minimize temperature fluctuations and bubble formation at the solvent pump mixing solenoid.

Analysis

The FAME samples were collected in scintillation vials and the solvents were evaporated. The fractions were analyzed on a Varian (Palo Alto, CA, USA) 3400 gas chromatograph equipped with a 30 m × 0.32 mm SP2380 (Supelco, Bellefonte, PA, USA) capillary column, flame ionization detector and utilizing helium as carrier gas. Thin-layer chromatography (TLC) was done on 3 × 1 in. (1 in. = 2.54 cm) silica gel 60A plates (Whatman, Maidstone, UK) impregnated with 10% silver nitrate. Ag-TLC was necessary for analysis of the eluted *cis/trans* fractions from methyl linolenate and arachidonate, since GC analysis of the former is difficult and has not been done for the latter. TLC solvent systems used were benzene for *cis/trans*-methyl linolenate and chloroform–acetone–acetic acid (96:4:0.5; v/v/v) for *cis/trans*-methyl arachidonate fractionation. TLC plates were sprayed with 2′,7′-dichlorofluorescein, dried and the spots were visualized by UV at 254 nm. TLC R_F values (5 cm solvent front migration) for isomerized methyl linolenate were 0.70 (three *trans*), 0.60 (two *trans*, one *cis*), 0.56 (one *trans*, two *cis*) and 0.44 (three *cis*). For isomerized methyl arachidonate, the values were 0.70 (four *trans*), 0.58 (three *trans*, one *cis*), 0.50 (two *trans*, two *cis*), 0.40 (one *trans*, three *cis*) and 0.38 (four *cis*). The FAMEs were separated only by the total number of *cis* and *trans* double bonds, not by the specific location of the *cis* and *trans* double bonds.

RESULTS AND DISCUSSION

Acetonitrile, the cosolvent of choice for increasing the eluting capability of solvents in silver ion chromatography [2], is soluble in hexane to *ca.* 1.5% (v/v) at 23°C. A binary system composed of hexane and 0.5% ACN in hexane was found to provide a wide range of solvent strengths and excellent baseline stability. However, elution times for methyl linoleate differed when we used a binary system [A–B (50:50) where A = hexane and B = 1.0% ACN in hexane] *vs.* a single solvent reservoir containing 0.5% ACN in hexane. Whether this was due to the HPLC pump, the solvent reservoirs or the solvent mixing solenoid was not investigated. Thus, an isocratic system with a single reservoir containing the appropriate % ACN in hexane mixture was more suitable for column characterization and reproducible FAME separations.

The separation of SFO–LSO methyl esters is shown in Fig. 1. FAME elution reproducibility, resolution and baseline stability were maintained at sample sizes of 17 to 170 μg, although capacity factors (k) decreased approximately 25% between the 17 and 170 μg sample sizes. The trend of longer retention times with smaller sample sizes was consistent throughout our studies. Peak distortion, such as observed when gas chromatographic columns are overloaded, was not observed in our system. Perhaps larger FAME samples compete for silver ion sites the same way the ACN cosolvent competes for those sites. Excellent peak shapes were obtained even with sample elution times of 1.5 to 2.0 h.

Separation of the *cis/trans* isomers of methyl 18:2 (4 isomers; Fig. 2), 18:3 (8 isomers; Fig. 3) and 20:4 (16 isomers; Fig. 4) are illustrated in Figs. 2–4. Baseline separation of the 9-*trans* and 9-*cis*-18:1 isomers was also achieved within 8 min

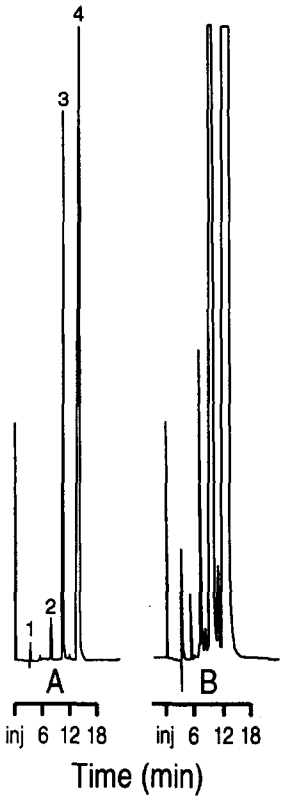

Fig. 1. Separation of SFO–LSO methyl ester standard. Flow-rate: 1.0 ml/min 0.2% ACN in hexane. UV detection at 210 nm. Sample size: A = 17 μg; B = 170 μg. Peaks: 1 = 16:0 and 18:0; 2 = 18:1; 3 = 18:2; 4 = 18:3.

