Gradients of *n*-Heptane and Acetonitrile in Silver-Ion High-Performance Liquid Chromatography Analyses of *cis* and *trans* Bonds in Lipids

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ABSTRACT: *Cis* and *trans* isomers of fatty acid methyl esters, fatty alcohols, and triacylglycerols were analyzed with a silver-ion high-performance liquid chromatography system. Gradients of *n*-heptane and acetonitrile were used to elute molecules with up to nine *cis* double bonds. The analyses were as fast and reliable and had a resolution similar to that of the best published analyses. However, published analyses were performed with chlorinated solvents, and these solvents are carcinogenic and mutagenic. The solvents we used, heptane and acetonitrile, are less dangerous to the analyst.
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KEY WORDS: Acetonitrile, *cis*-isomers, evaporative light-scattering detector, fatty acid methyl esters, fatty alcohols, gradient elution, *n*-heptane, silver-ion HPLC, *trans*-isomers, triacylglycerols.

Silver-ion chromatography is valuable in the analysis of lipids (1,2). This technique separates the molecules predominantly by the number of double bonds. It is also sensitive to the configuration of the double bonds, that is, *cis* or *trans* configuration.

Many reported separations on silver-ion columns have been performed with chlorinated solvents, mostly 1,2-dichloroethane and dichloromethane, which give good separations of fatty acid methyl esters (3–5) and triacylglycerols (6–8). However, 1,2-dichloroethane and dichloromethane are carcinogenic and mutagenic (9). Separations also have been performed with methanol and acetonitrile (6), but methanol is not suitable for the analysis of triacylglycerols because of risk of transesterification (10). Chlorinated solvents have been used to avoid this process (6).

Successful isocratic separations have been reported with *n*-hexane and small amounts of acetonitrile (11,12). Therefore, it was interesting to take it one step further and try out *n*-heptane and acetonitrile as substitutes for those chlorinated solvents. *n*-Heptane has a much lower neurotoxicity than *n*-hexane (13), which causes peripheral neuropathy (9,14,15).

For *n*-hexane and acetonitrile, only isocratic separations were published. Gradient separations were not successful owing to mixing problems. It was suggested that the mixing problems were attributable to the high-performance liquid chromatography (HPLC) system (11). Because gradient separations are more flexible, it would be interesting to see if a different HPLC system could give reproducible gradient separations.

n-Pentane and isohexane are also better solvents than *n*-hexane from the toxicological point of view. All four solvents are expected to give similar separations.

The purpose of this paper is to present good analyses of *cis* and *trans* fatty acid methyl esters, mixtures of fatty acid methyl esters and fatty alcohols, and finally triacylglycerols with gradients of *n*-heptane and acetonitrile in a silver-ion HPLC system.

EXPERIMENTAL PROCEDURES

Materials. *n*-Heptane, *n*-hexane, and acetonitrile were of HPLC grade, and isooctane was of analytical grade (Merck, Darmstadt, Germany). The fatty acid methyl esters: methyl stearate, methyl oleate (*cis*-9-18:1), methyl elaidate (*trans*-9-18:1), methyl linoleate (*cis*-9, *cis*-12-18:2), methyl linolelaidate (*trans*-9, *trans*-12-18:2), and methyl linolenate (*cis*-9, *cis*-12, *cis*-15-18:3); the fatty alcohols: stearyl alcohol, linoleyl alcohol (*cis*-9, *cis*-12-18:2), and linolenyl alcohol (*cis*-9, *cis*-12, *cis*-15-18:3); soybean oil and a mixture of triacylglycerol standards: tripalmitin, tristearin, triolein, trilinolein, and trilinolenin were purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Oleyl alcohol (*cis*-9-18:1) was purchased from Sigma Chemical Company (St. Louis, MO). Polyunsaturated fatty acid ethyl esters, mainly eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6), were of fish oil origin and were obtained from Pronova Biocare A/S (Sandefjord, Norway). All samples were dissolved in isooctane.

