Journal of Chromatography, 648 (1993) 221–232
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 345

Flame ionization detector responses to ethyl esters of sand eel (Ammodytes lancea) fish oil compared for different gas and supercritical fluid chromatographic systems

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(First received December 3rd, 1992; revised manuscript received April 19th, 1993)

ABSTRACT

A fish oil fatty acid ethyl ester mixture derived from the sand eel (Ammodytes lancea) has been analysed using various capillary gas chromatography (GC) and supercritical fluid chromatography (SFC) methods and 39 components including cholesterol have been identified. The results of the analytical SFC and GC experiments are compared showing a good reproducibility within methods and a fair agreement between methods. The advantages and disadvantages of the employment of manual and autosampler split injection, splitless injection, and cold on-column injection in GC as well as the use of polar and non-polar columns in GC and SFC are discussed.

INTRODUCTION

The annual world production of marine oil is about 1.5 billion kg [1]. The predominant part of it is used for the production of margarines and shortenings used to make pastries, bread, cakes, creams, and margarines and emulsifiers for human consumption. Other uses include feed for livestock, pets and fish farming, and industrial products like soaps, fuel oils, lubricants, greases, linoleum, protective coatings, etc. Fish is the most important source of long-chain ω-3 unsaturated fatty acids, specially the polyunsaturated EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), which are the most important and valuable of the ω-3 fatty acids. Recent reports indicate that ω-3 fatty acids may have medical effects in treatment of rheumatoid arthritis [2], heart diseases and strokes [3], atherosclerosis by lowering the cholesterol absorption [4], and cancer diseases in the colon region [5], and the use in the pharmaceutical industry may be the most important use of fish oils in the future.

Gas chromatography (GC) is a well-established method for the analysis and separation of fish oil esters [6–33]. Other methods, like high-performance liquid chromatography (HPLC) [18,21,28,29,34,35], and lately supercritical fluid chromatography (SFC) [27,36,37] have also been employed, but the method preferred for the analysis of fish oil esters is the capillary gas liquid chromatography (GLC) method using columns of moderate to high polarity. A standard method

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0021-9673/93/$06.00

for determination of fatty acid compositions of fish oil by GLC utilizing the split injection and the flame ionization detection techniques has been proposed [38]. This technique has been used by several authors, see, for instance, refs. 11, 23, and 27. Other choices may include employment of cold on-column injection [15,18,31] or mass spectrometric (MS) detection [29,30,33].

The primary use of HPLC and SFC for the examination of fish oil esters has focused on the separation and fractionation in a semi-preparative or preparative scale, followed by a GLC analysis of the fractions obtained. Recently, attempts to use HPLC [35] and SFC [36] as analytical tools for the fatty acid composition determination of fish oil ester mixtures have been published. However, J.M. Beebe *et al.* [35], using the HPLC method, report only the separation of 7 major compounds combined with a rather poor resolution, while Görner and Perrut [36], using the packed column SFC method, report the separation of 6 (2×3) methyl and ethyl esters present in fish oil. The main purpose of our study is to compare the well-established capillary GLC method with the rather untested capillary SFC method in the analysis of ethyl esters from fish oil using various columns. Although HPLC and SFC methods have less resolution when compared to GLC, they have the advantage of being ideal for on-line analysis coupling to processes like preparative HPLC or supercritical fluid extraction (SFE), where the automatic transfer of a representative sample to on-line coupled GC often is a major problem [39]. The results of this study will show that capillary SFC in many cases is sufficient as the analytical tool, when coupled directly to a continuous SFE process.

Analysis of the fatty acid composition of the sand eel or sand launce (Ammodytes lancea) has previously been presented by Laakso *et al.* [28] and Langholz *et al.* [25], who identified 19 and 14 components, respectively. The second aim of this work is to obtain more information about the fatty acid composition of the sand eel. We have analysed the composition of a sand eel fatty acid ethyl ester mixture using different capillary GC methods including various columns and injection techniques, and 39 components including cholesterol have been identified by retention time comparison. When comparing the fatty acid compositions of this work with compositions obtained by others, one should observe that the composition changes every year and from season to season depending on the feed the fish eat [10].

To sum up, the primary goal of this work was to compare the SFC technique with the GC technique using different columns. Additionally, more detailed information on the sand eel fatty acid composition was obtained, and reliability figures for GC analysis by various injection techniques and stationary phases were compared.

EXPERIMENTAL

Materials

Standards of fatty acid methyl esters were purchased from Sigma (St. Louis, MO, USA). Standard mixtures of methyl esters were purchased from Nu-Chek-Prep (Elysian, MN, USA). A qualitative standard mixture of fish oil methyl esters was from Larodan Fine Chemicals (Malmö, Sweden). *n*-Heptane LiChrosolv used as solvent for the fatty acid esters was obtained from Merck (Darmstadt, Germany). Helium, hydrogen, and atmospheric air were supplied by Hede Nielsen (Ballerup, Denmark). The stated purities are $>99.996\%$ of helium and $>99.8\%$ of hydrogen. Carbon dioxide was supplied by Linde (München, Germany) with a stated purity of $>99.995\%$, and sand eel ethyl esters were supplied by Grindsted Products (Århus, Denmark) with a stated purity of 98%. All materials were used without further purification.

GC methods

Six different gas chromatographic analyses were carried out under various experimental conditions.

