Argentation Supercritical Fluid Chromatography for Quantitative Analysis of Triacylglycerols

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A method for quantitative analysis of neutral lipids has been developed. Four different techniques have been combined for this purpose—supercritical fluid chromatography (SFC), silver ion chromatography, packed microcolumns and miniaturized evaporative light-scattering detection (ELSD). The development and optimization of the method are discussed. The separation of a series of vegetable, fish and hydrogenated oils was demonstrated. Application of eluent composition programming resulted in excellent separation of complex samples. Packed microcolumn argentation SFC provides at least as high a separation power as corresponding high-performance liquid chromatography methods. The combination of packed microcolumn SFC and miniaturized ELSD constitutes a powerful analytical system for the quantitative analysis of triacylglycerols.

KEY WORDS: Argentation chromatography, eluent composition programming, evaporative light-scattering detection, hydrogenated oils, packed microcolumns, supercritical fluid chromatography, triacylglycerols.

Supercritical fluid chromatography (SFC) was developed for packed columns in 1962 by Klesper and co-workers (1). However, for a long time the technique was studied and advanced only by a small group of scientists. It was not until 1981, when open tubular columns were proposed for the separation, that the technique gained a more widespread interest (2,3). During recent years, packed-column SFC has undergone a renaissance, this time promoted by companies such as Gilson Medical Electronics Co. (Villiers le Bel, France) (4) and Hewlett Packard (Wilmington, DE).

In SFC, the mobile phase is in a supercritical state, *i.e.*, temperature and pressure are above their critical values. Under these conditions, the density of the mobile phase is relatively high and provides the mobile phase solvatizing properties. As a consequence, solutes do not need to be vaporized to pass through the column, and separations can be performed at much lower temperatures than in gas chromatography (GC). This is, of course, a great advantage for the separation of thermally labile compounds.

In comparison with conventional high-performance liquid chromatography (HPLC), diffusion in supercritical media is faster and viscosities are lower. This leads to more efficient separations. SFC thus provides some distinctive advantages over GC and HPLC, but there is a general lack of polar mobile phases, which constitutes a fundamental limitation, *i.e.*, strongly polar solutes can only be chromatographed with difficulty. Detailed descriptions of SFC can be found in recent books (5–7).

The application of SFC to lipid analysis was recently summarized (8). It was pointed out that many of the published separations have been made with model mixtures rather than with realistic samples, and that a greater body of experience with real samples would be needed to make objective comparisons between different techniques. In the present work, a method for the quantitative analysis of neutral lipids is presented. The present method is based on work that has been published previously (9–15).

Argentation chromatography. Current types of open tubular columns for SFC do not provide the high separation efficiency sometimes desired. Several triacylglycerols (TAG) coelute when separation is attempted on an open tubular column coated with DB wax (Fisons, Loughborough, United Kingdom) (16), *e.g.*, PLL, OOO, SOL, (P, palmitate; S, stearate; O, oleate; L, linoleate; Ln, α-linolenate) PLLn and SOLn elute as a single peak.

In chromatography, a stationary phase that possesses a suitable selectivity may often lead to an improvement of difficult separations. For lipid separations, argentation chromatography or silver ion chromatography provides such a selectivity. Since its introduction in 1962 (17,18), this technique has been used extensively for the separation of lipids; its application was recently reviewed (19). Due to the nature of the interactions, selectivity is most effectively utilized in packed columns. Open tubular columns for argentation chromatography can be prepared, but their performance with lipids was inferior to that obtained with packed columns (20).

Argentation chromatography was first performed on silica impregnated with silver nitrate. However, the silver ions gradually leaked out of columns packed with this material. As an alternative support for the silver, macroreticular sulfonic acid ion-exchange resins were used in low-pressure liquid chromatography (21). For HPLC, Christie *et al.* (22–29) achieved excellent results when utilizing a silica-based cation exchanger as support for the silver. Columns packed with this type of stationary phase have been applied to SFC for fuel analysis (30–33) and the separation of neutral lipids (9–15). Christie (22) reported good column stabilities under HPLC conditions, although the resolution deteriorated slowly. It may be speculated that ionic substances in the mobile phase could displace the silver from the cation exchanger. Such a displacement is, however, quite unlikely with the supercritical medium used as mobile phase in the present work, which may contribute to the high column stability that has been experienced under SFC conditions.

