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Separation of triacylglycerols by supercritical-fluid argentation chromatography

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ABSTRACT

Microcolumns packed with a silica-based cation exchanger were used to separate triacylglycerols in vegetable oils. A supercritical mobile phase consisting of carbon dioxide, acetonitrile and isopropanol was used. Argentation chromatography results in separation according to the number of double bonds; in this work, separation was also obtained according to chain length and the nature of the double bonds. Full separation of molecular species of triacylglycerols in the investigated oils could be obtained after fractionation in reversed-phase liquid chromatography.

INTRODUCTION

Silver ion, or argentation, chromatography is a technique which has proved to be of great value in lipid analysis [1–5]. This method allows fractionation of triacylglycerols according to the number of double bonds they contain, and to some extent according to their geometrical configuration. The approach was first developed for thin-layer chromatography (TLC) [6]. Many attempts to adapt the technique to high-performance liquid chromatography (HPLC) have been made, these being for some time hampered by the lack of stable columns. However, it was shown by Christie and co-workers [7–9] that sufficient stability for use in HPCL could be achieved by using silica-based cation exchangers as support for the silver ions. In an earlier work, columns of this type were evaluated for group separation of triacylglycerols using supercritical fluids as the mobile phase (supercritical fluid chromatography, SFC) [10]. The chromatographic separation was performed on micropacked columns, and a supercritical mobile phase, consisting of carbon dioxide, 5.5% acetonitrile and 0.5% isopropanol, was used. Separation into groups according to the number of double bonds was sought, and the intention was to separate each group further by high-temperature gas chromatography (GC). However, such a system is not attractive for the analysis, since the recovery of highly unsaturated triacylglycerols with high-temperature GC [11,12] is uncertain. Another approach was taken by Takano and Kondoh [13], who used a two-dimensional liquid chromatography (LC) system for the separation of triacylglycerols. In that system, the sample first passes an argentation column and then a reversed-phase column. In the present work, another route

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was chosen for the separation of molecular species. Thus the vegetable oils were first fractionated according to the partition number by reversed-phase LC, and then separated further using microcolumn argentation SFC.

In this paper, it is shown that microcolumn argentation SFC can give separation of molecular species of triacylglycerols. This is an extension of our earlier results, in which separation into groups according to degree of unsaturation was achieved [10].

EXPERIMENTAL

The chromatographic system consisted of a Lee Scientific 600 Series SFC system and an Isco μLC-10 variable-wavelength absorbance detector. Detection was performed at 210 nm on a short length of 250 μm I.D. fused-silica tubing according to the method of Fields *et al.* [14]. The width of the slit on the detection capillary was about 1 mm. Fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ, U.S.A.), 11 μm I.D., was used as a restrictor in lengths of 20–25 cm.

Columns were prepared from fused-silica capillary tubing, 290 mm × 250 μm I.D. and 430 μm O.D. (Polymicro Technologies). All columns were packed with Nucleosil 5 SA (Macherey Nagel, Düren, Germany) and prepared as described previously [10]. After packing, the columns were washed, first with 400 μl of an aqueous solution of 10% ammonium nitrate and then with 400 μl of 0.1 *M* silver nitrate. Washing with ammonium nitrate was discontinued after preliminary experiments.

The mobile phase consisted of carbon dioxide–acetonitrile–isopropanol (92.8:6.5:0.7). The critical parameters were calculated from Lee Scientific software and ref. 15. The calculations indicated a critical temperature (T_c) of 62°C and a critical pressure (P_c) of 101 atm for the mixture. SFC-grade carbon dioxide (Scott Specialty Gases, Plumsteadville, PA, U.S.A.) was used. The mobile-phase mixture was prepared in the SFC pump as described previously [16]. Mobile phase velocity was 3.5 mm/s.

Reversed-phase HPLC was performed on columns, 250 mm × 10 mm, packed with Lichrosphere 100, RP 18, 5 μm (Merck), the mobile phase being HPLC-grade, degassed methanol–acetone (1:1).

Chromatographically purified oils, chromatographically purified (CPL) corn oil, CPL linseed oil, CPL palm oil, CPL soybean oil, CPL sunflower seed oil and standard substances (Larodan Fine Chemicals, Malmö, Sweden) were used. Solutes were dissolved in HPLC-grade pentane in concentrations of 30 mg/ml. Injection was performed with a split ratio of 1:1 and a time split of 0.2 s.

