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Journal of Chromatography A, 1033 (2004) 369–371
JOURNAL OF CHROMATOGRAPHY A
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Short communication

Separation of conjugated linoleic acid methyl esters by silver-ion high performance liquid chromatography in semi-preparative mode

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Received 6 June 2002; received in revised form 5 December 2002; accepted 23 January 2003

Abstract

Silver-ion HPLC (Ag-HPLC) has been utilized to separate a variety of unsaturated fatty acid methyl esters (FAMEs) by configuration, location or number of olefinic or acetylenic bonds. Two analytical Ag-HPLC columns connected in series and an isocratic solvent system of acetonitrile (ACN) in hexane were used to fractionate 10–15 mg samples of a mixture of two deuterium-labeled isomers of conjugated linoleic acid (Z9,E11- and Z9,Z11-octadecadienoic acid-17,17,18,18-d_4). "Baseline" (>95%) resolution of the two isomers, which decreased with increasing weights of sample injected, was maintained by careful adjustment of the percentage of ACN in the ACN/hexane solvent system. Chemical purities of the isolated FAME were >96%.

Keywords: Linoleic acid; Fatty acid methyl esters

1. Introduction

Silver-ion HPLC (Ag-HPLC), utilizing columns packed with 5–10 μm Nucleosil SA^{TM} (phenylsulfonic acid groups bonded to a silica substrate) or similar substrate in which the sulfonic acid protons have been exchanged with Ag ions has, over the last decade, proven to be a tremendously powerful technique for the analytical separation of *Z*- and *E*-geometric and positional fatty acid methyl ester (FAME) and triacylglycerol (TAG) isomers (see [1–3] for reviews). Ag-HPLC has been applied to the separation/quantization of *Z*- and *E*-FAME [4–7], FAME positional isomers from partially hydrogenated vegetable oils [7], conjugated FAME [8], FAME labeled with deuterium atoms on the double bond carbon atoms [9], TAG isomers [10–12] and to separate mixtures of FAME or TAG containing FAs of widely-differing chain lengths [13]. A single Ag-HPLC column (hexane/ACN as solvent) has been utilized [6] to resolve 15 of the 16 possible *Z*/*E* isomers of methyl arachidonate (Z5,Z8,Z11,Z14–20:4), a separation which far exceeds the capabilities of current GC, HPLC or other analytical methodologies.

Most analytical separations have used sample sizes of 0.5 to 150 μg. The application of analytical HPLC columns to the semi-preparative separation of FAME samples has been less-studied. While C_{18} reversed-phase HPLC has been utilized [14] to separate Z9-18:1 and E9-18:1 as FAME, the semi-preparative fractionation of *Z*- and *E*-polyunsaturated FAME and similar compounds by this system is limited. A HPLC system consisting of two analytical Ag-HPLC columns connected in series (referred to as "dual-column Ag-HPLC" in this manuscript) and an isocratic solvent system of acetonitrile (ACN) in hexane (with UV detection) was assembled and utilized for the semi-preparative separation of milligram quantities of two deuterium-labeled, conjugated linoleic acid (CLA; Z9, E11-18:2-17,17,18,18-d_4) isomers.

2. Materials and methods

2.1. Materials

Hexane (Allied Fisher Scientific, Orangeburg, NY) and ACN (E. Merck, Darmstadt, Germany) were used as received. The CLA mixture [78.8% E9,E11-18:2 and 21.2% Z9,E11-18:2 FAME (both 17,17,18,18-d4); purity by GC]

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doi:10.1016/j.chroma.2003.01.001

used for preparative separations was generated during the preparation of Z9,E11-18:2-d_4 [15] and was utilized in two concentrations: (a) 500 mg of sample diluted to 1.00 ml with isooctane and (b) the pure sample. Solution (a) was used for injection of 1.25, 2.5 and 5.0 mg samples and (b) for injection of 10 and 15 mg samples.

2.2. High-performance liquid chromatography

For preparative separations, a 50 μl injection loop, two columns connected in series and UV detection at 206, 212 or 215 nm were used. The ChromSpher Lipids© columns (4.6 mm i.d. × 250 mm stainless steel; 5 μm particle size; silver-ion impregnated) were purchased from Varian-Chrompack International, Middelburg, The Netherlands, and used as received. Solvent flow was standardized at 1.0 ml/min; new solvent mixtures were prepared every 2 days. Isocratic conditions (ACN in hexane; 23 °C) were used to minimize variations in FAME retention(s) and resolution(s). The void volume of each Lipids column was 2.1 ml.

The eluted CLA FAME samples were collected in scintillation vials and the solvents evaporated. The fractions were dissolved in a minimum volume of isooctane and analyzed on a Varian 3400 gas chromatograph (GC; Varian Instruments, Palo Alto, CA, USA) equipped with a 30 m × 0.32 mm SP2380 (Supelco Inc., Bellefonte, PA, USA) capillary column, FID and utilizing He as carrier gas (split ratio of 100:1). GC conditions were: 155 °C (10 min) to 200 °C at 3 °C/min.

