Supercritical Fluid Chromatographic Analysis of Fish Oils

Arne Staby[a], Christina Borch-Jensen[a], Steen Balchen[b] and Jørgen Mollerup[a,*]
[a]Department of Chemical Engineering, DTH, DK-2800 Lyngby, Denmark and [b]Project Fish Oil, Technological Laboratory, Danish Ministry of Fisheries, DTH, DK-2800 Lyngby, Denmark

Various natural and processed fish oil triglyceride mixtures have been analyzed by capillary supercritical fluid chromatography (SFC). The analyses were performed on nonpolar columns to separate the components by lipid class and by the number of carbon atoms. The compounds separated included free fatty acids, squalene, α-tocopherol, cholesterol, wax esters, cholesteryl esters, di- and triglycerides. This kind of analysis is not possible by gas chromatography or high-performance liquid chromatography methods without prior treatment of the fish oil, making SFC superior for this application. Applications of SFC to fish oils are given, including a control analysis of the various process steps in the refining of a fish oil, analysis of a lipase-catalyzed transesterification of a fish oil and the detection of polymeric artifacts.

KEY WORDS: Carbon dioxide, cholesterol, chromatography, fatty acid, fish oil, SFC, supercritical, triacylglycerol, triglyceride, wax ester.

In the characterization of natural products, like fish oil, the qualitative content of various lipid classes and the quantitative distribution of triglycerides (triacylglycerols) (TGs) are of great importance. Papers presenting separation of lipid class components or TGs derived from fish oil have been published, and a variety of chromatographic techniques have been applied. These include thin-layer chromatography (TLC) (1–3), gas chromatography (GC) (4), high-performance liquid chromatography (HPLC) (5–11), and supercritical fluid chromatography (SFC) (12–19).

Of these techniques, GC provides the highest resolution in the shortest analysis time, but high temperatures are needed. The application of high-temperature GC to the separation of TGs obtained from fish oil may be difficult because the molecular weight of some TGs is 1000 or more. Temperatures up to 400°C may therefore be necessary to elute the TGs, leading to enhanced risk of thermal degradation of the highly unsaturated components. Thus, full or partial hydrogenation is necessary to elute the TGs, which causes loss of information on the structure of the fish oil. The "state of the art" of GC analysis of TGs from fish oil is described by Myher *et al.* (20), who analyzed Grignard-degraded diacylglycerols (DGs) derived from TGs of menhaden oil. On a polar GC column at a maximum operating temperature of 260°C, they identified 72 DGs, but they were not able to detect DGs with molecular weights higher than approximately 650 with 10 double bonds.

The liquid-chromatography methods, TLC and HPLC, provide much lower resolution in often longer analysis times than the GC method. The TLC method with silica or silver ion stationary phases does not afford proper resolution of a TG mixture derived from fish oil (1), and the method is merely employed to separate a fish oil into its lipid classes (15). The HPLC method, both normal and reversed-phase, has been increasingly used in recent years to separate TGs from fish oil (6,9), especially employing the method of Christie (5) with a silver ion column and mass spectra detection. The method primarily separates the TGs by degree of unsaturation and to some extent, by the molecular weight and the position of the unsaturation. This method has also been used to fractionate the TGs on a semi-preparative scale, followed by a GC analysis of the fatty acid methyl esters (FAMEs) of the separate fractions obtained (7). Another, more detailed study of fish oil TGs may be obtained by combining reverse-phase HPLC, which separates merely by molecular weight, and silver-ion HPLC (8).

The SFC technique provides almost the same high resolution and fairly short analysis time as the GC method. The work on separation of fish oil constituents by SFC has focused on group separation of marine oils, often with poor resolution obtained on nonpolar columns (12,13,16–18). Kallio's group (14,15), however, obtained a fair resolution of their silver-ion TLC fractions of TGs derived from baltic herring oil on a nonpolar DB-5 column. In addition, Blomberg *et al.* (19) have recently reported on separation of individual TGs from a fish oil by argentation SFC.

In this work, the molecular weight distributions of several constituents of natural and processed fish oils, derived from sand eel (sand launce) and salmon, have been analyzed by capillary SFC. The scope of this work is to show some new examples of SFC application. In some of these applications, SFC is superior to HPLC and GC techniques.