RESULTS AND DISCUSSION

Columns

Some columns were rinsed with ammonium nitrate before the treatment with silver nitrate. Such a rinsing was shown to be unnecessary, since no effect on the chromatographic properties was observed. Further, in some cases, plugging of the columns resulted. For the application of Ag^+, a large excess of reagent was applied, as it was believed this would lead to the highest yield. Relatively poor results were reported when using small amounts of silver nitrate for the impregnation [17]. Final-

ly, in order to provide a smooth change over from water to the relatively non-polar SFC mobile phase, the columns were rinsed with methanol after Ag^+ impregnation. However, observations made during the early stages of this work showed that such a rinsing could be omitted.

Mechanical column stability presented a problem in part I of this work [10], the columns often exploding when high pressure was applied. This problem was completely solved by the use of thick-walled fused silica capillary tubing, by which means a good stability was achieved. The columns could be used at temperatures up to 160°C. Moreover, under the conditions used in this work, the columns have now been used for more than one year without deterioration of the performance. It should be noted, however, that the injection of oxidizing substances may lead to the formation of silver oxide in the column.

Separation of vegetable oils

A series of chromatograms demonstrating the separation of vegetable oils is shown in Figs. 1–5. Note that the acyl groups are given in an arbitrary order and do not represent any specific positions within the triacylglycerol molecules. Two different pressure–temperature programmes were used, a relatively fast programme for oils containing highly unsaturated triacylglycerols, and a slow programme for oils having moderately unsaturated triacylglycerols. Peak identifications are tentative, and are based on comparisons with results from reversed-phase LC, from which the main components are known. Further, since the retention times obtained on an argentation column are very stable, the retention order can be mapped by comparison of the retention times of the main components of different oils. Moreover, peak identities have been further established by the separation of fractions obtained on reversed-phase LC. These fractions have been collected according to partition number, and

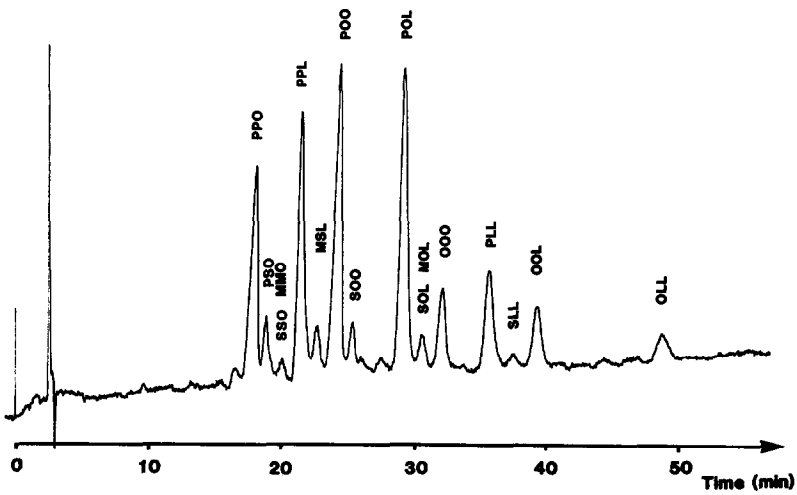

Fig. 1. Supercritical fluid chromatogram of palm oil. Injection at 95°C and 240 atm; after 2 min, programmed at −0.5°C/min to 75°C and 0.5 atm/min to 300 atm. UV detection at 210 nm. See the Experimental section for practical details and Table I for abbreviations.