The separation in argentation chromatography is sometimes considered to be based on a single property of the lipid molecules—the nature of their unsaturation. In practice, however, several retention mechanisms are active. Separation according to the position of the fatty acid moieties in a triacylglycerol, *e.g.*, PPO from POP, was originally only reported on columns packed with silica and impregnated with Ag^+ (34,35). Interaction with the silica was obviously of crucial importance in this case. However, Chrompack (Middelburg, The Netherlands) recently introduced columns that, in HPLC mode, can separate PPO from POP (36). These columns are packed with a silica-based cation exchanger in the Ag^+ ionic form. A further example of the simultaneous activity of different

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retention mechanisms involves the separation according to chainlength that is sometimes observed. Such separations were achieved on silver-impregnated cation exchanger columns under SFC conditions (9–15). The bonded phenylpropyl moieties may contribute to this separation.

Packed microcolumns. Packed microcolumns have been employed in the present analysis system. These columns provide a low flow of mobile phase that matches the geometry of the miniaturized evaporative light-scattering detector (ELSD) that was used. Further, the microcolumns are quite cheap to prepare and run. Finally, due to the low flow rates and low thermal mass of packed microcolumns, efficient thermal gradients can be applied in SFC with such columns. Fused-silica capillary tubing may be used as column material, although glass-lined metal tubing is easier to handle. Such packed columns are commercially available from SGE (Ringwood, Victoria, Australia).

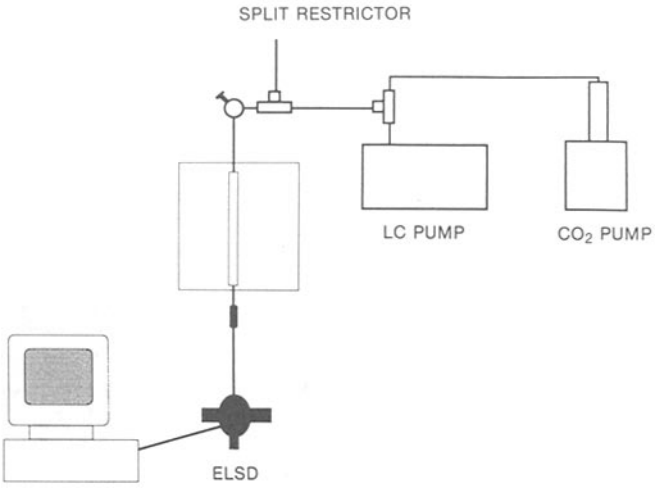

FIG. 1. System for eluent composition programming. LC, liquid chromatography; ELSD, evaporative light-scattering detector.

EXPERIMENTAL PROCEDURES

Columns. Fused-silica capillaries, 0.25 mm i.d. (Polymicro Technologies, Phoenix, AZ), or glass-lined metal tubing, 0.7 mm i.d. (SGE) were used. The columns were slurry-packed with Nucleosil 5 SA or 4 SA (Macherey Nagel, Düren, Germany) and impregnated with silver nitrate as described earlier (9,10). Fused-silica capillary tubing (Polymicro Technologies), 9 or 10 μm i.d., was used as a restrictor.

Chromatography. Chromatography was performed on a Lee Scientific 600 Series SFC (Salt Lake City, UT). The mobile phase consisted of a mixture of carbon dioxide, acetonitrile and isopropanol (92.8:6.5:0.7, mol%) for isocratic elution of TAGs, and 97.1:2.6:0.3 mol% for fatty acid methyl esters. SFC-grade carbon dioxide (Scott Spec. Gases, Plumsteadville, PA) was used; however, less expensive 99.9% carbon dioxide could be used as well (4). Negative temperature and positive pressure programming, as well as compositional gradients, were applied. For eluent compositional gradients, a second pump (LC-10AD; Shimadzu, Tokyo, Japan) was connected *via* a mixing chamber, TCMA0120113T (The Lee Company, Westbrook, CT) to the SFC (Fig. 1). The second pump was run in a constant-flow mode, whereas the first pump had to be run in a constant-pressure mode. The split ratio was 1:4. Mobile phase velocity was *ca.* 5 mm/s. Isocratic elution was performed at *ca.* 3.5 mm/s.

Detection. Separation was followed by detection on a Lee Scientific-UV (ultraviolet) detector at 210 nm or by a miniaturized light-scattering detector (14). The construction of the miniaturized light-scattering detector is shown in Figure 2.

System evaluation. Chromatographically purified oils were obtained from Larodan Fine Chem. (Malmö, Sweden). Cohune oil was obtained from LipidTeknik (Stockholm, Sweden), and hydrogenated oils were from Karlshamns Oils and Fats AB (Karlshamn, Sweden). Solutes were dissolved in HPLC-grade pentane in concentrations of 30 mg/mL. When necessary, addition of chloroform was made. When UV-detection and columns with an i.d. of 0.25 mm were used, injection was with a split ratio of 1:1 and a timed split of 0.2 s. Under these conditions, *ca.* 60 nL was allowed to enter the column. Sample injection was