EXPERIMENTAL PROCEDURES

Equipment. An SFC-3000 system (Carlo Erba Instruments, Milan, Italy), equipped with a flame-ionization detector (FID) and a pneumatic Valco valve for time-split injections, was used in all the SFC experiments outlined below. The mobile-phase pump cylinder was thermostatted by circulation of ethylene glycol from a Hetofrig CB 12 cooling bath (Heto Lab Equipment A/S, Birkerød, Denmark) at −5°C to ensure flow rate reproducibility. The pressure drop over the chromatographic system was obtined by integral restrictors made of uncoated fused-silica tubing by the method of Guthrie and Schwartz (21) and connected to the chromatographic column described below. Integration and control of the chromatographic run were carried out *via* a personal computer with MAXIMA chromatography software (Dynamic Solutions, Ventura, CA). Further details of the equipment are described elsewhere (22).

Materials. Standards of cholesterol (CHO), α-tocopherol (TOC) and squalene (SQU) and standard mixtures of saturated and unsaturated mono-, di and triglycerides were purchased from Sigma Chemical Company (St. Louis, MO). Standards of free fatty acids (FFAs), wax esters (WEs), cholesteryl esters (CEs) and FAMEs were purchased from Nu-Chek-Prep Inc. (Elysian, MN). *n*-Heptane LiChrosolv and chloroform (trichloromethane) LiChrosolv, utilized as solvents for the fish oil components, were obtained from Merck (Darmstadt, Germany). Helium, hydrogen and atmospheric air were supplied by Hede Nielsen A/S (Ballerup, Denmark). The stated purities were

*To whom correspondence should be addressed at The Technical University of Denmark, Department of Chemical Engineering, Building 229, DK-2800 Lyngby, Denmark.

99.996%+ for helium and 99.8%+ for hydrogen. Carbon dioxide was supplied by Linde AG (München, Germany) with a stated purity of 99.995%+. The crude oil of sand eel (*Ammodytes* sp.) was obtained from a commercial production by Thyborøn Andels Fiskeindustri A. m. b. a. (Thyborøn, Denmark). The crude and treated fish oils of salmon (*Oncorhynchus* sp.) were supplied by Claus Becker of The Technical University of Denmark, Department of Biochemistry and Nutrition (Lyngby, Denmark). Finally, the treated sunflower oil polymer mixture was supplied by M.C. Dobarganes of Instituto de la Grasa y sus Derivados, C.S.I.C. (Sevilla, Spain). All materials were used without further purification.

Methods. Two separate SFC analysis methods of the fish oils and the sunflower polymer mixture were carried out at different conditions. The Valco valve with a 0.2-μL loop and an injection time of 0.2 s were utilized in both experiments. The injection temperature was 60°C, and the detector temperature was 300°C. Carbon dioxide was employed as the carrier gas, and the hydrogen and air pressures for the FID were 55 kPa and 100 kPa, respectively.

Method 1: A nonpolar 5%-phenyl-methylpolysiloxane DB-5 (J&W Scientific, Folsom, CA) fused-silica capillary column (20 m × 0.1 mm × 0.4 μm) was employed for all fish oil analyses. The chromatographic runs were performed isothermally at 170°C, where the best selectivity of the TGs were obtained. The initial carbon dioxide density of 0.300 g/mL was immediately increased at a rate of 0.004 g/mL/min to 0.468 g/mL, where it was held constant for 14 min. The density was then increased at a rate of 0.001 g/mL/min to 0.502 g/mL, where it was held constant for 70 min. The initial and final carbon dioxide pressures were 20.3 and 34.6 MPa, respectively, and the initial carrier-gas flow was 1 mL/min. The total analysis time was 160 min.

Method 2: A nonpolar 5%-phenyl-methylpolysiloxane SE-52 (Carlo Erba Instruments) fused-silica capillary column (8.5 m × 0.1 mm × 0.4 μm) was employed for the sunflower polymer analysis. The chromatographic runs were performed isothermally at 50°C to obtain maximum density of CO_2. The initial carbon dioxide density of 0.700 g/mL was held for 30 min, then increased at a rate of 0.003 g/mL/min to 0.900 g/mL. Subsequently, the density was increased at a rate of 0.001 g/mL/min to 0.920 g/mL, where it was held constant for 233 min. The initial and final carbon dioxide pressures were 14.9 and 39.1 MPa, respectively, and the initial carrier-gas flow was 1 mL/min. The total analysis time was 350 min.

