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Separation of unsaturated fatty acid methyl esters by packed capillary supercritical fluid chromatography

Comparison of different column packings

Mustafa Demirbüker, Ingela Hägglund and Lars G. Blomberg

Department of Analytical Chemistry, Stockholm University, Arrhenius Laboratory, S-106 91 Stockholm (Sweden)

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ABSTRACT

Packed capillary supercritical fluid chromatography with ultraviolet detection was applied to the separation of methyl esters of unsaturated fatty acids obtained from a fish oil. Separation was attempted on three different types of column packing: cation exchanger impregnated with silver nitrate, silica and anion exchanger treated with potassium permanganate. Separation on the argentation column resulted in relatively good resolution of geometric isomers of dienes and trienes. Further, this column performed well in the separation of fatty acid methyl esters from a fish oil. Separatiaon of the same sample on a column packed with silica was somewhat less complete. Finally, a permanganate-treated anion exchanger separated the fatty acid methyl esters into groups according to the degree of unsaturation.

INTRODUCTION

Fatty acids are the distinctive structural components of lipids, and methods for their separation and analysis are of great significance. Gas chromatographic (GC) [1] as well as high-performance liquid chromatographic (HPLC) [2] methods have been successfully used. Silver ion or argentation chromatography has been of particular interest for the separation of complex mixtures of *cis/trans* isomers [3,4]. In this method, fatty acids are separated according to the number, position and geometrical configuration of their double bonds. Argentation columns for HPLC were orginally prepared by impregnation of silica with silver nitrate. The silver nitrate was, however, rapidly leached out from such columns, thus contaminating samples and reducing the working life of the column. It was demonstrated by Christie [5] that columns in which a silica-based cation exchanger has been impregnated with silver nitrate can be stable under HPLC conditions. Recently, it was shown that such columns also show excellent stability under supercritical fluid chromatographic (SFC) conditions [6,7].

Argentation columns of this stable type have been applied to the separation of derivatized fatty acids by HPLC using conventional column dimensions [1–4]. Two different approaches may be distinguished. First, the most complete separation may be sought, *e.g.* of *cis/trans* isomers [3]. Second, the conditions may be changed to give a separation into groups which subsequently will be further separated using a complementary chromatographic method, *e.g.* GC [1,4].

Oxides of transition metals, such as chromium [8], vanadium [9,10] and manganese [11], have been

Correspondence to: Dr. L. G. Blomberg, Department of Analytical Chemistry, Stockholm University, Arrhenius Laboratory, S-106 91 Stockholm, Sweden.

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chemically attached to silica in order to be used as catalysts. This type of modification often results in decreased silanol activity [12]. It was recently shown that silanol groups on silica and silica-based anion exchangers could be deactivated by treatment with potassium permanganate [13]. Under SFC conditions, manganese acts as a weak electron acceptor, and triacylglycerols could be separated into groups according to their degree of unsaturation.

Few separations of fatty acid derivatives using normal-phase HPLC have been reported, but it is considered that there is some potential for further development [2].

In this work, separation of fatty acid methyl esters (FAMEs) was attempted on packed capillary columns, using supercritical fluids as mobile phase. The performance of different types of column packing—silica, Ag^+/cation exchanger and potassium permanganate/anion exchanger—was evaluated.

EXPERIMENTAL

SFC was performed on a Lee Scientific 600 Series SFC system equipped with a Lee Scientific variable-wavelength absorbance detector. Detection was executed at 210 nm. Fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ, USA), 11 μm I.D., was used as restrictors in lengths of 20–25 cm.

Columns were prepared from fused-silica capillary tubing, 250 or 330 mm × 0.25 mm I.D. and 0.43 mm O.D. (Polymicro Technologies). Three types of packing were used: Nucleosil 4 SA and 5 SB (Macherey Nagel, Düren, Germany) and Superspher Si 60 4 μm (Merck, Darmstadt, Germany). Slurry packing and impregnation with silver nitrate were performed as described previously [6,7]. For the modification with permanganate, some columns were, after packing with Nucleosil 5 SB, washed successively with methanol, 400 μl of distilled water, 400 μl of 0.2 *M* potassium permanganate and 400 μl of distilled water. Finally, the columns were dried by flushing with carbon dioxide at 115°C and 275 atm [13].