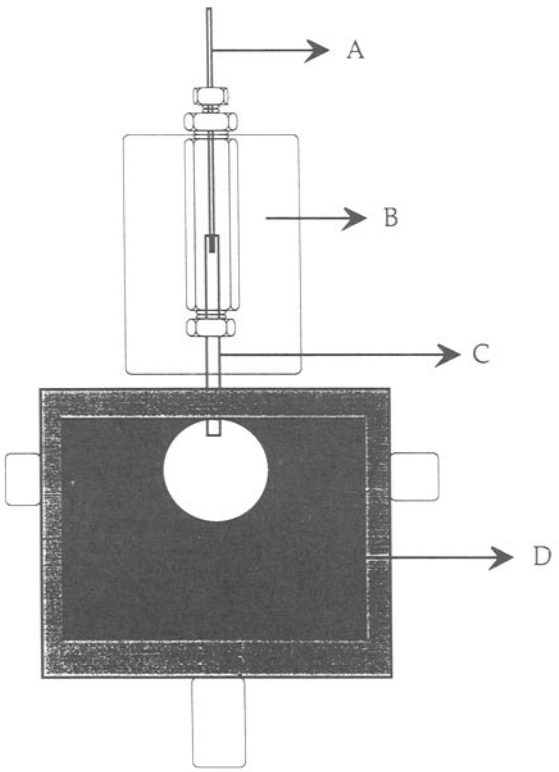

FIG. 2. Schematic diagram of the evaporative light-scattering detector (14). A, Restrictor; B, heating block; C, drift tube; D, detector cell.

200 nL without split when columns with an i.d. of 0.7 mm and ELSD were used. Peak identifications were tentative and were based on the retention of standards and the separation of fractions obtained on reversed-phase HPLC (10). Further, the retention times of different TAG were established by the comparison of chromatograms from a number of different vegetable oils. The composition of these oils had first been determined by means of reversed-phase HPLC. The acyl groups are given in an arbitrary

sequence and do not denote any specific position at the glycerol moiety.

RESULTS AND DISCUSSION

Mobile phase. It was necessary to add acetonitrile to the carbon dioxide to facilitate elution. Acetonitrile modifies the retentive properties of the stationary phase and improves the solubility of analytes in the mobile phase. A minor amount of isopropanol was added to achieve a mobile phase that is homogeneous under the applied conditions. It should be pointed out that reproducible retention times can be obtained only when the mobile phase consists of one single phase.

Addition of modifiers to the carbon dioxide generally results in an increase in critical temperature (T_c) and pressure (P_c). Calculation of the critical parameters for the mixture used for separation of TAGs under isocratic conditions gave $T_c = 62°C$ and $P_c = 101$ atm (10). When operating under conditions below critical, this technique is not SFC in *sensu strictu;* however, liquid carbon dioxide also provides relatively high diffusion, and the viscosity is lower than for liquids ordinarily used as mobile phases for HPLC. Thus, chromatography with subcritical carbon dioxide as mobile phase may also provide faster and more efficient separations than conventional HPLC.

Retention in argentation SFC. Retention characteristics in argentation TLC and HPLC have been extensively discussed (19,37,38). For a given column, the elution order is influenced by the nature of the mobile phase, and it seems that some mobile phases can reduce the double bond effect. In SFC, a dienoyl residue is retained less than two monoenes in the same molecule, and a triene is less retained than a monoene and a diene, *e.g.,* SOL is eluted before OOO (Fig. 3). Christie (23,28) reported a reversed-elution order for HPLC (23,28). Some degree of chain-length separation is sometimes observed in HPLC (19); in SFC, this type of separation can be extensive (14). Separation of TAGs differing only in the position of the double bonds in one fatty acid moiety is demonstrated in Figure 4. The chromatogram shows the separation of a fraction of borage oil, obtained by means of reversed-phase HPLC. Separation of TAG differing from each other only in the position of a double bond has been achieved also by argentation HPLC (26,27). A chromatogram of borage oil is shown in Figure 5. This oil contains a relatively high proportion of long-chain monoenes (39). Such monoenes greatly complicate the elution pattern. Separation can, as shown here, be obtained by means of a two-step procedure.

Separation of geometrical isomers of methyl linolenate by argentation SFC has been reported previously (13). Such a mixture should contain eight components, and eight peaks were distinguished. The separation was more complete than that achieved by HPLC (25). Partial hydrogenation of an oil will result in a great number of *trans* isomers; group separation of partially hydrogenated oils is shown in Figure 6.