Sample preparation and peak identification. Three samples of sand eel oil were analyzed: A) crude, B) alkali-refined, bleached and deodorized, and C) alkali-refined, bleached and randomly interesterified by use of sodium methoxide as catalyst. The processing methods of the treated sand eel oils are based on common methods (23), slightly modified by Project Fish Oil at the Technological Laboratory, Danish Ministry of Fisheries. Two samples of salmon oil were analyzed: D) crude and E) transesterified with decanoic acid methyl ester by means of a 1,3-specific lipase. Oil E was obtained in the absence of solvents in a continuous reactor and purified by column chromatography on silicic acid (C. Becker, private communication, complete methodology to be published). The sunflower oil polymer mixture F was the polar fraction of a thermoxidized sunflower oil obtained by a method developed at Instituto de la Grasa y sus Derivados (M.C. Dobarganes, unpublished method).

The fish oil samples were dissolved in *n*-heptane to a concentration of 70 to 100 mg fish oil per mL, while the concentration of the sunflower polymer mixture was 14 mg/mL in *n*-heptane. The standards and the standard mixtures of mono-, di and triglycerides were dissolved in 1:1 mixtures of *n*-heptane and chloroform to various concentrations, depending on the number of components in the standard samples.

Comparison of the retention times found from the chromatographic runs of the fish oil samples with those of the commercially available standards and standard mixtures formed the basis for peak identification.

RESULTS AND DISCUSSION

Fish oils. The experimental chromatogram peak numbers, retention times and peak area% with their deviation of the various compounds of the three sand eel oils, obtained from the SFC analyses, are presented in Table 1. The subscript numbers following the various group compounds, FAME, FFA, DG, CE and TG, refer to the number of acyl carbon atoms of the molecule, while for WEs the subscript number refers to the total number of carbon atoms. Each of the chromatographic experiments was performed three times and showed similar results in retention times as well as in composition of the separate oils. Chromatograms of oils A, B and C are given in Figures 1 through 3, respectively.

Figures 1, 2 and 3 show that the nonpolar DB-5 column primarily separated the fish oils into lipid groups, and within a lipid group by the molecular weight, as might be expected. Little separation was observed by the position or the degree of unsaturation. The lipid groups present in the crude fish oil were FFAs, WEs, DGs, CEs, TGs, along with the single components CHO, TOC and SQU. The presence of CEs was difficult to determine, because they coeluted with the uneven numbered TGs. The WEs and the uneven numbered DGs also coeluted. The FAME of oil C in Figure 3 showed a slightly lower retention than the corresponding FFA due to higher solubility of the FAME in supercritical CO_2 (24). The mixtures were also checked for monoglycerides (MGs) but none were found. The retention times of MGs are somewhat higher than the corresponding FFAs, that is, MG_{16} has a higher retention time than FFA_{18} but is lower than FFA_{20}. The three chromatograms display a good separation of the various components and roughly baseline separation of the TGs within an analysis time of less than 2 h.

A comparison of the three chromatograms demonstrates the influence of processing on the components present. From Figure 1 to Figure 2, the FFA had been removed, and the amount of CHO was decreased. Table 1 shows that the amounts of all other components were fixed and not influenced by the deodorization process. In Figure 3, the interesterification process led to the formation of small amounts of FAMEs and larger amounts of the heavy TGs. Examining Table 1, one will notice that the quantity of TG_{56} was constant, while the quantities of lighter and heavier TGs were less and larger, respectively. Therefore, by subsequent fractionation of the TGs,

TABLE 1

Compounds, Chromatogram Peak Numbers (No.), Retention Times (t_R) and Composition of the Three Sand Eel Oils[a]