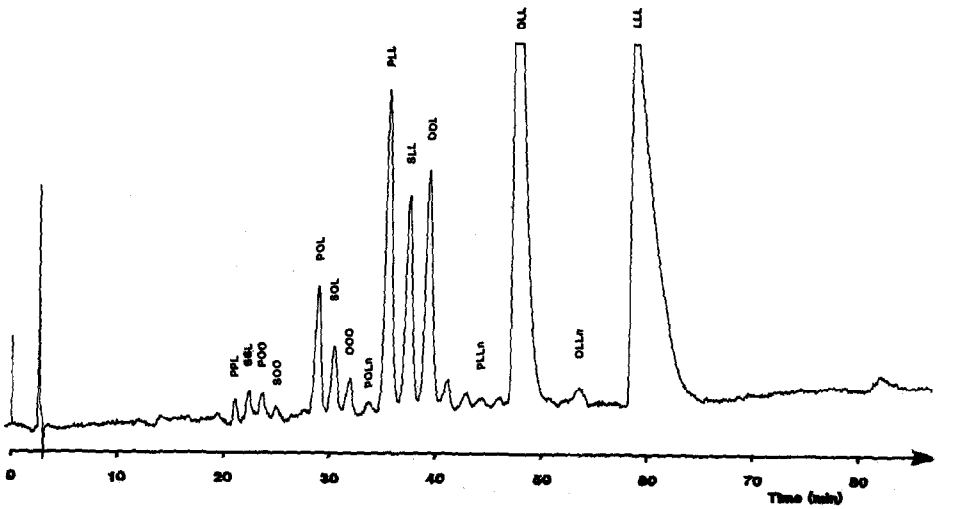

Fig. 2. Supercritical fluid chromatogram of sunflower seed oil. Conditions as in Fig. 1.

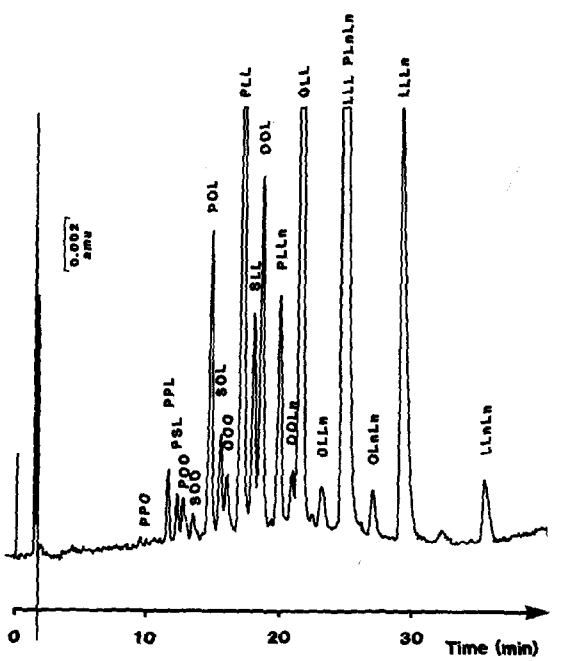

Fig. 3. Supercritical fluid chromatogram of soybean oil. Injection at 115°C and 260 atm; after 2 min, programmed at −1°C/min to 75°C and 1 atm/min to 300 atm. See Table I for abbreviations.

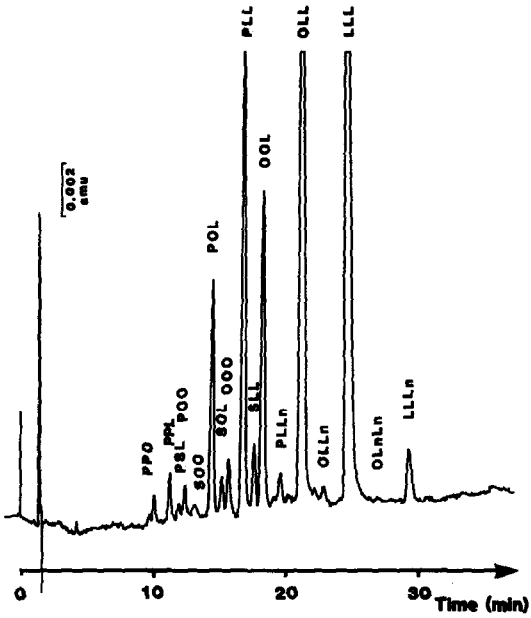

Fig. 4. Supercritical fluid chromatogram of corn oil. Conditions as in Fig. 3.