The mobile phase consisted of carbon dioxide–acetonitrile–isopropanol. Different contents of modifier were used for the different column types. SFC-grade carbon dioxide (Scott Specialty Gases, Plumsteadville, PA, USA) was used. The content of isopropanol was *ca.* 10% of that of acetonitrile. The mobile phase mixture was prepared in the SFC pump as described previously [14]. Mobile phase velocity was 3.5 mm/s. Analytes were dissolved in HPLC-grade pentane at concentrations of 30 mg/ml. The injector was equipped with an internal loop, 200 nl, and injection was performed using a timed split of 0.2 s. In addition, a split ratio of 1:1 was applied. Using these conditions, *ca.* 60 nl were allowed to enter the column.

GC was performed on a Mega gas chromatograph (Carlo Erba). Open tubular columns coated with the stationary phase Sila C were prepared as described previously [15]. Hydrogen was used as mobile phase.

FAME standards and CPL fish oil 30 were obtained from Larodan Fine Chemicals (Malmö, Sweden). Saponification and methylation of triacylglycerols were performed according to Christie [16]. Mixtures of geometrical isomers were prepared from the parent compounds by nitric oxide-catalysed isomerzation [17].

RESULTS AND DISCUSSION

The separation of a mixture of geometrical isomers of dienes is shown in Fig. 1A. Isomers 9*t*,12*c*-18:2 and 9*c*,12*t*-18:2 could not be resolved, which was observed also for argentation HPLC [3]. Fig. 1B illustrates the separation of the geometrical isomers obtained from linolenic acid. This mixture should contain eight components. In argentation chromatography, *trans* isomers always migrate ahead of *cis* isomers [1], and the first peak in Fig. 1B thus ought to be the *t,t,t* isomer and the last peak the *c,c,c* isomer. Then there remain two groups of peaks; the first group ought to be the *t,t,c* isomers and the second group the *t,c,c* isomers. In the separation of the same type of sample with argentation HPLC, six peaks were obtained, and the analysis time was much longer, 40 min [3].

Separation of *t,t*-18:2 and *c,c*-18:2 was attempted on capillary columns packed with anion exchanger and modified *in situ* with potassium permanganate: an α-value of 1.02 was obtained.

Fish oil FAMEs

The SFC separation of the FAMEs from a fish oil on an argentation column is shown in Fig. 2, the

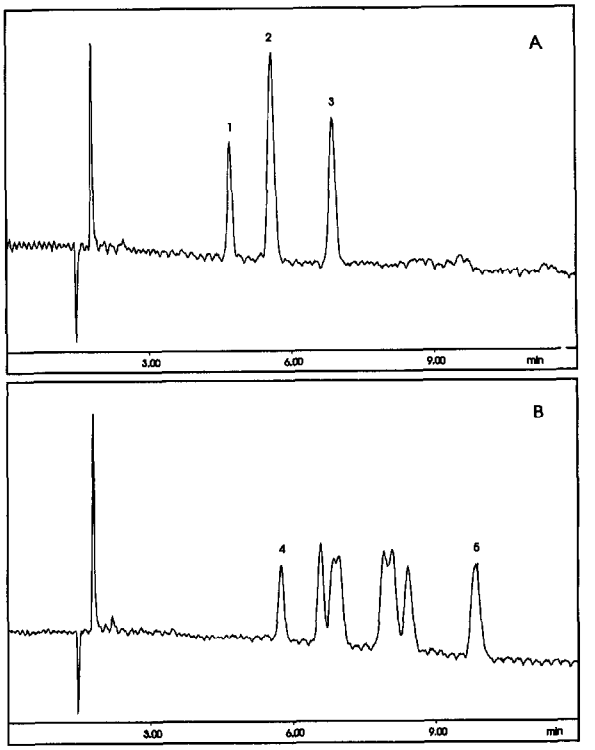

Fig. 1. Supercritical fluid chromatogram of methyl esters of geometrical isomers of (A) linoleic acid and (B) linolenic acid. Capillary column, 250 mm × 0.25 mm I.D., packed with 4-μm cation exchanger and impregnated *in situ* with silver nitrate. Injection at 115°C and 280 atm; after 2 min, programmed at −1°C/min and 2 atm/min to 75°C and 360 atm. Mobile phase: carbon dioxide–acetonitrile–isopropanol (97.1:2.6:0.3, mol%). UV detection at 210 nm. See the Experimental section for practical details. Peaks: 1 = 9*t*,12*t*-18:2; 2 = 9*c*,12*t*-18:2 + 9*t*,12*c*-18:2; 3 = 9*c*,12*c*-18:2; 4 = *t*,*t*,*t*-18:3; 5 = *c*,*c*,*c*-18:3.