Compound	No.	t_R (min)	Oil A (area %)	Oil B (area %)	Oil C (area %)
$FAME_{14}$	1	19.11	n.p.[b]	n.p.	0.24 ± 0.02
FFA_{14}	2	19.36	0.08 ± 0.01	n.d.[c]	0.06 ± 0.01
$FAME_{16}$	3	20.52	n.p.	n.p.	0.14 ± 0.01
FFA_{16}	4	20.94	0.35 ± 0.01	n.d.	0.05 ± 0.02
$FAME_{18}$	5	21.99	n.p.	n.p.	0.13 ± 0.02
FFA_{18}	6	22.54	0.34 ± 0.02	n.d.	0.05 ± 0.01
$FAME_{20}$	7	23.65	n.p.	n.p.	0.16 ± 0.02
FFA_{20}	8	24.35	0.38 ± 0.02	n.d.	0.06 ± 0.01
$FAME_{22}$	9	25.65	n.p.	n.p.	0.21 ± 0.02
FFA_{22}	10	26.47	0.58 ± 0.02	n.d.	0.07 ± 0.00
SQU	11	30.73	0.03 ± 0.00	0.03 ± 0.01	0.02 ± 0.01
TOC	12	35.64	0.01 ± 0.01	0.01 ± 0.01	n.d.
CHO	13	37.69	0.74 ± 0.01	0.59 ± 0.01	0.02 ± 0.01
DG_{28}	14	38.25	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.00
WE_{32}(+ DG_{29})	15	39.34	0.06 ± 0.00	0.06 ± 0.01	n.d.
DG_{30}	16	41.07	0.03 ± 0.00	0.03 ± 0.01	0.08 ± 0.02
WE_{34}(+ DG_{31})	17	42.12	0.08 ± 0.01	0.08 ± 0.01	0.04 ± 0.01
DG_{32}	18	43.89	0.10 ± 0.01	0.10 ± 0.01	0.17 ± 0.02
WE_{36}(+DG_{33})	19	45.09	0.07 ± 0.01	0.08 ± 0.02	0.03 ± 0.01
DG_{34}	20	46.72	0.17 ± 0.01	0.18 ± 0.02	0.26 ± 0.02
WE_{38}(+ DG_{35})	21	48.06	0.06 ± 0.00	0.07 ± 0.01	0.03 ± 0.01
DG_{36}	22	49.79	0.20 ± 0.01	0.20 ± 0.02	0.39 ± 0.04
WE_{40}(+DG_{37})	23	51.31	0.05 ± 0.00	0.05 ± 0.00	0.02 ± 0.01
DG_{38} + TG_{42}	24	53.13	0.28 ± 0.01	0.27 ± 0.02	0.48 ± 0.04
WE_{42}(+ DG_{39})	25	54.33	0.04 ± 0.01	0.02 ± 0.00	0.01 ± 0.01
DG_{40} + TG_{44}	26	56.13	0.50 ± 0.02	0.50 ± 0.03	0.66 ± 0.04
WE_{44}(+ DG_{41})	27	57.94	0.02 ± 0.02	0.04 ± 0.02	0.02 ± 0.01
TG_{46} + DG_{42}	28	59.87	1.63 ± 0.02	1.68 ± 0.08	1.36 ± 0.03
TG_{47} + DG_{43}	29	61.72	0.23 ± 0.02	0.11 ± 0.02	0.12 ± 0.06
TG_{48}(+ DG_{44})	30	63.81	4.62 ± 0.01	4.68 ± 0.14	2.75 ± 0.08
CE_{14} + TG_{49}	31	65.10	0.61 ± 0.05	0.52 ± 0.11	0.66 ± 0.06
TG_{50}	32	67.92	9.10 ± 0.03	9.26 ± 0.05	5.45 ± 0.17
CE_{16} + TG_{51}	33	69.83	0.95 ± 0.03	0.95 ± 0.08	0.85 ± 0.26
TG_{52}	34	72.28	14.66 ± 0.09	15.00 ± 0.04	9.40. ± 0.03
CE_{18} + TG_{53}	35	74.16	c.w.TG_{52}[d]	c.w.TG_{52}	1.06 ± 0.25
TG_{54}(+ CE_{20})	36	76.83	16.17 ± 0.17	16.55 ± 0.26	14.00 ± 0.13
TG_{56}(+ CE_{22})	37	81.43	15.67 ± 0.05	16.08 ± 0.10	16.31 ± 0.21
TG_{58}	38	86.01	13.40 ± 0.07	13.71 ± 0.12	15.89 ± 0.26
TG_{60}	39	90.57	9.31 ± 0.12	9.40 ± 0.09	12.96 ± 0.19
TG_{62}	40	95.28	5.20 ± 0.19	5.39 ± 0.01	8.31 ± 0.20
TG_{64}	41	100.07	2.79 ± 0.12	2.81 ± 0.08	5.00 ± 0.15
TG_{66}	42	105.45	1.10 ± 0.07	1.20 ± 0.07	2.24 ± 0.27
TG_{68}	43	111.47	0.17 ± 0.03	0.14 ± 0.03	0.35 ± 0.11
TG_{70}	44	117.91	0.02 ± 0.01	n.d.	n.d.