Composition gradients. An advantage of SFC is that a great number of gradients affecting the properties of the mobile phase are applicable. Most commonly, gradients resulting in increased mobile-phase densities are applied. Such gradients, however, result in a decrease in solute diffusion coefficients, which leads to impaired chromatographic performance. The optimal mobile-phase velocity, u_{opt}, will thus be decreased, and the slope of the high-velocity branch of the van Deemter curve will be steeper. Moreover, pressure programming, without application of constant flow regulation, results in increased mobile-phase velocities, leading to decaying separation efficiencies. On the contrary, it would be beneficial to apply a program that decreases the mobile-phase flow rate (40). When applying positive pressure and negative temperature gradients, we have thus been obliged to employ quite slow mobile-phase velocities, typically 2–3 mm/s at the start of a run, to maintain the highest separation efficiency throughout the entire analysis. In addition, negative temperature programming is not really optimal in connection with argentation chromatography because the strength of the olefin/silver ion complex will thereby be strengthened.

Application of moderate modifier gradients on packed-column SFC is an attractive approach. With such gradients, the mobile-phase elution strength will be greatly enhanced, while diffusion coefficients will be only moderately increased. Thereby, it will be possible to apply higher mobile-phase velocities without appreciable losses in separation efficiency. Furthermore, when no constant flow regulation is employed, u will be decreased during the composition program, which may be an advantage because late-eluting compounds, in general, have a lower u_{opt}. Moreover, it may be advantageous to combine a compositional gradient with a positive temperature gradient (41). Such temperature gradients could be valuable in argentation chromatography. The selectivity will, however, be lost at temperatures above 125°C.

The chromatographic system applied was not optimal for accurate compositional gradient elution. The accuracy of the reciprocating Shimadzu pump was only ±2 μL/min. This could partly be overcome by the application of a split, as in Figure 1. To obtain a fast response to changing mobile-phase composition, the dead volume between split and column must be as low as possible. Furthermore, due to the low mobile-phase flow rate, at least 30 min of system equilibration was needed between runs to obtain retention time reproducibility. The retention time relative SD of peak 5 (Fig. 6A) was 4.35 (four runs). With the current instrumentation, columns with a somewhat larger i.d. would result in improved gradient reproducibility. For narrow-bore columns, a flow-regulated syringe pump for the modifier should lead to improved precision. Columns with an i.d. of 1–2 mm can be employed when a split is inserted between the column and the detector. Such a split could, when it is easily regulated, be opened after a programmed run to achieve a rapid equilibration before the following injection. Moreover, a second detector, *e.g.,* UV or mass spectrometry, could be applied to the split line (42).

Application of a mobile-phase gradient is beneficial for separation of oils containing components that have a widely differing degree of unsaturation. For isocratic elution, a relatively high concentration of modifier is required to elute the more highly unsaturated species. Under such conditions, species having few unsaturations elute quickly without being well-separated, *cf.,* separation of fish oil (9,12) and of linseed oil (10). A great improvement was achieved when applying a mobile-phase composition gradient, as compared to pressure/temperature gradients