3. Results

Figs. 1 and 2 depict the Ag-HPLC elution patterns for 1.25 mg/2.5 mg and 5.0 mg/10.0 mg samples of the 78.8% E9,E11-18:2/21.2% Z9,E11-18:2 FAME mixture on a dual-column Ag-HPLC system. Resolution of the two main components, which decreased with increasing weights of samples injected, was maintained by decreasing

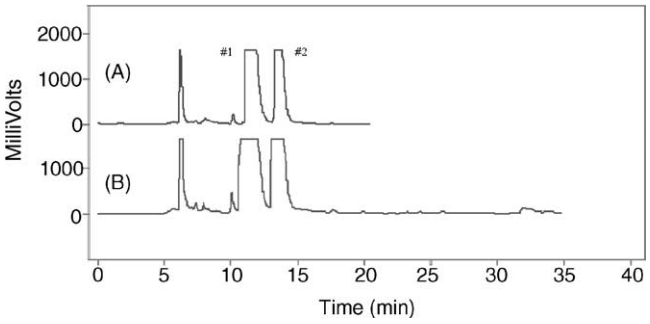

Fig. 1. Fractionation of CLA sample by dual-column Ag-HPLC. Sample sizes: 1.25 mg (A) and 2.5 mg (B). Flow rate: 1.0 ml/min 0.30% ACN in hexane. UV detection at 206 nm. Peak 1: E9,E11-18:2; peak 2: Z9,E11-18:2.

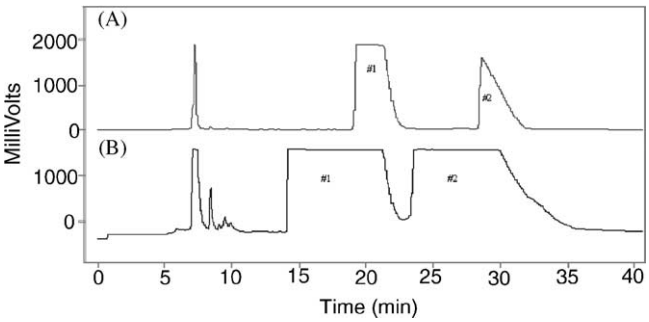

Fig. 2. Fractionation of CLA sample by dual-column Ag-HPLC. Sample sizes: 5 mg (A) and 10 mg (B). Flow rate: 1.0 ml/min 0.15% ACN in hexane. UV detection at 215 and 212 nm, respectively. Peak 1: E9,E11-18:2; peak 2: Z9,E11-18:2.

the percentage of ACN in the isocratic hexane/ACN solvent system. By careful adjustment of solvent composition, "baseline" resolution could be maintained for injected samples of 15 mg. Purities of the isolated fractions were found to be >96% (by GC).

4. Discussion

Two Lipids© columns connected in series were utilized to increase column capacity and improve peak-to-peak resolution. The isocratic solvent system was chosen since it did not require the 20–30 min equilibration times between injections necessary with programmed solvent systems and a UV detector could be used. A single solvent reservoir was used to minimize the effects of differences in instrument configurations (number of solvent pumps, reservoirs, mixing chambers, valves) between different HPLC systems. The solvent mixtures could be stored for up to a week in tightly sealed bottles and still be used with little (<5%) change in FAME retention volumes. While the volatility of the hexane did result in some bubble formation in the pump, initial degassing of the solvents in an ultrasonic bath, addition of a helium sparger and use of a small fan to cool the solvent pump mixing solenoid eliminated the problem.

For preparative separations by Ag-HPLC, the percentages of ACN in the hexane were adjusted to maintain >95% baseline resolution of the two CLA isomers. Calculating sample retention times from the centers of each over-ranged peak, a small decrease in retention times and loss of resolution was noted even when sample sizes were increased from 1.25 to 2.5 mg (Fig. 1). Loss of resolution between the two peaks was more evident when the sample size was increased from 2.5 to 5 mg (chromatograms not shown). Resolution was restored at a sample loading of 5 mg when the % ACN in the solvent was decreased from 0.3 to 0.25%. [Excellent peak shapes were obtained even with sample elution times of 1.5–2.0 h (data not shown).] No loss of Ag^+ ions (often a significant problem with silver nitrate/silica systems) was observed; >400 injections (dual-column system) over

a 1-year period resulted in no significant (<10%) loss of resolution.

One can continue to compensate for losses in peak-to-peak resolution for larger sample sizes by decreasing the percentage of ACN in the solvent but, under these conditions, the total time required per run increases. A comparison of sample sizes and retention times demonstrates a practical limit to the capacity of the dual-column system (for this CLA FAME example) at a sample size of <15 mg. Fractionation of a 15 mg sample of the CLA isomer mixture required ca. 80 min to elute the two CLA isomers (versus 20 min or 35 min for elution of the 5 and 10 mg samples, respectively). Two 10 mg samples could actually be fractionated within 70 min (2 × 35 min), less time than that required to separate one 15 mg sample. The isocratic solvent system also allowed us to fractionate 10 mg samples (Fig. 2B) by injecting a sample every 30 min.

Since sample size, column temperature (see ref. [16])/condition and batch-to-batch variations in silver loading of the ChromSpher Lipids© columns (private communications) can effect elution times, the data presented in Figs. 1 and 2 should be viewed only as a guide. Some adjustment of solvent composition(s) may be required to reproduce the separations shown. The separations shown in Figs. 1 and 2 were reproduced on two different dual-column Ag-HPLC systems; only minor changes in solvent composition were required to achieve >95% resolution. UV detector wavelengths were adjusted from 206 to 215 nm (higher to lower response) to better visualize the peak-to-peak separation efficiency of the system.

As we have demonstrated, the ACN/hexane solvent system and the commercially available ChromSpher Lipids column(s) are a useful combination for the semi-preparative separation of CLA isomers. Alltech Associates, Deerfield, IL, USA recently introduced a Ag-HPLC column [16] and development of standard methods is underway [17]. The development of new Ag-HPLC substrates and solvent systems should further enhance the importance of this technology in the analysis and semi-preparative isolation of TAG and FAME isomers [18–20], pheromones, and other polyunsaturated substrates.

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