HPLC. An HPLC system from Shimadzu (Tokyo, Japan) was used. It consisted of two pumps (model LC-6A), an autoinjector (model SIL-6B), and a system controller (model SCL-6B). The detector was an evaporative light scattering detector (model 750/14, Applied Chromatography Systems Ltd., Macclesfield, Cheshire, England). The column (Chrom-Spher Lipids, 5 μm, 4.6 × 250 mm) was purchased from

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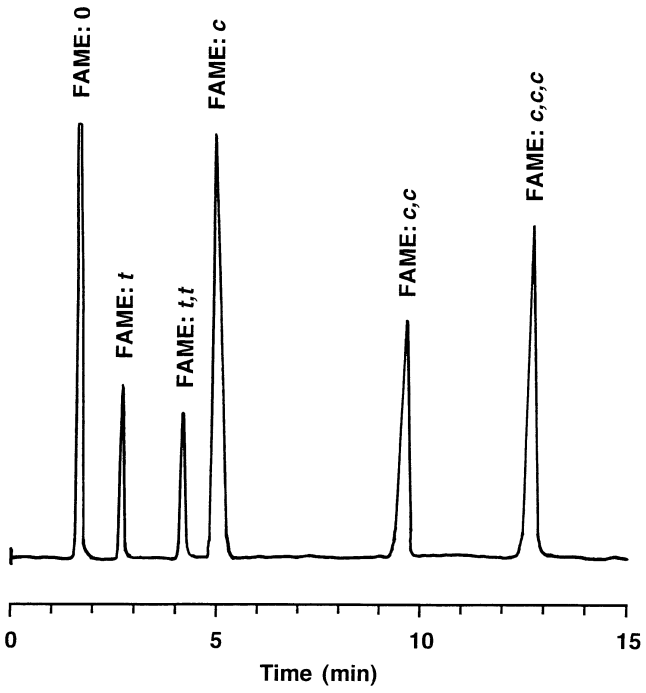

FIG. 1. Separation of FAME standards with zero to three double bonds. Gradient: See Table 1 (FAME–FOH). Sample size: 117 µg. Abbreviations: FAME, fatty acid methyl esters; FOH, fatty alcohols; 0, no double bond; *t, trans* double bond; *c, cis* double bond. The substances are specified in the Materials section.

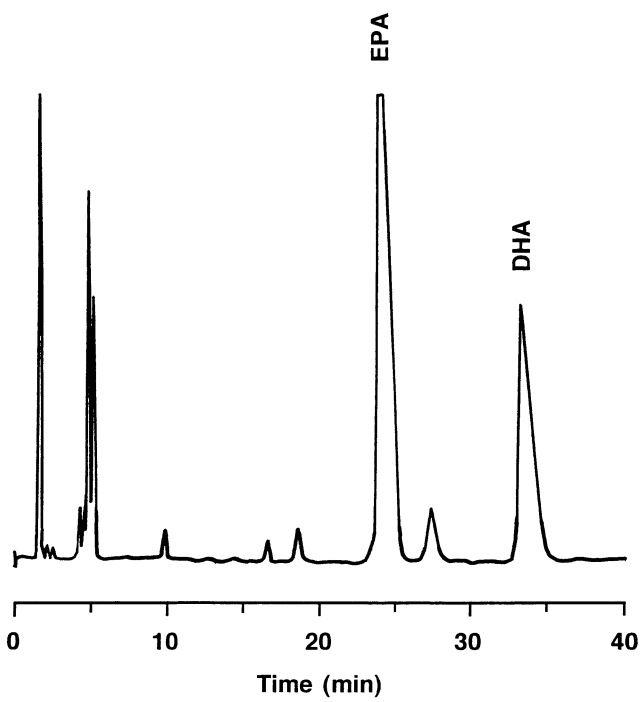

FIG. 2. Separation of polyunsaturated fatty acid ethyl esters (FAEE) from fish oil. Gradient: See Table 1 (FAEE). Sample size: 395 µg. Abbreviations: EPA, eicosapentaenoic acid (20:5); DHA, docosaheptaenoic acid (22:6).

Chrompack Ltd. (Middelburg, The Netherlands). The column temperature was 23–25°C. The solvents were mixed in the reservoir with a magnetic stirrer for at least 5 min before use. Thorough mixing of the solvents was important for reliable results. Two solvent reservoirs were used: (A) heptane and (B) heptane/acetonitrile 100:2 (vol/vol). The design of the gradients is presented in Table 1.

TABLE 1
Gradients for Separation of FAME–FOH, FAEE, and TG

FAME and FOH 0–3 double bonds		FAEE 0–6 double bonds		TG 0–9 double bonds	
Time (min)	Comp. (%A)	Time (min)	Comp. (%A)	Time (min)	Comp. (%A)
0	94.9	0	94.9	0	88
5	83.5	3	90.4	30	72.2
8.5	83.5	20	85.8	40	49.5
10	61.7	30	85.8	50	49.5
15	56.9				
18[a]	47.5				
25	47.5				
25.2[b]	94.9[b]	30.2[b]	94.9[b]	50.2[b]	94.9[b]
35[b]	94.9[b]	40[b]	94.9[b]	60[b]	94.9[b]

[a]If only FAME are analyzed, the gradient could be stopped at 18 min. [b]Reconditioning. Flow rate: 3.0 mL/min. (A) heptane and (B) heptane/acetonitrile 100:2 (vol/vol). Abbreviations: FAME, fatty acid methyl esters; FOH, fatty alcohols; FAEE, fatty acid ethyl esters; TG, triacylglycerols.