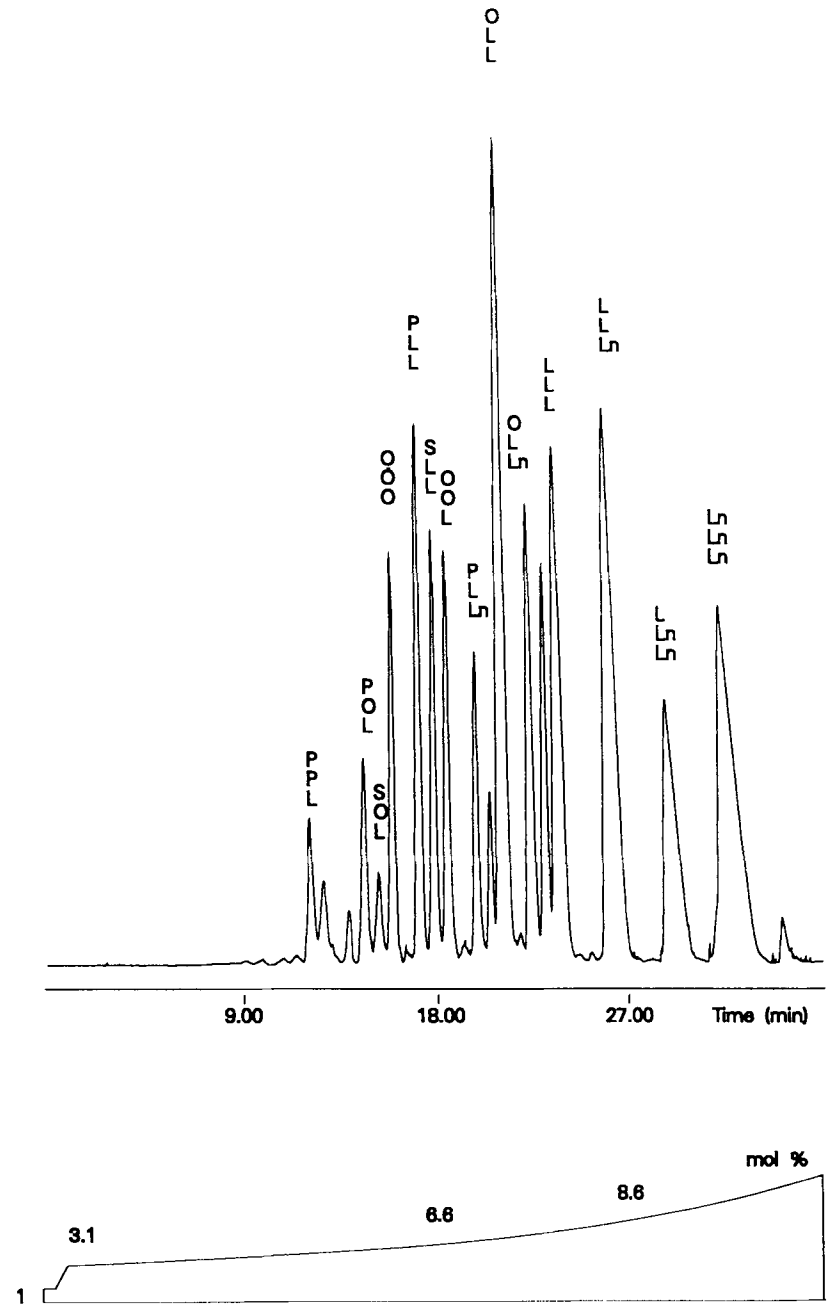

FIG. 3. Supercritical fluid chromatogram, evaporative light-scattering detection of linseed oil. Column: glass-lined metal tubing, 150 mm × 0.7 mm, packed with Nucleosil 4 SA and impregnated with $AgNO_3$. Conditions: temperature, 100°C; pressure, 340 atm.; mobile phase, gradient of CO_2/acetonitrile/isopropanol, 90:10 mol%; restrictor, fused-silica capillary tubing 130 mm × 10 μm. Peaks, triacylglycerols, P, palmitate; L, linoleate; S, stearate; O, oleate; Ln, α-linolenate.

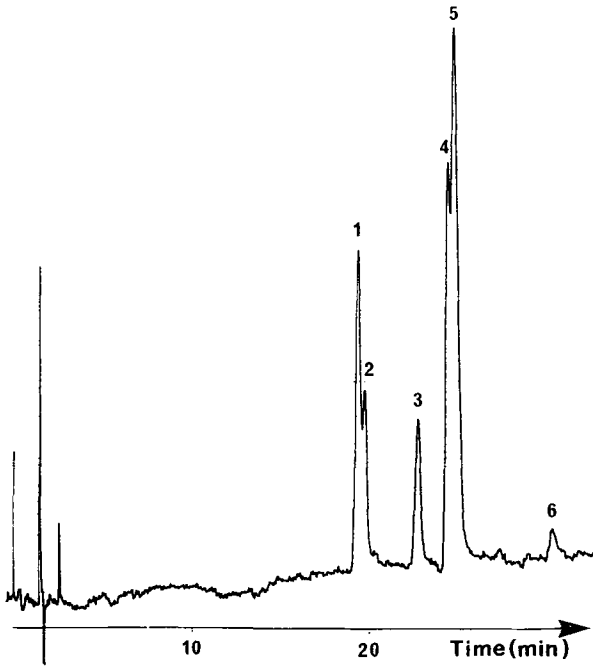

FIG. 4. Supercritical fluid chromatogram, ultraviolet detection, of a fraction, partition number 42, of borage oil obtained by reversed-phase high-performance liquid chromatography. Column, fused-silica, 290 mm × 0.25 mm, packed with Nucleosil 5 SA and impregnated with $AgNO_3$. Conditions: injection at 115°C and 260 atm, after 2 min, programmed at −1°C/min to 75°C and at 1 atm/min to 300 atm. Mobile phase: carbon dioxide/acetonitrile/isopropanol (92.8:6.5:0.7) mol%. Peaks: 1, PLG; 2, PLLn; 3, SGG; 4, OLG; 5, LLL; 6, GGGo. Abbreviations as in Figure 3, and G, γ-linolenate; Go, gondoate.

(Figs. 3 and 7). Furthermore, a general advantage of eluent composition programming is that the analytes are focused at the column head, which results in improved performance.

ELSD. Detection is often a problem with packed-column SFC. Flame-ionization detection (FID) can be used when neat carbon dioxide is being employed as mobile phase. However, for most samples it is necessary to add a polar modifier to the mobile phase, and this precludes the use of FID. Detection by means of UV is therefore often employed. For the present application, this is not suitable because the UV response is proportional to the number of double bonds, and saturated TAG cannot be detected at all. An ELSD solves this problem.