Method 1

A Carlo Erba SFC-3000 Instrument (Carlo Erba Instruments, Milan, Italy) was set up as a gas chromatograph. The chromatograph was equipped with a flame ionization detector and a manual cold on-column injection port connected to a retention gap ($1.5\ m \times 0.32$ mm) and a

terephthalic acid (TPA) modified polyethylene glycol HP-FFAP (Hewlett-Packard, Avondale, PA, USA) fused-silica capillary column (25 m × 0.2 mm × 0.33 μm). 1 μl of a 0.05% (w/w) solution of ethyl esters in *n*-heptane was injected. The detector temperature was 270°C. The initial oven temperature of 95°C was held for 2 min, then increased at a rate of 3°C/min to 170°C followed by an increase of 1°C/min to 210°C, where it was held constant for 52 min. Helium carrier gas flow was set at 26 cm/s and the helium make-up gas flow at 30 ml/min. The hydrogen and air gas flows for the FID were 30 ml/min and 230 ml/min, respectively, and secondary cooling pressure (nitrogen or air) of the injection system was 500 kPa. Integration and control of the chromatographic run were carried out via a personal computer with MAXIMA chromatography software (Dynamic Solutions, Ventura, CA, USA).

Method 2

An HP 5880A gas chromatograph was equipped with a manual capillary injection port and a flame ionization detector was used. The split injection mode with a split ratio of 1:100 and a TPA modified polyethylene glycol HP-FFAP fused-silica capillary column (25 m × 0.2 mm × 0.33 μm) was employed. The fast injected volume was 2 μl of a 2.7% mixture of ethyl esters in *n*-heptane. The injection and detection temperatures were 250°C. The initial oven temperature of 140°C was held for 15 min, then increased at a rate of 3°C/min to 170°C followed by an increase of 1°C/min to 240°C, where it was held constant for 10 min. The linear velocity of the helium carrier gas was 53 cm/s. The hydrogen and air flow rates for the FID were 40 ml/min and 500 ml/min, respectively. Integration and control of the chromatographic run were carried out by an HP 5880A Series GC Terminal.

Method 3

The chromatographic runs of this method were carried out as described in Method 2, except for the use of the splitless injection mode with a fast injected volume of 1 μl of a 1.0% mixture of ethyl esters with *n*-heptane, and a different oven temperature program. The initial oven temperature of 80°C was held for 1 min, then increased at a rate of 5°C/min to 200°C followed by an increase of 3°C/min to 230°C, where it was held constant for 15 min.

Method 4

The chromatographic runs of this method were carried out as described in Method 2, except for the use of a dimethylpolysiloxane HP-1 fused-silica capillary column (12 m × 0.2 mm × 0.33 μm), a fast injected volume of 1 μl of a 2.7% solution of ethyl esters in *n*-heptane, and a different oven temperature program. The initial oven temperature of 140°C was held for 1 min, then increased at a rate of 3°C/min to 186°C followed by an increase of 4.5°C/min to 270°C, where it was held constant for 1 min.

Method 5

An HP 5890 gas chromatograph equipped with a capillary injection port, a HP-7673A autosampler and a flame ionization detector was used. The column was a 68% cyanopropylphenylpolysiloxane SP-2330 (Supelco, Bellefonte, PA, USA) fused-silica capillary column (30 m × 0.32 mm × 0.2 μm). Injection in split mode with a split ratio of 1:50 was used. The injected volume was 0.2 μl of a 4.6% solution of ethyl esters in *n*-heptane. The injection and detection temperatures were 250°C and 240°C, respectively. The initial oven temperature of 140°C was immediately raised at a rate of 3°C/min to 200°C, held for 1 min, and further raised at 3°C/min to 220°C, where it was held for 9 min. The helium carrier gas flow was 21 cm/s, nitrogen make-up gas flow was set at 25 ml/min, and hydrogen and air supplies for the FID were set at 33 ml/min and 500 ml/min, respectively. Integration was carried out by an HP-3396A integrator and the data were transferred to a computer by HP-3393A/3396A contributed file server software.

Method 6

This method differed from Method 5 in the choice of column, which was a polyethylene glycol Omegawax 320 (Supelco) fused-silica capillary column (30 m × 0.32 mm × 0.25 μm).

The split ratio was 1:30, and the injected volume of a 4.1% mixture of ethyl esters in *n*-heptane was 0.2 μl. The temperature program began with an oven temperature of 160°C, immediately followed by an increase at 3°C/min to 200°C, where it was held for 1 min, followed by an increase at 3°C/min to 220°C, where it was held constant for 12 min. The helium carrier gas flow was 30 cm/s, while the other conditions were as for Method 5.

SFC methods

Three different supercritical fluid chromatographic analyses were carried out at different conditions. A Carlo Erba SFC-3000 system equipped with a flame ionization detector and a pneumatic Valco valve with a 0.2 μl sample loop was used in all SFC experiments. The injection temperature was 60°C and the detector temperature was 300°C. Hydrogen and air gas pressures for the FID were 55 kPa and 100 kPa, respectively. Carbon dioxide was employed as the carrier gas. The pump cylinder was thermostatted by circulation of ethylene glycol from a Hetofrig CB 12 cooling bath (Heto Lab Equipment, Birkerød, Denmark) at −5°C to ensure carbon dioxide flow rate reproducibility. The pressure drop over the chromatographic system was obtained by integral restrictors made of uncoated fused-silica tubing by the method of Guthrie and Schwartz [40] and connected to the chromatographic column. Integration and control of the chromatographic run were carried out via a personal computer with MAXIMA chromatography software.

Method 7

A 50% cyanopropylphenyl–methylpolysiloxane DB-225 (J&W Scientific, Folsom, CA, USA) fused-silica capillary column (20 m × 0.1 mm × 0.1 μm) was employed. The injection time was 0.2 s. The concentration of the samples injected was 0.5% of ethyl esters in *n*-heptane. The chromatographic runs were performed isothermally at 140°C. The initial carbon dioxide density of 0.15 g/ml was held for 25 min, then increased at a rate of 0.001 g/ml/min to 0.225 g/ml followed by an increase of 0.002 g/ml/min to 0.355 g/ml, where it was held constant for 30 min. The initial and final carbon dioxide pressures were 9.8 MPa and 19.3 MPa, respectively, and the initial linear velocity was 1.7 cm/s.