[a]Abbreviations: FFA, free fatty acids; SQU, squalene; TOC, tocopherol; CHO, cholesterol; DG, diacylglycerol; WE, wax ester; TG, triglyceride; CE, cholesteryl ester.
[b]n.p., Not present. Fatty acid methyl esters (FAMEs) are usually not constituents of nonesterified fish oils.
[c]n.d., Not detected, below detection limit.
[d]c.w.TG_{52}, co-elutes with TG_{52}.

some concentration of the long-chain, mainly polyunsaturated fatty acids, could be achieved.

The chromatogram of the crude salmon oil, D, in Figure 4 is similar to the chromatogram of the sand eel in Figure 1, though the amounts of FFAs and CHO were less. As these components are important in determining the overall quality of a fish oil, the salmon oil was considered to be of a better quality than the sand eel oil. Figure 5 shows the chromatogram of the lipase-catalyzed transesterified salmon oil, E, where the fatty acids in the *sn*-1,3 positions of the TGs had been displaced by decanoic acid. The abscissa scales of Figures 4 and 5 are identical and, therefore, the two chromatograms can be compared directly. Comparison of the two figures indicates that the lipase-treated salmon oil of Figure 5 contained more FFAs than the crude oil, and almost all the original TGs were transesterified. The TGs, TG_{36}, TG_{38}, TG_{40} and TG_{44} refer to the TGs with fatty acids of 16, 18, 20, 22 and 24 carbon atoms in the *sn*-2 position, respectively, and account for approximately 5, 13, 33, 35 and 1%, respectively, of the total oil. The amount of unreacted TGs was approximately 6%, and the amount of TG_{30} was less than 0.5%. This reflects the high 1,3-specificity of the lipase, and indicates that the heavy fatty acids are preferably positioned in the *sn*-2 position. Further analysis would undoubtedly show that these heavy fatty acids primarily account for the highly unsaturated fatty acids like docosahexaenoic acid and eicosapentaenoic acid (20,25).

As shown, the SFC method separated the miscellaneous lipid components, including CHO, TOC and SQU, within a fairly short analysis time. In fact, the five SFC chromatograms exhibit equal or better separation of the

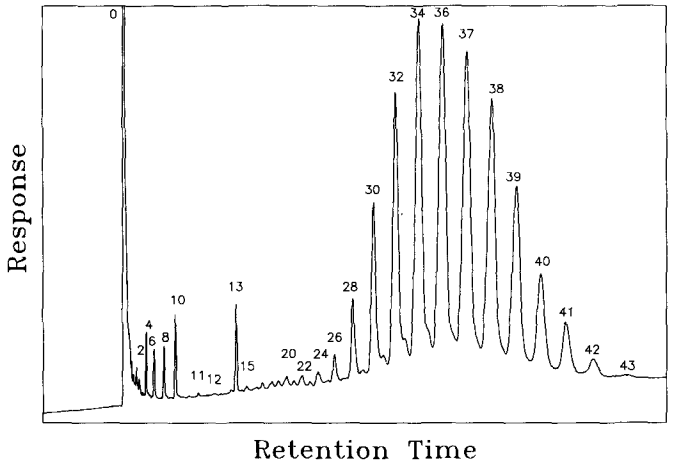

FIG. 1. Supercritical chromatogram of fluid crude sand eel oil A. Conditions as in Method 1 of the text. Marked peaks: (0) solvent (*n*-heptane), others as in Table 1.

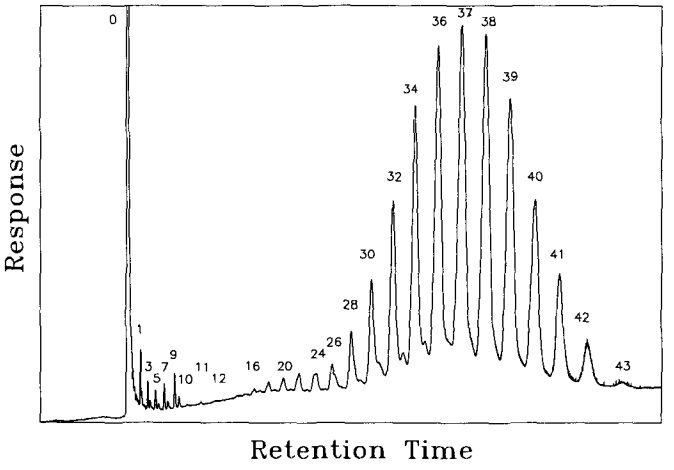

FIG. 3. Supercritical fluid chromatogram of randomly interesterified sand eel oil C. Conditions as in Method 1 of the text. Marked peaks: (0) solvent (*n*-heptane), others as in Table 1.