of injection (0.2% ACN in hexane; chromatogram not shown). The order of elution for the methyl linoleate isomers (Fig. 2) was *trans*-9,*trans*-12-; *trans*-9,*cis*-12-; *cis*-9,*trans*-12- and *cis*-9,*cis*-12-18:2. The observed elution order differs from that obtained with capillary GC (SP 2330, SP 2340 or SP 2560 stationary phases) in which *cis*-9,*trans*-12-18:2 elutes before *trans*-9, *cis*-12-18:2.

The separation of all eight *cis*/*trans* isomers of methyl 18:3 (Fig. 3) was similar to, but with better resolution than, the separation obtained on a 50 m CP Sil 88 capillary GC column [10] or by packed capillary supercritical fluid chromatography [11]. The FAMEs eluted in four peaks or sets of peaks corresponding to the total number of *cis* and *trans* double bonds. These are marked as A (three *trans*), B (two *trans*, one *cis*), C (one *trans*, two *cis*) and D (three *cis*).

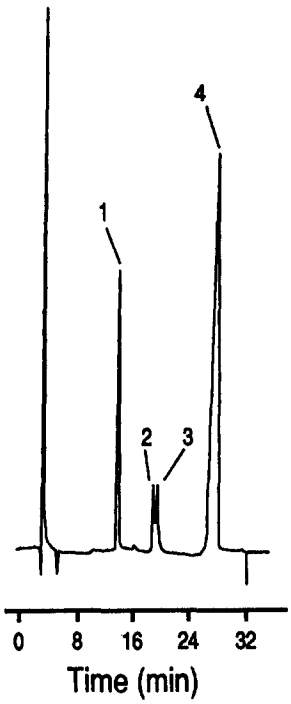

Fig. 2. Separation of isomerized methyl linoleate. Sample size: 20 μg. Flow-rate: 1.0 ml/min 0.125% ACN in hexane. UV detection at 210 nm. Peaks: 1 = *trans*-9, *trans*-12; 2 = *trans*-9, *cis*-12; 3 = *cis*-9, *trans*-12; 4 = *cis*-9, *cis*-12.

The resolution of 15 of the 16 possible *cis*/*trans* isomers of methyl 20:4 (Fig. 4) far exceeded the capabilities of current GC, other HPLC or other analytical methodology. Again, the eluted isomers are grouped by total *cis* and *trans* double bonds, with an elution pattern of A (four *trans*), B (three *trans*, one *cis*), C (two *trans*, two *cis*), D (one *trans*, three *cis*) and E (four *cis*). At 0.15% ACN in hexane, the 20:4 isomers could be separated within 25 min; with this solvent system, all four (three *trans*, one *cis*) isomers can be separated, but one (two *trans*, two *cis*?) of the other isomers is unresolved. These results may be compared to the work by Lanser and Emken [12], who separated isomerized methyl arachidonate into only five fractions on an XE254 silver ion column. As in silver ion TLC, separation was based on total number but not on specific location of the *cis* and *trans* double bonds. Work is currently underway to further identify the 18:3 and 20:4 isomers and to develop solvent systems to minimize *cis*/*trans*