content of acetonitrile in the mobile phase being 2.6 mol% in this case. A gas chromatogram of the same sample is shown in Fig. 3. Fractions were collected from the SFC eluate for further separation by open tubular GC, a relatively complete separation of the components thus being obtained. Further, the analysis of the fractions aided in the identification of the peaks in the SFC chromatogram.

The separation obtained on an anion exchanger treated *in situ* with potassium permanganate is shown in Fig. 4. Although the mobile phase contained only 0.6% acetonitrile in this case, the retention times were much shorter than for the Ag^+ column. Fig. 5 shows the separation obtained on a column packed with silica. The most complete separation was obtained with the argentation column, but the separation achieved with the column packed with pure silica was almost as good. Columns that had been treated with potassium permanganate gave inferior separations. Here, separation into groups according to the degree of unsaturation was achieved rather than separation of molecular species. The potential practical values of such a group

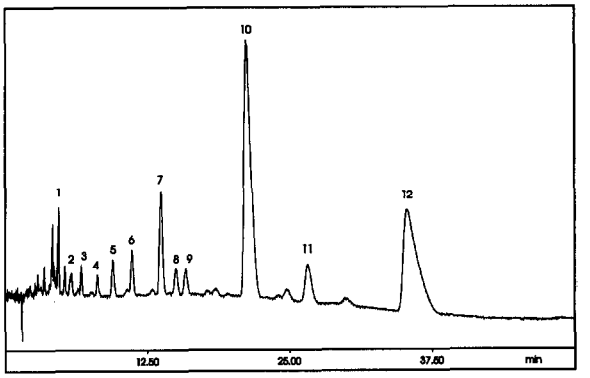

Fig. 2. Supercritical fluid chromatogram of FAMEs of CPL fish oil 30. Column, mobile phase and conditions as in Fig. 1. Peaks: 1 = 18:1; 2 = 20:1; 3 = 18:2; 4 = 22:1; 5 = 18:3; 6 = 16:4; 7 = 18:4; 8 = 20:4(*n*−6); 9 = 20:4(*n*−3); 10 = 20:5; 11 = 22:5; 12 = 22:6.

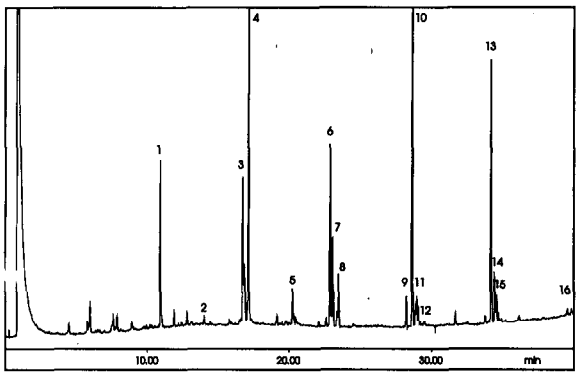

Fig. 3. Gas chromatogram (flame ionization detection) of FAMEs of CPL fish oil 30 on a 25 m × 0.25 mm I.D. fused-silica capillary column coated with Polymer S. Conditions: split injection at 130°C; after 2 min, programmed at 3°C/min to 250°C. Peaks: 1 = 14:0; 2 = 15:0; 3 = 16:1*c* + 16:4; 4 = 16:0 + 16:1*t* + 16:2; 5 = 17:1; 6 = 18:1*c* + 18:2*c*,*c* + 18:4 + 18:5; 7 = 18:1*t*; 8 = 18:0 + 18:2*t*,*t* + 18:3; 9 = 20:4(*n*−6); 10 = 20:3 + 20:5; 11 = 20:1 + 20:2*c*,*c* + 20:4(*n*−3); 12 = 20:0; 13 = 22:6; 14 = 22:5 + 22:1*c*; 15 = 22:1*t*; 16 = 24:1.