Method 8

A dimethylpolysiloxane CP-Sil 5 CB (Chrompack Instruments, Greve, Denmark) fused-silica capillary column (20 m × 0.05 mm × 0.2 μm) was employed. The injection time was 0.2 s. The concentration of the samples injected was 2.7% of ethyl esters in *n*-heptane. The chromatographic runs were performed isothermally at 70°C. The initial carbon dioxide density of 0.15 g/ml was held for 20 min, then increased at a rate of 0.011 g/ml/min to 0.22 g/ml, followed by an increase of 0.001 g/ml/min to 0.455 g/ml, and then followed by an increase of 0.003 g/ml/min to 0.63 g/ml, where it was held constant for 10 min. The initial and final carbon dioxide pressures were 7.2 MPa and 19.6 MPa, respectively, and the initial linear velocity was 3.6 cm/s.

Method 9

A 5% phenyl–methylpolysiloxane DB-5 (J&W Scientific) fused-silica capillary column (20 m × 0.1 mm × 0.4 μm) was employed. The injection time was 0.1 s. The concentration of the samples injected was 2.5% of ethyl esters in *n*-heptane. The chromatographic runs were performed isothermally at 60°C. The initial carbon dioxide density of 0.65 g/ml was held for 40 min and then increased at a rate of 0.001 g/ml/min to 0.83 g/ml, where it was held constant for 60 min. The initial and final carbon dioxide pressures were 16.4 MPa and 29.9 MPa, respectively, and the initial linear velocity was 1.9 cm/s.

Sample preparation and peak identification

The fish oil ethyl esters were transesterified to the corresponding methyl esters by a base-catalyzed transesterification followed by a boron trifluoride-catalyzed esterification according to the AOCS method Ce 1b-89 [38]. In the transesterification step the molar concentration of methanol was about 300 times that of the ethyl esters, thus minimizing the occurrence of un-

changed ethyl esters in the methyl ester chromatograms.

The methyl ester standards were dissolved in *n*-heptane to a concentration of each component of 0.001% (w/w) for the cold on-column injections, of 0.14% for the GC split injections, and 0.026% for the splitless injections. The fish oil methyl ester standard mixture was dissolved in *n*-heptane to a total concentration of 2.0% for use in the autosampler split injections. For the SFC injections the concentration of each component of the methyl ester standards was 0.14% on the CP-Sil 5 CB and the DB-5 columns, and 0.05% on the DB-225 column.

Comparison of the retention times found in these chromatograms with those of commercially available standard mixtures (*cf.* Materials) formed the basis for peak identification of the ethyl esters derived from the fish oil.

RESULTS AND DISCUSSION

GC

The 39 components found and the experimental peak area% of the gas chromatographic analyses are presented in Table I. Each chromatographic experiment was performed three times, except Method 1 which was performed six times. For each of the six methods the repeatability was quite good. The relative standard deviation of major compounds (>1 area%) was occasionally as large as 2.5%, but generally it was less than 1%, while for minor compounds (<1 area%) in a few instances it was as large as 10%, but usually less than 3%. The elution sequence shown in Table I is as for the FFAP column.

A comparison of the two methods using autosampler split injection and polar columns, methods 5 and 6 in Table I, displays good agreement except for a few compounds. The temperature program used in Method 6 was set so that the components $C_{10:0}$ and $C_{12:0}$ eluted together with the solvent. The amount of $C_{18:1\omega7}$ clearly deviates between the two methods and to some extent also the amounts of $C_{22:6\omega3}$ and $C_{24:1\omega9}$ and their total sum. The amounts of $C_{22:1\omega11}$ and $C_{22:1\omega9}$ differ due to poor separation using both methods, but the total sum of $C_{22:1}$ of the two methods is in balance.

Examining the results of Method 2 using the manual split injection and the polar FFAP column, a rather good agreement with the results of methods 5 and 6 can be seen. The components $C_{16:2}$, $C_{17:0}$, $C_{16:3}$, $C_{18:3\omega6}$, $C_{20:1\omega9}$ and $C_{22:4\omega6}$ show significant differences in peak area%. The differences of the components $C_{16:2}$, $C_{17:0}$ and $C_{16:3}$ could be due to the presence of phytanic acid ethyl ester, which elutes close to or together with $C_{17:0}$ on polyglycol columns [41]. The difference in area% between the components $C_{22:1\omega11}$ and $C_{22:1\omega9}$ is caused by peak overlapping and probably the presence of other $C_{22:1}$ isomers, while the difference between $C_{22:6\omega3}$ and $C_{24:1\omega9}$ is mainly caused by the peak overlapping when using polyglycol columns, methods 2 and 6 [17].