The ELSD has been developed for connection to conventional HPLC (43), but it has also been used for packed-column SFC with wide- (44–48) or narrow-bore columns (14,49,50). The response of the detector is highly dependent on the mobile-phase flow rate. Carraud *et al.* (44) thus obtained a maximum in a plot of peak area *vs.* mobile-phase flow. It may be speculated that the ascending part of the curve is caused by losses of solutes on the walls of the drift tube at low flow rates, whereas the descending part may be explained by the formation of smaller drops at high flow rates. It was deduced that pressure programming in SFC could not be combined with connection to ELSD (44). In SFC, pressure programming is of crucial importance for optimization of the separation, and it was decided to develop an ELSD that would give a similar response over a range of mobile-phase flow rates. This would, of course, also improve detector performance

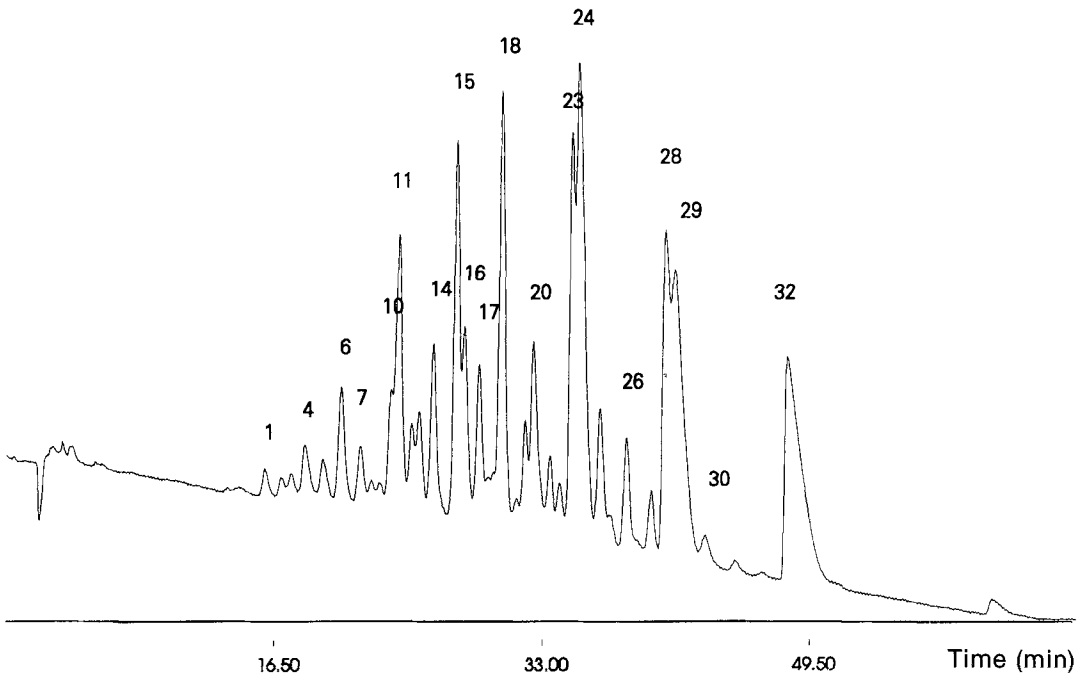

FIG. 5. Supercritical fluid chromatogram, ultraviolet-detection, of borage oil. Column, fused-silica, 330 mm × 0.25 mm, packed with Nucleosil 4 SA and impregnated with $AgNO_3$. Conditions: injection at 115°C and 260 atm, after 2 min, programmed at −0.5°C/min to 85°C and at 1 atm/min to 320 atm. Mobile phase as in Figure 4. Peaks: 1, PPL; 4, PPG; 6, POL; 7, SOL; 10, POG; 11, PLL; 14, OOL; 15, PLG; 16, PLLn; 17, OOG; 18, OLL; 20, SGG; 23, OLG; 24, LLL; 26, GLEr; 28, GGO; 29, GLL; 30, GGGo; 32, LGG. Abbreviations as in Figures 3 and 4.