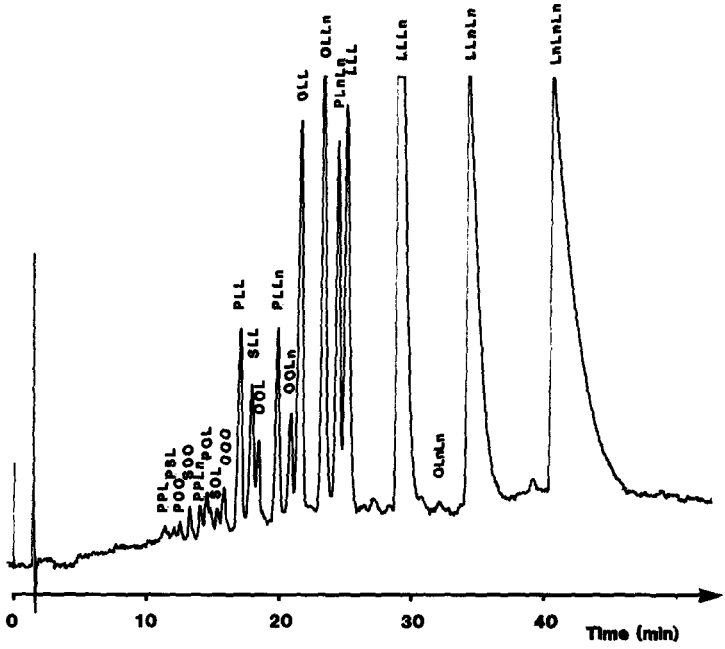

Fig. 5. Supercritical fluid chromatogram of linseed oil. Conditions as in Fig. 3.

thus each fraction contained a limited number of triacylglycerols, *e.g.* having partition number 46.

Separation of triacylglycerols in corn oil by means of SFC has been attempted on microcolumns packed with cyanopropyl-bonded silica [18]. However, the separation thus achieved was quite poor.

Separation of triacylglycerols

The ability of argentation TLC and HPLC to separate *trans* and *cis* isomers of unsaturated triacylglycerols is well recognized [3–5, 19,20]. The *cis* double bonds are thus retained more than *trans*. Such a separation was also achieved with microcolumn argentation SFC. An α value of 1.24 was thus obtained for the separation of 1,2,3-tri-[(*trans*)-9-octadecenoyl]glycerol and 1,2,3-[(*cis*)-9-octadecenoyl]glycerol under the conditions given in Fig. 1.

Elution of mono-, di- and triolein is shown in Fig. 6; it is evident that diolein complexes with Ag^+ to a lesser extent than triolein, while for mono-olein retention is determined mainly by residual silanol groups. Hammond and Irwin [4] reported longer retention times for diacylglycerols than for triacylglycerols when using silver nitrate-impregnated silica columns for HPLC. The silanol activity is obviously much higher in this type of silica than in silica which has been modified with cation-exchange moieties.

Separation of positional isomers, *e.g.* SOS from SSO (see Table I), was obtained by Smith *et al.* [21] using agentation HPLC. It was observed that the separation was favoured by a high silver content. The interaction between Ag^+ and double bonds increases with decreasing temperatures, and the separation of posi-

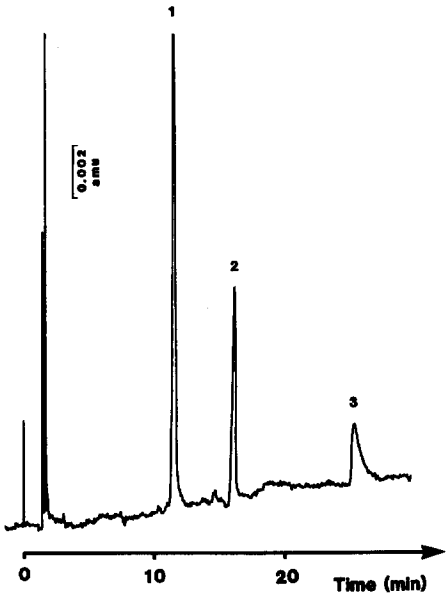

Fig. 6. Supercritical fluid chromatogram of mono-, di- and triacylglycerols. Conditions as in Fig. 3. Peaks: 1 = diolein; 2 = triolein, 3 = monoolein.

TABLE I

ABBREVIATION OF FATTY ACID MOIETIES

Fatty acid moiety	Abbreviation
Myristate	M
Palmitate	P
Stearate	S
Oleate	O
Linoleate	L
α-Linolenate	Ln

tional isomers was achieved at 6.8°C. However, a separation could also be obtained at 25°C [13]. Separation of positional isomers could not be achieved in this work, even when using subcritical conditions at 30°C.

Two-dimensional separations and elution orders

Although the argentation columns show a relatively high separation power, full separation of molecular species cannot be achieved. For example, in palm oil, the presence of myristate leads to many isomers (Fig. 1), and the presence of palmolein in olive oil results in separation problems. In Fig. 7 is shown the separation of a fraction of corn oil collected at partition number 46 from reversed-phase LC, and in Fig. 8 is shown the separation of the fraction having partition number 48.