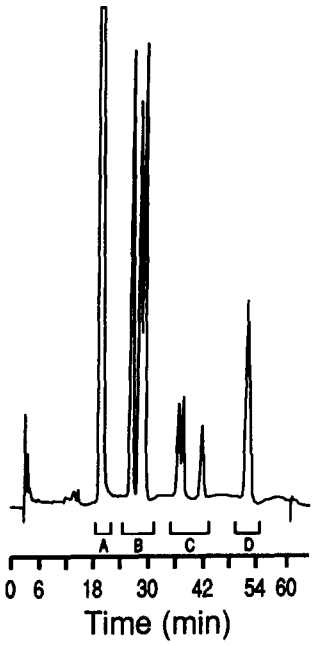

Fig. 3. Separation of isomerized methyl linolenate. Sample size: 20 μg. Flow-rate: 1.0 ml/min 0.125% ACN in hexane. UV detection at 210 nm. Groups: A = three *trans*); B = two *trans*, one *cis*; C = one *trans*, two *cis*); D = three *cis*.

isomer overlap during the analysis of mixtures of 18:2, 18:3, and 20:4 isomers.

Christie and Breckenridge [5], reported a gradual loss in resolution after sometimes only six months of column use. Whether this loss of resolution is due to silver ion removal by the ACN [5,13] or is due to the use of chlorinated solvents (silver salt formation?) is unclear. Another point of interest was the time required to equilibrate the system after changes were made in solvent composition. While the ChromSpher Lipids column had a column volume of *ca.* 3 ml, an increase in ACN concentration was not noted until the introduction of 7 to 8 ml of solvent (determined with refractive index detector). The problem of ACN–silver ion interaction and subsequent ACN retention is not new and may be noted in all forms of chromatography employing silver ions in the stationary phase. In our isocratic system, the column was equilibrated with the appropriate solvent mix for at least 0.5 h before sample injection. Since ACN dissolves very slowly into hexane, the ACN–hexane solvent mix was thoroughly stirred

Fig. 4. Separation of isomerized methyl arachidonate. Sample size: 100 μg. Flow-rate: 1.0 ml/min 0.125% ACN in hexane. UV detection at 210 nm. Groups: A = four *trans*; B = three *trans*, one *cis*; C = two *trans*, two *cis*; D = one *trans*, three *cis*; E = four *cis*.

for 5 min before use. To obtain reproducible retention times, thorough mixing of the ACN and hexane is essential.

A solvent system containing 0.125 to 0.15% ACN in hexane can provide good resolution in reasonable time for FAMEs containing up to four double bonds (15–30 min for mono- and dienoic fatty acids, 40–60 min for tri- and tetraenoic fatty acids). Up to 200 μg of sample can be applied to the column without severe peak distortion or significant (>25%) loss of retention with resolutions often exceeding the capabilities of capillary GC. Development of this and other solvent systems and utilizing commercially available silver ion HPLC columns should enhance the importance of this technology in the analysis of triacylglycerides, phospholipids and other unsaturated molecules.

REFERENCES

1 R.O. Adlof and E.A. Emken, *J. Am. Oil Chem. Soc.*, 58 (1981) 99.
2 W.J. DeJarlais and R.O. Adlof, *J. Am. Oil Chem. Soc.*, 60 (1983) 975.
3 R.O. Adlof and E.A. Emken, *J. Am. Oil Chem. Soc.*, 62 (1985) 1592.
4 W.W. Christie, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 148.
5 W.W. Christie and G.H. McG. Breckenridge, *J. Chromatogr.*, 469 (1989) 261.
6 B. Nikolova-Damyanova, B.G. Herslof and W.W. Christie, *J. Chromatogr.*, 609 (1992) 133.
7 W.W. Christie, E.Y. Brechany and K. Stefanov, *Chem. Phys. Lipids*, 46 (1988) 127.
8 J.M. Snyder and C.R. Scholfield, *J. Am. Oil Chem. Soc.*, 59 (1982) 469.
9 T.W. Gibson and P. Strassburger, *J. Org. Chem.*, 41 (1976) 791.
10 R.L. Wolf, *J. Chromatogr. Sci.*, 30 (1992) 17.
11 M. Demirbüker, I. Hägglund and L.G. Blomberg, *J. Chromatogr.*, 605 (1992) 263.
12 A.C. Lanser and E.A. Emken, *J. Chromatogr.*, 256 (1983) 460.
13 W.W. Christie, personal communication.