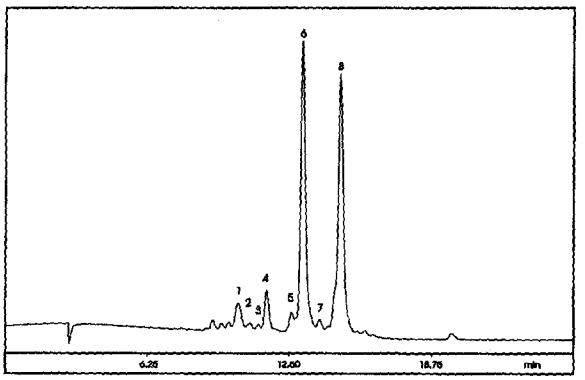

Fig. 4. Supercritical fluid chromatogram of FAMEs of CPL fish oil 30. Capillary column, 330 mm × 0.25 mm I.D., packed with 5-μm anion exchanger and modified *in situ* with potassium permanganate. Injection at 115°C and 220 atm; after 2 min, programmed at −2°C/min and 4 atm/min to 75°C and 300 atm. Mobile phase: carbon dioxide–acetonitrile–isopropanol (99.3:0.6:0.06, mol%). See Experimental section for practical details. Peaks: 1 = 18:1; 2 = 18:2; 3 = 18:3; 4 = 18:4; 5 = 20:4; 6 = 20:5; 7 = 22:5; 8 = 22:6.

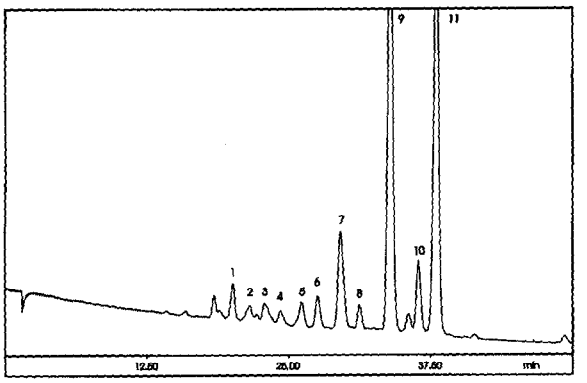

Fig. 5. Supercritical fluid chromatogram of FAMEs of CPL fish oil 30. Capillary column, 250 mm × 0.25 mm I.D., packed with 4-μm silica. Mobile phase and conditions as in Fig. 4. Peaks: 1 = 18:1; 2 = 20:1; 3 = 18:2; 4 = 22:1; 5 = 18:3; 6 = 16:4; 7 = 18:4 + 20:4($n-6$); 8 = 20:4($n-3$)?; 9 = 20:5; 10 = 22:5; 11 = 22:6.

separation may involve the use in hyphenated systems, where each group is being separated further in another dimension. Moreover, permanganate-treated columns could be of value in preparative work.

A relatively high content of acetonitrile in the mobile phase, 2.8%, is needed for the elution of FAMEs from Ag^+ columns. For elution on silica and permanganate-treated columns, 0.8% acetonitrile was sufficient. It seems that, for argentation columns, the main function of the acetonitrile is to partially deactivate the silver ions. Permanganate-treated columns were less retentive than silica columns, and it seems that such a treatment serves to deactivate residual silanol groups. However, silanol groups aid in the separation of molecular species of FAMEs. Triacylglycerols, on the other hand, are relatively poorly separated on columns packed with silica [13]. For the separation of such compounds, elimination of residual silanols on the column packing seems to be beneficial.

The column packing material became light brown on treatment with potassium permanganate. After rinsing with a solution of potassium persulphate, the packing was again white, and the colour of the eluate was purple. Further, the presence of managanese on the silica surface was detected by means of atomic absorption spectroscopy. Permanganate has an oxidizing effect on unsaturated lipids; such an effect would here lead to a consumption of permanganate ions and thus a reduction in retention times. However, the permanganate-treated columns showed excellent reproducibility of the retention under the conditions applied in this work. Further, the columns showed high stability at relatively high temperatures, the retention properties being unchanged after 12 h at 180°C. It is not known in what form the managanese occurs on the surface, but it seems unlikely that it is as permanganate.

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