Only slight separation of triacylglycerols according to chain length has been

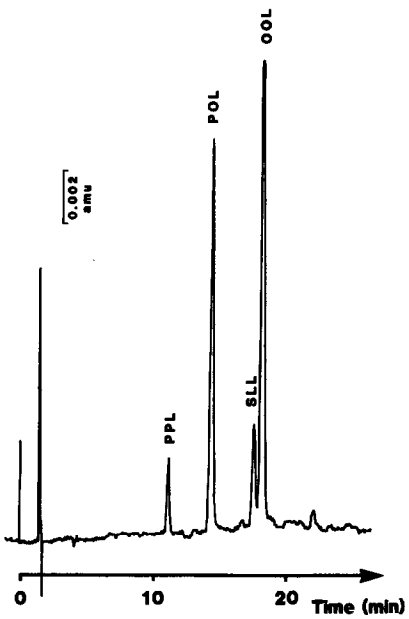

Fig. 7. Supercritical fluid chromatogram of a fraction of corn oil. Conditions as in Fig. 3. Fraction collected from reversed-phase LC at partition number 46.

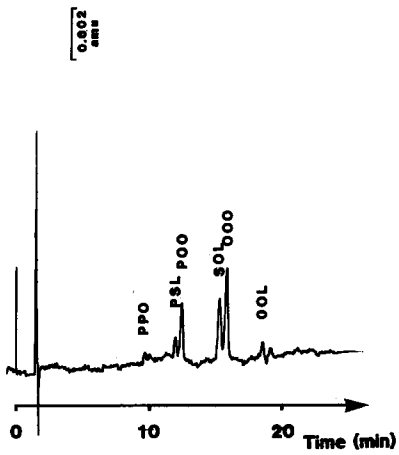

Fig. 8. Supercritical fluid chromatogram of a fraction of corn oil. Conditions as in Fig. 3. Fraction collected from reversed-phase LC at partition number 48.

obtained on silver-loaded silica in HPLC [4]. A notable chain length separation was obtained in this work: compare the difference in retention of POL and SOL (see Table I) in corn oil fractions (Figs. 7 and 8). Further, from the analysis of the fractions obtained from palm oil, an elution order — PPO, PSO, SSO, MMO — could be

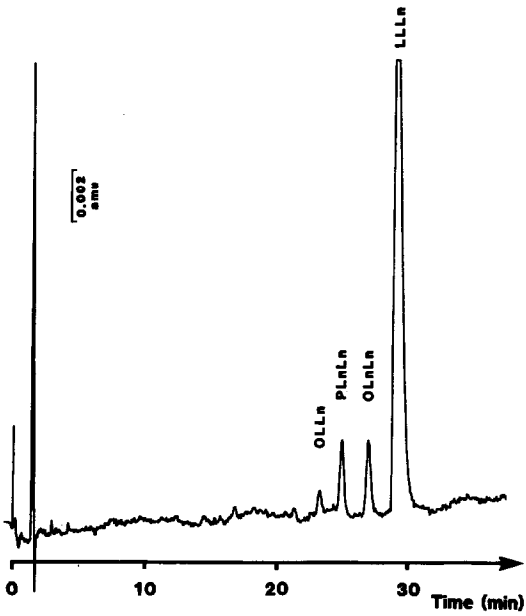

Fig. 9. Supercritical fluid chromatogram of a fraction of soybean oil. Conditions as in Fig. 3. Fraction collected from reversed-phase LC at partition number 40. The presence of OLLn is due to overlap between fractions.

established. Moreover, SOL and MOL were eluted at the same retention time. Depending on the aim of the analysis, such separations may be considered either as an advantage or as an unnecessary complication of the elution pattern.

A linoleic acid-containing triacylglycerol does not have a retention exactly equal to that of one containing two oleic acids [22]. In this work, the presence of one L-unit in the triacylglycerol results in lower retention than two O-units (compare PPL/POO and LLS/LOO in Fig. 1). The same elution order was shown for argentation LC by Hammond and Irvin [4], Aitzetmüller [3], and Takano and Kondoh [13]. Using an argentation TLC system, Gunstone and Padley [23] determined that LLS showed higher complexing powers than LOO. Similarly, as reported by Christie [8], LLS was shown to have a somewhat longer retention time than LOO. Further, as shown in this work, one α-linolenic acid unit gives a lower retention than three oleic acid units (compare the elution of PPLn and OOO in Fig. 5). A reversed elution order was reported by Christie [8]. The retention of α-linolenic-containing triacylglycerols is further demonstrated in Fig. 9, which shows separation of a fraction of soybean oil having partition number 40. Clearly, different mobile phase compositions may lead to different elution orders [5].