The cold on-column injection operation with the FFAP column, Method 1, demonstrates significant differences in peak area% for the major light and heavy compounds, indicating some kind of discrimination compared to Method 2. Peak area% of light compounds like $C_{16:0}$ and $C_{16:1\omega7}$ are greater than those of Method 2, while of heavy compounds like $C_{20:5\omega3}$ and $C_{22:6\omega3}/C_{24:1\omega9}$ they are less. Traitler [42] has noted that when using the cold on-column injection technique, discrimination of low-volatility compounds is much less pronounced than in heated injection port systems, which is directly contrary to what we experience. Even though on-column injection is considered the method of choice for optimal quantitative analysis of complicated mixtures [43,44], careful optimization of the splitting injector will lead to accurate quantitation [45]. As we find good agreement between results by the three split injection methods, and as for the cold on-column injection method we find the same low relative standard deviations as the other methods, we must assume a systematic, yet unexplained, error of either the cold on-column injection method or of the three split injection methods. A chromatogram of the ethyl ester mixture using Method 1 is presented in Fig. 1. The figure shows the good separation of the isomers $C_{18:1\omega9}$ and $C_{18:1\omega7}$, $C_{22:1\omega11}$ and $C_{22:1\omega9}$, and a partial peak overlap between $C_{22:6\omega3}$ and $C_{24:1\omega9}$ which might be expected [17]. The sepa-

TABLE I

GAS CHROMATOGRAPHIC PEAK AREAS OF THE SAND EEL ETHYL ESTER MIXTURE USING VARIOUS COLUMNS AND INJECTION TECHNIQUES

The experimental conditions are given in the text. The relative standard deviations of each of the six methods are generally less than 1% for major compounds (> 1 area%) and less than 3% for minor compounds, but they may be as large as 2.5% and 10%, respectively. n.a.: not analyzed for, elutes before the integration start. n.d.: not detected.

Method no.	1	2	3	4	5	6
Column	FFAP	FFAP	FFAP	HP-1	SP-2330	SP-320
Injection	on-column, manual	split, manual	splitless, manual	split, manual	split, auto	split, auto
Component	Peak areas (%)					
$C_{10:0}$	0.3	0.3	0.3	0.3	0.3	n.a.
$C_{12:0}$	0.2	0.2	0.2	0.2	0.2	n.a.
$C_{14:0}$	7.1	6.3	6.8	6.2	6.2	6.1
$C_{14:1\omega5}$	0.5	0.3	0.3	n.d.	0.2	0.3
$C_{15:0}$	0.5	0.5	0.5	0.5	0.5	0.5
$C_{15:1\omega5}$	0.1	0.2	0.2	n.d.	0.1	n.d.
$C_{16:0}$	17.9	16.5	17.1	16.3	16.6	16.3
$C_{16:1\omega7}$	12.1	11.1	11.9	11.9	10.9	11.2
$C_{16:2}$	1.4	1.4	1.4	0.6	0.8	0.6
$C_{17:0}$	0.2	0.2	0.2	0.2	0.9	0.4
$C_{16:3}$	0.6	0.6	0.7	0.9	(0.0)[a]	0.4
$C_{16:4\omega3}$	0.8	0.8	0.8	(0.0)[b]	0.9	0.8
$C_{18:0}$	2.1	2.2	2.2	2.2	2.4	2.3
$C_{18:1\omega9}$	10.1	10.0	10.3	9.9	10.2	10.3
$C_{18:1\omega7}$	2.3	2.3	2.3	2.5	2.9	2.4
$C_{18:2\omega6}$	2.9	3.0	3.1	4.3	3.0	3.0
$C_{19:0}$	0.1	0.2	0.2	n.d.	n.d.	n.d.
$C_{18:3\omega6}$	0.4	0.4	0.4	(0.0)[c]	0.2	0.2
$C_{18:3\omega3}$	1.3	1.4	1.4	(0.0)[c]	1.4	1.5
$C_{18:4\omega3}$	3.8	4.0	4.1	4.4	4.2	4.0
$C_{19:1\omega9}$	0.1	0.1	0.1	n.d.	0.1	0.2
$C_{20:0}$	0.2	0.1	0.2	0.1	0.2	0.1
$C_{20:1\omega9}$	4.2	4.4	4.3	4.9	4.8	4.9
$C_{20:2\omega6}$	0.3	0.4	0.3	0.3	0.3	0.3
$C_{21:0}$	0.1	0.1	0.1	n.d.	n.d.	0.0
$C_{20:3\omega6}$	n.d.	n.d.	n.d.	n.d.	0.1	0.1
$C_{20:4\omega6}$	0.3	0.3	0.3	n.d.	0.4	0.4
$C_{20:3\omega3}$	0.1	0.1	0.1	(0.0)[d]	(0.0)[e]	0.2
$C_{20:4\omega3}$	0.7	0.7	0.7	1.2	0.8	0.8
$C_{20:5\omega3}$	10.3	11.2	10.7	11.1	11.1	11.0
$C_{22:1\omega11}$	6.6	7.0	6.3	7.5	6.4	7.4
$C_{22:1\omega9}$	1.1	0.7	0.7	0.2	1.9	0.9
$C_{21:5\omega3}$	0.4	0.5	0.5	0.5	0.5	0.6
$C_{22:4\omega6}$	0.1	0.1	0.1	n.d.	n.d.	0.3
$C_{22:5\omega6}$	0.1	0.1	0.1	n.d.	(0.0)[f]	0.1
$C_{22:5\omega3}$	0.5	0.7	0.6	0.8	0.7	0.7
$C_{22:6\omega3}$	9.2	11.8	9.8	11.3	10.7	11.1
$C_{24:1\omega9}$	0.8	(0.0)[g]	0.6	0.9	0.7	0.9
$C_{27}H_{44}OH$	n.d.	n.d.	n.d.	0.8	n.d.	n.d.
Identified	95.9	96.5	94.6	94.2	94.5	93.4

[a] Possible peak coincidence with component $C_{17:0}$.
[b] Peak coincidence with component $C_{16:3}$.
[c] Peak coincidence with component $C_{18:2\omega6}$.
[d] Peak coincidence with component $C_{20:2\omega6}$.
[e] Peak coincidence with component $C_{20:4\omega6}$.
[f] Possible peak coincidence with component $C_{21:5\omega3}$.
[g] Peak coincidence with component $C_{22:6\omega3}$.