RESULTS AND DISCUSSION

Fatty acid methyl esters. Adlof (11) has reported isocratic separations of *cis* and *trans* methyl oleate and of the four methyl linoleate *cis/trans* isomers with *n*-hexane and acetonitrile. These separations were repeated successfully. Changing from *n*-hexane to *n*-heptane gave the same results.

On the Shimadzu HPLC system, we used gradients of heptane and acetonitrile (see Table 1). These were found to be stable and gave reproducible separations. This is a clear improvement compared to previous investigations (11). Fatty acid methyl esters with up to three *cis* double bonds were easily eluted, and good separation between *cis* and *trans* isomers was achieved (see Fig. 1).

With a slightly different gradient, it was also possible to elute fatty acid ethyl esters with up to six *cis* double bonds in 35 min (see Fig. 2). This is faster than in separations performed with gradients of 1,2-dichloroethane, dichloromethane, methanol, and acetonitrile. In an analysis of fatty acid methyl esters from plasma lipids (4), the fatty acid with six double bonds was eluted in 40 min, and in another analysis of fatty acid composition of the sponge *Dysidea fragilis* (5), the same peak was eluted in 43 min.

Fatty acid methyl esters and fatty alcohols. The developed gradient could also be used for the separation of fatty acid methyl ester and fatty alcohol mixtures. The silver ions in the

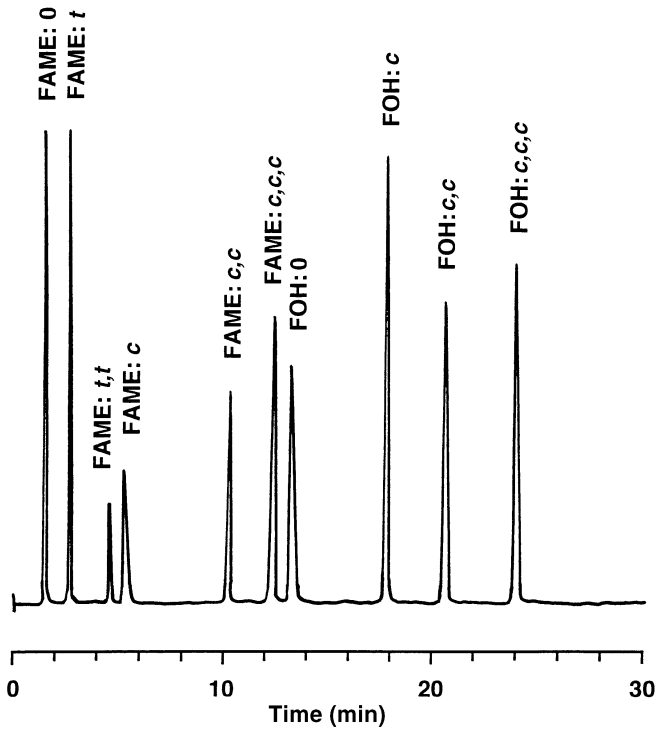

FIG. 3. Separation of FAME standards and FOH standards with zero to three double bonds. Gradient: See Table 1 (FAME–FOH). Sample size: Approx. 55 µg/substance. Abbreviations: 0, no double bond; *t, trans* double bond; *c, cis* double bond. The substances are specified in the Materials section. See Figure 1 for other abbreviations.