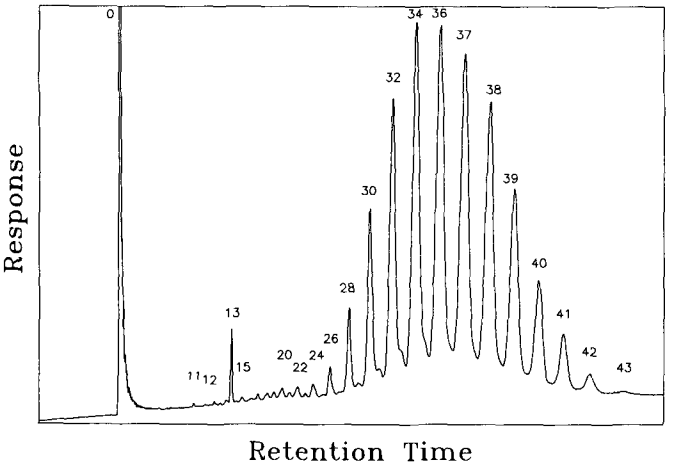

FIG. 2. Supercritical fluid chromatogram of deodorized sand eel oil B. Conditions as in Method 1 of the text. Marked peaks: (0) solvent (*n*-heptane), others as in Table 1.

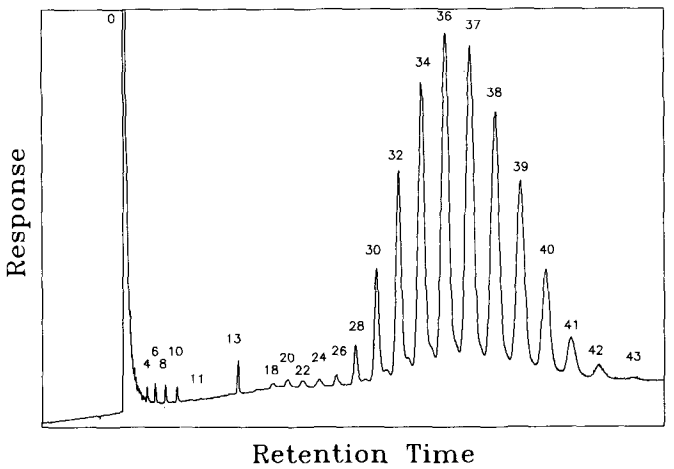

FIG. 4. Supercritical fluid chromatogram of crude salmon oil D. Conditions as in Method 1 of the text. Marked peaks: (0) solvent (*n*-heptane), others as in Table 1.

various compounds than any chromatogram of fish oil TG products published in the literature. A similar separation cannot be obtained by GC without prior treatment of the fish oils, that is, partial or full hydrogenation of the double bonds to prevent polymerization at the high injection and/or elution temperatures needed. Further, HPLC methods do not offer sufficient separation of the fish oil TGs, making SFC superior for this kind of separation.

Sunflower polymers. Figure 6 presents a HPSEC (high-performance size-exclusion chromatography) chromatogram of a sunflower polymer mixture prepared by M.C. Dobarganes. The technique utilizes one 100-Å HPSEC column and one 500-Å HPSEC column connected in series. Further details of the method are described elsewhere (26). The figure shows the separation of the TG monomers, dimers and polymers of the mixture obtained in an analysis time of 20 min.

The corresponding SFC chromatogram of the sunflower polymer mixture is shown in Figure 7 for an elution time