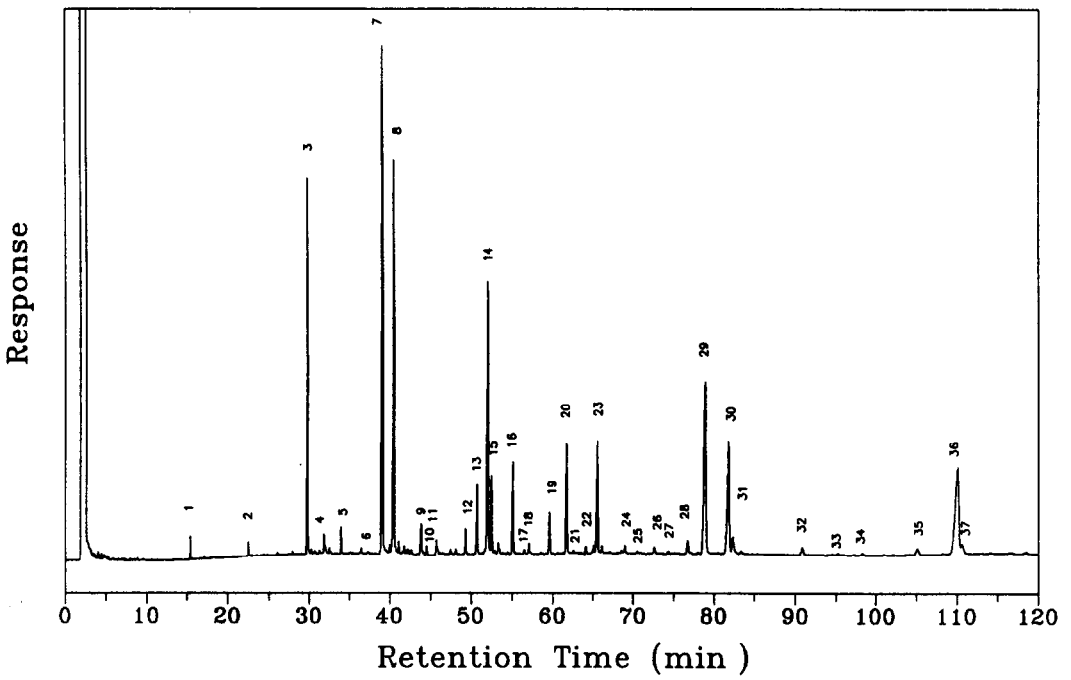

Fig. 1. GLC of sand eel fatty acid ethyl ester mixture. Conditions: column, HP-FFAP (25 m × 0.2 mm × 0.33 μm); injection technique, cold on-column; conditions moreover as in Method 1 of the text. Peaks: 1 = $C_{10:0}$; 2 = $C_{12:0}$; 3 = $C_{14:0}$; 4 = $C_{14:1\omega5}$; 5 = $C_{15:0}$; 6 = $C_{15:1\omega5}$; 7 = $C_{16:0}$; 8 = $C_{16:1\omega7}$; 9 = $C_{16:2}$; 10 = $C_{17:0}$; 11 = $C_{16:3}$; 12 = $C_{16:4\omega3}$; 13 = $C_{18:0}$; 14 = $C_{18:1\omega9}$; 15 = $C_{18:1\omega7}$; 16 = $C_{18:2\omega6}$; 17 = $C_{19:0}$; 18 = $C_{18:3\omega6}$; 19 = $C_{18:3\omega3}$; 20 = $C_{18:4\omega3}$; 21 = $C_{19:1\omega9}$; 22 = $C_{20:0}$; 23 = $C_{20:1\omega9}$; 24 = $C_{20:2\omega6}$; 25 = $C_{21:0}$; 26 = $C_{20:4\omega6}$; 27 = $C_{20:3\omega3}$; 28 = $C_{20:4\omega3}$; 29 = $C_{20:5\omega3}$; 30 = $C_{22:1\omega11}$; 31 = $C_{22:1\omega9}$; 32 = $C_{21:5\omega3}$; 33 = $C_{22:4\omega6}$; 34 = $C_{22:5\omega6}$; 35 = $C_{22:5\omega3}$; 36 = $C_{22:6\omega3}$; 37 = $C_{24:1\omega9}$; 38 = $C_{27}H_{44}OH$.

ration of component $C_{22:6\omega3}$ and $C_{24:1\omega9}$ using Method 2, however, is much poorer, possibly due to the different temperature program.

Method 3 involves the application of a splitless injection with the FFAP column. The use of the splitless injection method is, of course, not the suitable method for the analysis of fatty acid esters, but is usually used for detection and determination of components present in trace amounts [43]. Still, it is included here for reasons of comparison, and when compared to Method 2 it exhibits significant differences in peak area% for the major light and heavy compounds, indicating an expected discrimination of the low-volatility compounds.

The employment of the non-polar HP-1 column and the manual split injection, Method 4, exhibited the expected pattern of peak coincidences between compounds of different degree of unsaturation [17]. The results of Method 4 are in rather good agreement with the results of Method 2 except for components of C_{16} chain lengths and $C_{20:4\omega3}$, but using the HP-1 column it is possible to determine the amount of cholesterol ($C_{27}H_{44}OH$) of the ester mixture in the same chromatographic run. This was not possible with the more polar columns. A chromatogram of the ethyl ester mixture using Method 4 is presented in Fig. 2. The figure shows the peak coincidence of several compounds, indicative of the unsuitability of a non-polar column for the separation of a complex mixture of unsaturated fatty acid ethyl esters. For an uncomplicated mixture, however, this type of column may have some merit as it allows determination of cholesterol simultaneously with the fatty acid ester composition.