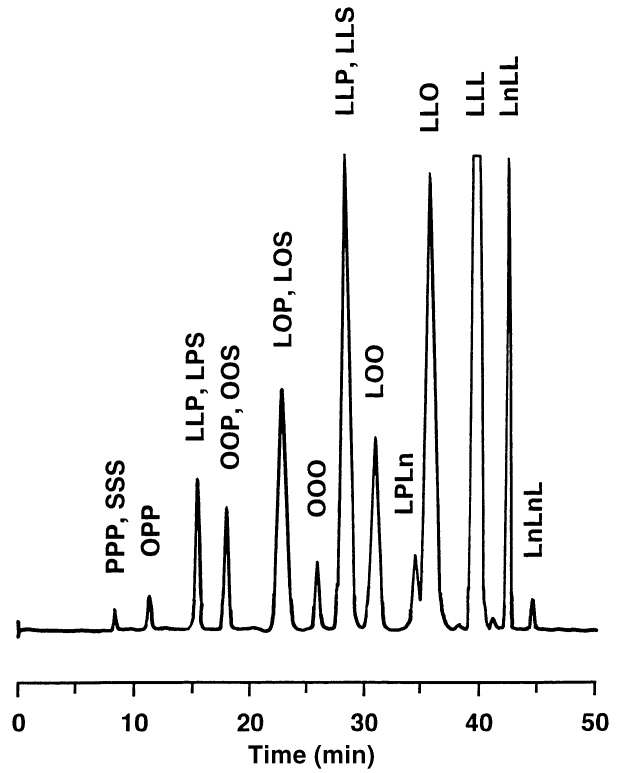

FIG. 4. Separation of triacylglycerols from soybean oil. Gradient: See Table 1 (TG). Sample size: 1200 µg. Abbreviations: TG, triacylglycerols; P, palmitin; S, stearin; O, olein; L, linolein; Ln, linolenin.

column interact, besides with the double bonds, also with the free electron pairs of the polar alcohol group (16). Therefore, fatty alcohols elute after similar unsaturated fatty acid methyl esters (Fig. 3). The polarity of the mobile phase has to be increased considerably to elute the polyunsaturated alcohols. Appropriate separation of methyl linolenate and stearyl alcohol is troublesome. However, with the described gradient, a fast and good separation of fatty acid methyl ester and fatty alcohol mixtures was accomplished.

Triacylglycerols. A third procedure was used to separate triacylglycerols with up to nine *cis* double bonds (trilinolein). Figure 4 shows an analysis of the triacylglycerols in soybean oil. Identification of the peaks in this chromatogram was made from the analysis of a mixture of triacylglycerol standards (tripalmitin, tristearin, triolein, trilinolein, trilinolenin; Fig. 5) and by comparison with the product specification from the manufacturer Larodan Fine Chemicals AB (Malmö, Sweden).

Christie (7) has presented separations of linseed oil and evening primrose oil (8). The analyses were made with 1,2-dichloroethane, dichloromethane, acetone, and acetonitrile in a gradient. Triacylglycerols with up to nine double bonds were separated in about 52 min. With our gradient, the triacylglycerol with nine double bonds was eluted in 45 min (Fig. 5). The resolution of Christie's separations is nearly in the same range as ours, but in the separation of linseed oil (7), the

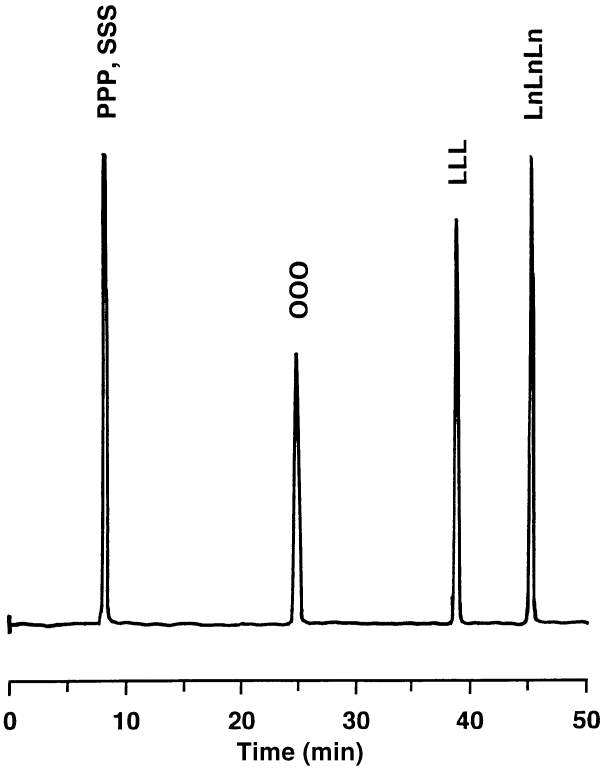

FIG. 5. Separation of triacylglycerol standards. Gradient: See Table 1 (TG). Sample size: 250 µg. See Figure 4 for abbreviations.

triacylglycerol with one saturated, one dienoic, and one trienoic fatty acid coelutes with the triacylglycerol with three dienoic fatty acids. In our chromatogram, they seem to be clearly separated (peak LPLn and LLL in Fig. 4), but this needs to be further investigated because our identification of the peaks in Figure 4 can be questioned.

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