CONCLUSIONS

A technique for separation of molecular species of triacylglycerols was developed, and the retention characteristics of silver-modified cation-exchange columns in SFC were studied. Some separation according to chain length was found; triacylglycerols containing palmitin were thus eluted before those containing stearin. Unexpectedly, triacylglycerols containing myristin were found to be retained more than those containing stearin. The selectivity is thus different from what has been presented for argentation HPLC, where there is little or no chain-length separation. The separation of *cis* and *trans* isomers seems to be equal in HPLC and SFC. The separation of positional isomers has been reported for HPLC, but such separations could not be achieved with SFC. Further, some differences in elution orders were observed. The general resolving power of argentation SFC, as presented here is, however, higher than that achieved on argentation HPLC: compare the separation of linseed oil and sunflower seed oil in this work and in ref. 8. The improvement of the separations may be explained by the relatively high diffusion in supercritical media. Colum stability is of crucial importance, and it was excellent under the conditions applied. A high stability, in terms of silver ion leakage, was also reported for HPLC [8], however, the mobile phase should not contain polar solvents [24].

For SFC separation of triacylglycerols, the use of packed argentation columns makes a polar mobile phase modifier necessary. The flame ionization detector cannot be used in combination with such mobile phases, and we have thus resorted to detection with UV. The response is, at the wavelength used, proportional to the number of double bonds; saturated triacylglycerols could not be detected using this system. The use of a light-scattering detector would solve this problem. A further improvement of the system, leading to shorter analysis times, could be achieved by the introduction of gradient elution.

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REFERENCES

1 L. J. Morris, *J. Lipid Res.*, 7 (1966) 717.
2 C. Litchfield, *Analysis of Triglycerides*, Academic Press, New York, 1972.
3 K. Aitzetmüller, in H. Engelhardt (Editor), *Practice of High Performance Liquid Chromatography*, Springer, Berlin, 1986, p. 287.
4 E. W. Hammond and J. W. Irwin, in R. Macrae (Editor), *HPLC in Food Analysis*, Academic Press, London, 1988, p. 95.
5 W. W. Christie, *HPLC and Lipids*, Pergamon Press, Oxford, 1987.
6 L. J. Morris, *Chem. Ind. (London)*, (1962) 1238.
7 W. W. Christie, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 148.
8 W. W. Christie, *J. Chromatogr.*, 454 (1988) 273.
9 W. W. Christie and G. H. McG. Breckenridge, *J. Chromatogr.*, 469 (1989) 261.
10 M. Demirbüker and L. G. Blomberg, *J. Chromatogr. Sci.*, 28 (1990) 67.
11 E. G. Hammond, *Trends Anal. Chem.*, 8 (1989) 308.
12 I. Hägglund, K. Janák, L. Blomberg, S. Claude and R. Tabacchi, in preparation.
13 S. Takano and Y. Kondoh, *J. Am. Oil Chem. Soc.*, 64 (1987) 380.
14 S. M. Fields, K. E. Markides and M. L. Lee, *Anal. Chem.*, 60 (1988) 802.
15 R. C. Reid, J. M. Prausnitz and B. E. Poling, *The Properties of Gases and Liquids*, McGraw-Hill, New York, 4th ed., 1987, Chapter 5 and Appendix A.
16 S. Schmidt, L. G. Blomberg and E. R. Campbell, *Chromatographia*, 25 (1988) 775.
17 F. Ulberth and E. Achs, *J. Chromatogr.*, 504 (1990) 202.
18 H.-C. K. Chang and L. T. Taylor, *J. Chromatogr. Sci.*, 28 (1990) 607.
19 W. W. Christie, *Gas Chromatography and Lipids*, The Oily Press, Ayr, 1989.
20 W. W. Christie, *Lipid Analysis*, Pergamon Press, Oxford, 2nd ed., 1982.
21 E. C. Smith, A. D. Jones and E. W. Hammond, *J. Chromatogr.*, 188 (1980) 205.
22 K. Aitzetmüller, *Chem. Ind. (London)*, (1988) 452.
23 F. D. Gunstone and F. B. Padley, *J. Am. Oil Chem. Soc.*, 42 (1965) 957.
24 N. U. Olsson, *Dissertation*, University of Stockholm, Stockholm, 1991.