All the gas chromatographic experiments showed a good reproducibility and many compounds have been identified, corresponding to

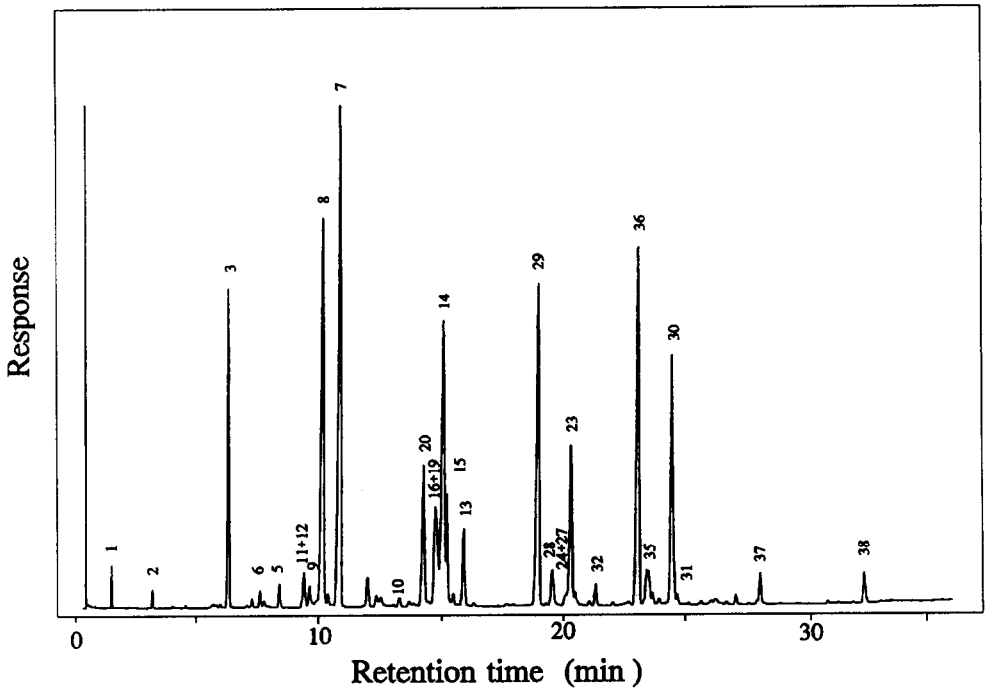

Fig. 2. GLC of sand eel fatty acid ethyl ester mixture. Conditions: column, HP-1 (12 m × 0.2 mm × 0.33 μm); injection technique, manual split; conditions moreover as in Method 4 of the text. Peaks as in Fig. 1.

93–97% of the integrated area. Unidentified components account primarily for various isomers of $C_{16:1}$, $C_{18:1}$, $C_{20:1}$ and $C_{22:1}$.

SFC

The experimental peak area% obtained from the supercritical fluid chromatographic analyses are presented in Table II. Each chromatographic experiment was performed three times. The relative standard deviation for the three methods concerned may be as large as 10%, but generally it is less than 4%. Chromatograms of the methods 6, 7, and 8 are given in Figs. 3, 4, and 5, respectively. The elution sequence shown in Table II is as for the DB-225 column. The fatty acid structures ($\omega - x$) have not been given for some components in Table II due to the peak coincidence of some of the various isomers.

Examining Table II and Figs. 3–5, it can be observed that Method 7, using the polar DB-225 column, separates the ester mixture better than the two other methods, 8 and 9, using the non-polar columns CP Sil 5 and DB-5. The better separation observed of Method 8 using the CP Sil 5 column compared to Method 9 using the DB-5 column is due to the smaller diameter of the CP Sil 5 column, which causes the number of theoretical plates to be higher. Differences in composition obtained by the three methods occurred for some major compounds like $C_{16:0}$, $C_{16:1}$ and $C_{22:6}$, and for peak coincidence of the non-polar columns between polyunsaturated compounds of C_{16} and C_{18} chain lengths. All in all, the optimum choice of column seems to be a polar column like the DB-225 or similar with a diameter of 0.05 mm or less to increase the number of theoretical plates compared to the one employed in this work.

All the supercritical fluid chromatographic experiments showed a good reproducibility with mean relative standard deviations of the identified compounds of approximately 3%. Analysis of cholesterol with all the SFC methods is possible. 95–98% of the integrated area has been identified and unidentified components possibly account for various higher chain length esters.