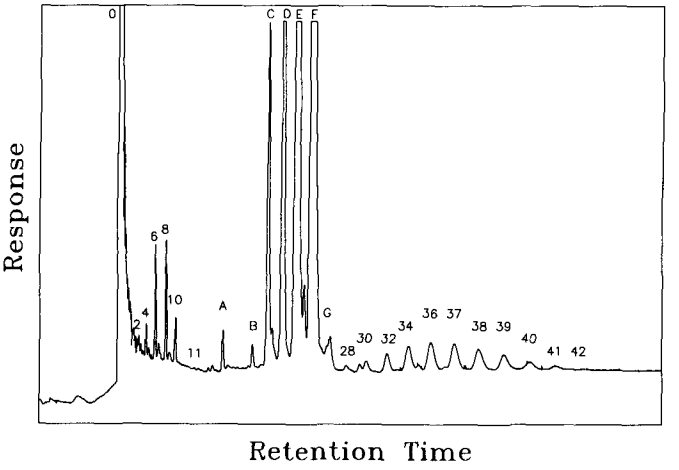

FIG. 5. Supercritical fluid chromatogram of lipase-catalyzed transesterified salmon oil E. Conditions as in Method 1 of the text. Marked peaks: (0) solvent (*n*-heptane), (A) TG_{30}, (B) TG_{34}, (C) TG_{36}, (D) TG_{38}, (E) TG_{40}, (F) TG_{42}, (G) TG_{44}, others as in Table 1. TG, triglyceride.

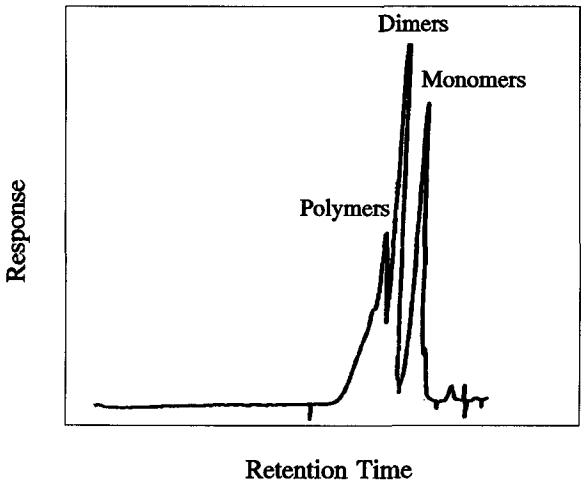

FIG. 6. High-performance size exclusion chromatogram of sunflower oil polymer mixture F. Conditions as described in the text and by Dobarganes *et al.* (Ref. 26).

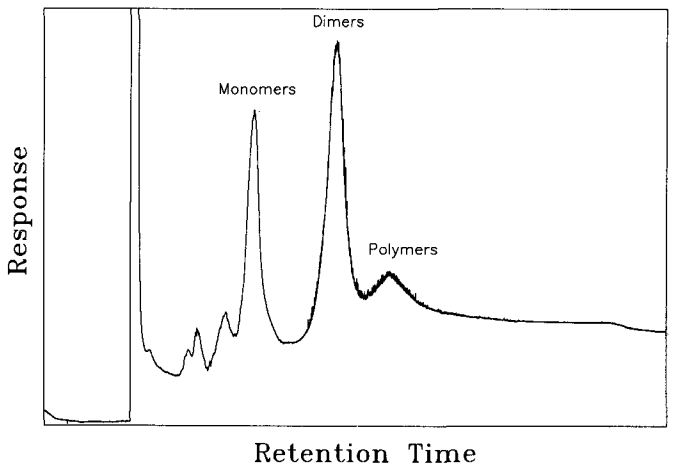

FIG. 7. Supercritical fluid chromatogram of the sunflower oil polymer mixture F. Conditions as described in Method 2 of the text.

of 200 min. The chromatogram displays a somewhat better separation of the various groups than that achieved by the HPSEC method, although the TG polymers (probably those heavier than the trimers) eluted as a broad tailing of the polymer peak. Whether it is expressive of heavier TG polymer components or simply ordinary peak tailing is difficult to say when comparing to the HPSEC chromatogram, where the heavy polymers were eluted together on the front of the polymer peak. The addition of a polar entrainer, such as methanol, to the CO_2 mobile phase would probably lead to significantly shorter analysis times for the SFC method. Unfortunately, the addition of entrainers is not possible with the current chromatograph (due to lack of an extra pump and use of the FID). The inadequacy of CO_2 is indicated by the extensive spiking of the dimer and polymer peaks of Figure 7, which would also be removed by the polar entrainer.

Direct comparison of the two methods for detection and determination of polymeric artifacts in natural oils, including fish oils, reveals an advantage of the SFC method in providing equal or better separation on only one capillary column compared to the HPSEC method (27,28), which uses two to three expensive columns connected in series. The HPSEC analysis time of 25–50 min (27,28) could undoubtedly also be approached by SFC if the mobile phase is modified with a polar entrainer.

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