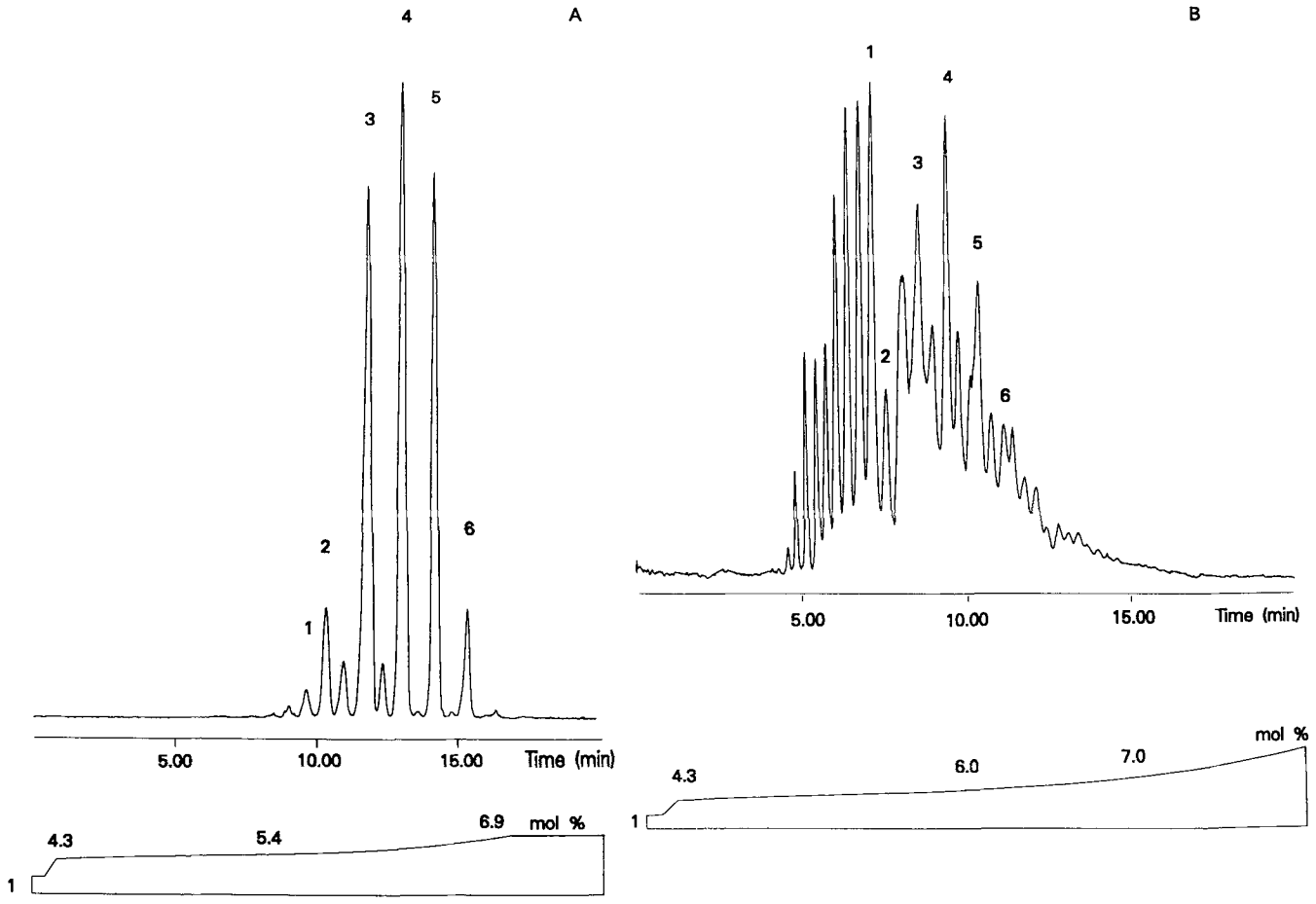

FIG. 6. Supercritical fluid chromatograms, evaporative light-scattering detection, of partially hydrogenated rapeseed oil (Lobra) (A) and fish oil (B). Column, conditions and abbreviations as in Figure 3. Peaks: triacylglycerols, 1, P (two *trans*-monoenes); 2, S (two *trans*-monoenes); 3, three *trans*-monoenes; 4, one *cis*-, two *trans*-monoenes; 5, two *cis*-, one *trans*-monoene; 6, three *cis*-monoenes.

when applying flow programming. The approach taken was to try to reduce the losses of solutes in the drift tube at low mobile-phase flow rates. For this purpose, we took advantage of the low mobile-phase flow from microcolumns, where a miniaturized drift tube would be sufficient for the evaporation of the mobile phase. Detector dimensions were thus optimized for connection to a packed microcolumn with an i.d. of 0.7 mm. As indicated above, wider columns can also be employed, but a split should then be installed between column and detector. The application of 4.6-mm i.d. columns would allow split ratios of *ca.* 1:100, thus providing a convenient method for fractionation.

The detector response was relatively constant over a range of flow rates of expanded mobile phase, 8–16 mL/min, and also at flow rates above 18 mL/min (14). The limit of detection for trimyristin, triolein and trilinolein was less than 6 ng when the lower range of flow rates was applied. This is in the same range as reported for other ELSD systems. There is, however, a potential for further improvement of the limit of detection of our detector. However, at the higher flow rates, the response was *ca.* 20 times lower. Detection by means of ELSD at the higher flow rates was applied in Figures 3, 6 and 7.