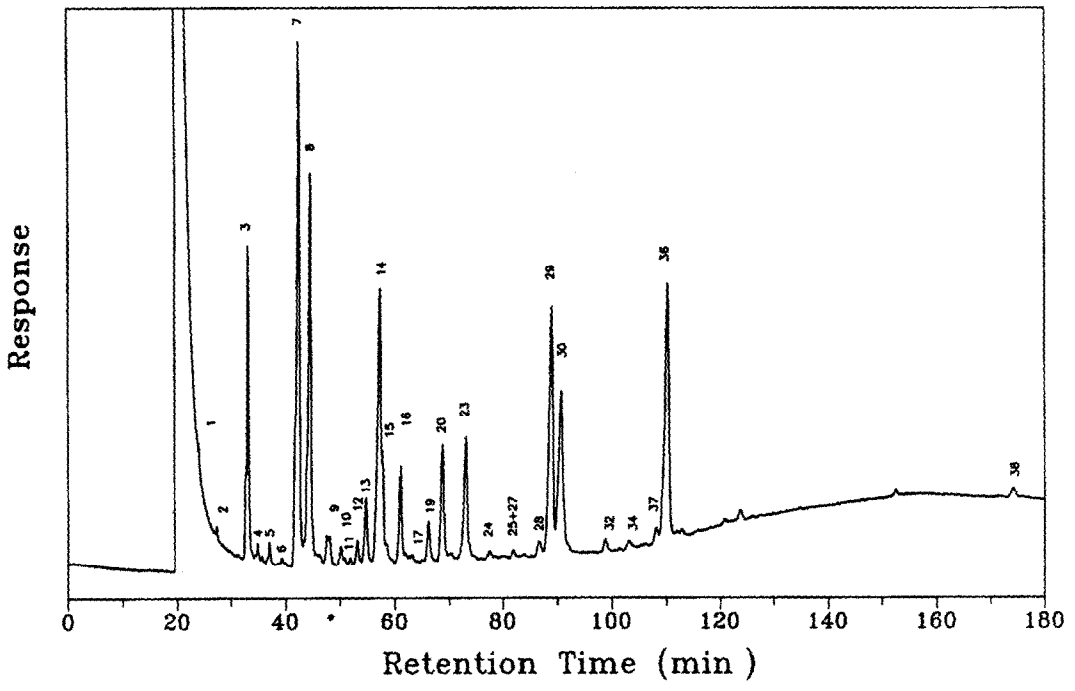

Fig. 3. SFC of sand eel fatty acid ethyl ester mixture. Conditions: column, DB-225 (20 m × 0.1 mm × 0.1 μm); conditions moreover as in Method 7 of the text. Peaks as in Fig. 1.

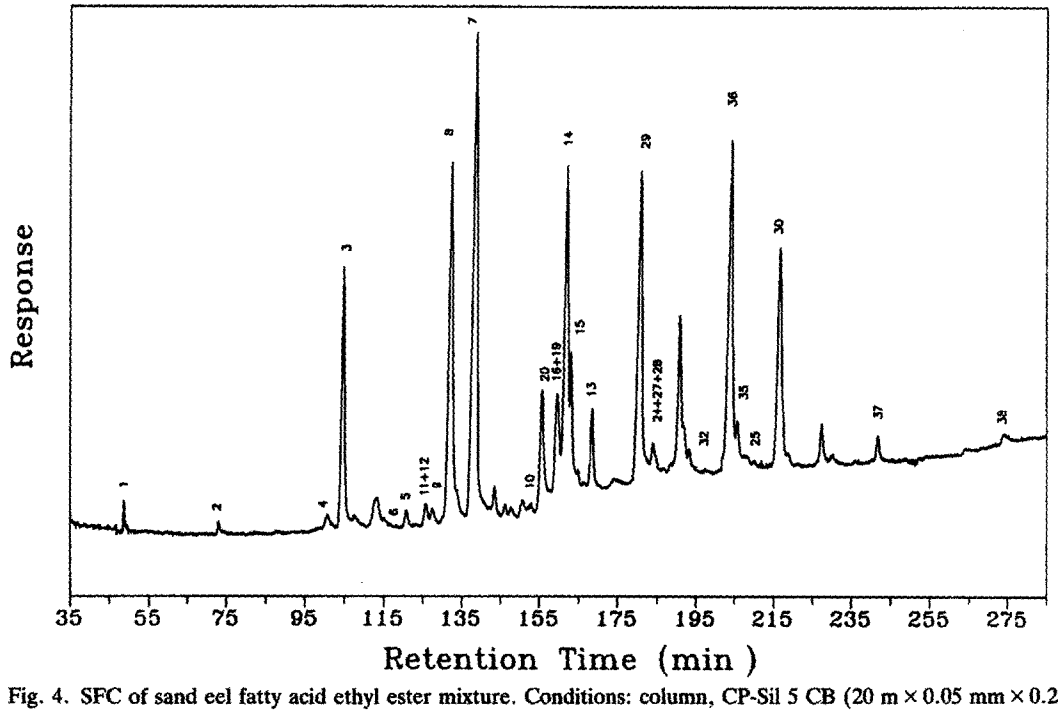

Fig. 4. SFC of sand eel fatty acid ethyl ester mixture. Conditions: column, CP-Sil 5 CB (20 m × 0.05 mm × 0.2 μm); conditions moreover as in Method 8 of the text. Peaks as in Fig. 1.

TABLE II

SUPERCRITICAL FLUID CHROMATOGRAPHIC PEAK AREAS OF THE SAND EEL ETHYL ESTER MIXTURE USING VARIOUS COLUMNS

The experimental conditions are given in the text. The relative standard deviations of each of the three methods are generally less than 4% of each compound, but they may be as large as 10%. n.d.: not detected.

Method no.	7	8	9
Column	DB-225	CP Sil 5	DB-5
Component	Peak areas (%)		
$C_{10:0}$	0.1	0.3	0.3
$C_{12:0}$	0.1	0.2	0.2
$C_{14:0}$	6.2	6.4	6.1
$C_{14:1\omega5}$	0.4	0.4	n.d.
$C_{15:0}$	0.4	0.4	0.5
$C_{15:1\omega5}$	0.2	0.2	0.1
$C_{16:0}$	17.1	17.4	16.2
$C_{16:1}$	12.6	11.8	11.5
$C_{16:2}$	1.3	0.3	1.2
$C_{16:3}$	0.6	0.5	(0.0)[a]
$C_{17:0}$	0.1	0.7	0.4
$C_{16:4\omega3}$	0.6	(0.0)[b]	(0.0)[a]
$C_{18:0}$	1.9	2.2	2.2
$C_{18:1\omega9}$	9.4	9.6	16.6
$C_{18:1\omega7}$	2.6	4.2	(0.0)[c]
$C_{18:2\omega6}$	3.0	3.9	(0.0)[c]
$C_{19:0}$	0.3	n.d.	n.d.
$C_{18:3}$	1.2	(0.0)[d]	4.1
$C_{18:4\omega3}$	4.1	3.8	(0.0)[e]
$C_{20:1}$	4.6	5.2	5.2
$C_{20:2\omega6}$	0.2	1.4	1.0
$C_{20:3}$	0.2	(0.0)[f]	(0.0)[f]
$C_{20:4}$	0.5	(0.0)[f]	(0.0)[f]
$C_{21:0}$	n.d.	0.2	n.d.
$C_{20:5\omega3}$	10.4	10.2	11.0
$C_{22:1}$	8.1	7.6	8.1
$C_{21:5\omega3}$	0.5	0.7	1.1
$C_{22:5}$	0.3	1.3	0.4
$C_{24:1}$	0.5	0.7	0.9
$C_{22:6\omega3}$	11.4	10.5	12.2
$C_{27}H_{44}OH$	0.4	0.4	0.7
Identified	98.4	95.2	96.7

[a] Peak coincidence with component $C_{16:2}$.
[b] Peak coincidence with component $C_{16:3}$.
[c] Peak coincidence with component $C_{18:1\omega9}$.
[d] Peak coincidence with component $C_{18:2}$.
[e] Peak coincidence with component $C_{18:3}$.
[f] Peak coincidence with component $C_{20:2}$.

Comparisons

In this study, the composition of a sand eel fatty acid ethyl ester mixture has been analysed using gas and supercritical fluid chromatography. A comparison of the best SFC method of this work for separation of long chain fatty acid esters, Method 7, with the GC methods 5 and 6, generally displays a good concordance except for a few compounds, especially $C_{16:1}$, $C_{18:1\omega9}$, $C_{20:4}$ and $C_{22:5}$. The results of $C_{10:0}$ and $C_{12:0}$ of Method 7 are less satisfactory due to their presence in the solvent peak. An investigation of Figs. 1 and 3 shows almost the same peak elution order of the FFAP and the DB-225 columns, while Figs. 2, 4 and 5 show a similar pattern of the non-polar columns. The injection technique of the SFC experiments may cause some discrimination of heavy compounds, but the alternatives are few because of the high pressure involved [46].

Not all of the GC and SFC experiments have been optimized regarding the analysis time, but generally the SFC experiments have longer analysis times than the GC experiments. The standard deviation of peak area% of the GC experiments are lower than those of the SFC experiments, and the GC method using a polar column must be the choice of analysis method for the determination of fatty acid ester compositions of fish oils. On the other hand, the SFC method using a polar column offers an easy simultaneous determination of both fatty acid esters and cholesterol and operating temperatures considerably lower than that of the GC methods minimizing the potentiality of irreversible thermal degradation of the polyunsaturated compounds.

The results of this work show that SFC can be used for the analysis of fatty acid esters and legitimate the use of SFC directly coupled on-line to high-pressure equipment for analytical purposes.

ACKNOWLEDGEMENTS

This work has been supported by grants from Nordisk Industrifond and the Danish Research Council. The authors thank Grindsted Products

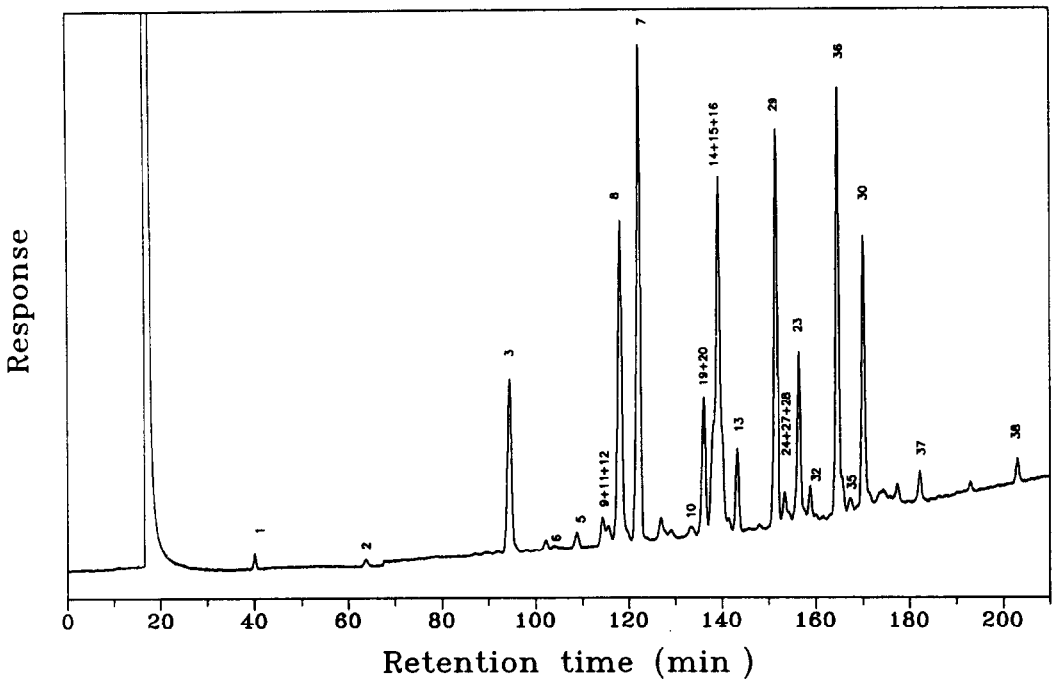

Fig. 5. SFC of sand eel fatty acid ethyl ester mixture. Conditions: column, DB-5 (20 m × 0.1 mm × 0.4 μm); conditions moreover as in Method 9 of the text. Peaks as in Fig. 1.

A/S for the supply of the fish oil ester mixture, M. Sc. Torben Forskov for the set up of the cold on-column injection method, Mr. Ruddy Perriard for performing the GC experiments using splitless injection, and Ms. Trang Thu Vu for performing the GC experiments using autosampler injection.

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