The suitability for quantitative analysis was demonstrated for corn oil (14). The relative SD in peak areas was generally less than 4% (n = 6). Minor components had peak areas with higher relative SD. Under isocratic conditions, r^2 of a log/log plot of peak area *vs.* sample amount was 0.9940 over a range of 11 to 200 ng. Also, mobile-phase compositional gradients may affect the detector response. This has been attributed to changes in droplet size due to alterations of mobile-phase surface tension and viscosity (51). The approach taken here does not counteract this effect.

A method where SFC is applied for the quantitative analysis of TAGs has been described. The decisive point is the comparison of the performance for the particular application with that obtained with other chromatographic techniques. First, the advantage over GC is that polyunsaturated TAG are stable under SFC conditions, but not at the high temperatures necessary for separation by means of GC. Second, SFC possesses a higher intrinsic separation power than HPLC, which we have attempted to demonstrate in this article. Separations can thus be performed more efficiently, and analysis times are shorter. It could be argued, however, that chain-length separations are not always desirable because they

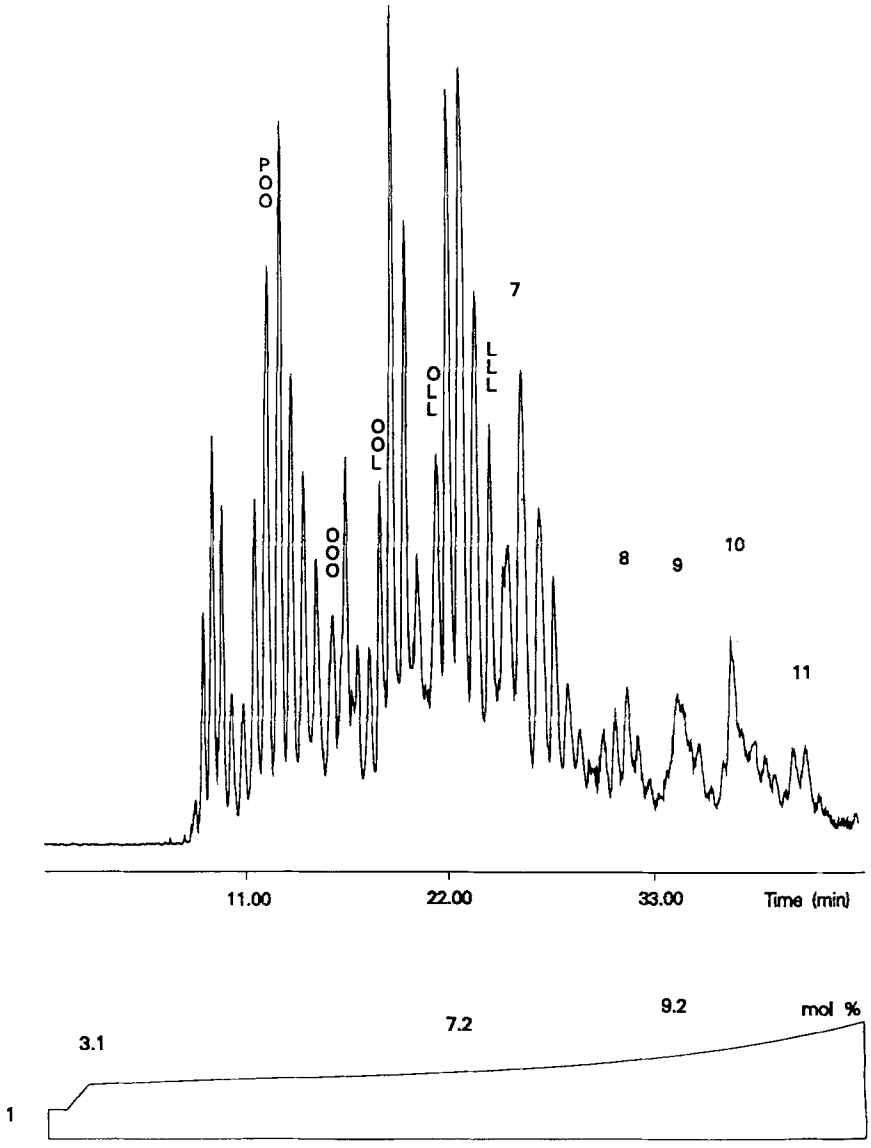

FIG. 7. Supercritical fluid chromatogram, evaporative light-scattering detection, of CPL fish oil 30. Column conditions and abbreviations as in Figure 3. Peak numbers refer to the number of double bonds.

complicate relatively simple traces. A second factor of decisive importance is the commercial availability of the necessary equipment. It seems that the recent generation of SFC instruments, dedicated to packed-column operation, in combination with commercially available ELSD, would be suitable for the quantitative analysis of TAGs.

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