Journal of Chromatography, 187 (1980) 307–317

CHROM. 12,316

FRACTIONATION OF FISH OIL FATTY ACID METHYL ESTERS BY MEANS OF ARGENTATION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, AND ITS UTILITY IN TOTAL FATTY ACID ANALYSIS

M. ÖZCIMDER* and W. E. HAMMERS**

Laboratory for Analytical Chemistry, State University, Croesestraat 77a, 3522 AD Utrecht (The Netherlands)

(First received June 28th, 1979; revised manuscript received August 13th, 1979)

SUMMARY

The utility of reversed-phase and argentation high-performance liquid chromatography (HPLC) as pre-fractionation methods in fatty acid analysis is discussed. Both HPLC modes were applied to cod liver oil fatty acid methyl esters. Apart from positional isomers, the fractions obtained by reversed-phase HPLC were analysed by gas–liquid chromatography and appear to be free of the usually occurring "critical pairs". The mechanism of retention of the fatty acid methyl esters on low-loaded silver nitrate-impregnated silicas is discussed. It is shown that argentation HPLC is a rapid semi-preparative pre-fractionation method for highly unsaturated fatty esters with 3–6 double bonds.

INTRODUCTION

Thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) are important analytical methods in lipid research. The latter has found widespread use in the analysis of apolar lipids such as fatty acid esters. It has become common practice to use very polar stationary phases in GLC in order to obtain a good separation of saturated and unsaturated esters. However, even for simple mixtures not all peaks in the chromatogram are completely resolved.

Usually, the position of a peak in a gas chromatogram is characterized by the equivalent chain length (*ECL*) of the fatty acid ester. On a polar GLC column

$$ECL = n + xn_{C=C}$$

where n and $n_{C=C}$ are the numbers of carbon atoms and double bonds, respectively, in

* On leave from the Analytical Department, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey.

** To whom correspondence should be addressed.

the fatty acid. The magnitude of x depends on the number, position and the *cis/trans* geometry of the double bonds and on the polarity of the stationary phase. Generally, it decreases from 1 to about 0.5 as $n_{C=C}$ increases from 1 to 6. Fatty acid esters that have similar *ECL* values (critical pairs) cannot be separated completely. Some of these ester pairs can be resolved on apolar columns but then other peaks may overlap to various extents. Several workers have attempted to separate geometrical and positional isomers by means of GLC[1–9]. Generally, the selectivity towards positional isomers increases with the polarity of the stationary phase. However, as the polarity is increased, overlap with a preceding and/or next eluting ester species may occur. When also *cis/trans* isomers are present, the problems become difficult to solve, even with capillary columns[2,7].

In this paper the utility of high-performance liquid chromatography (HPLC) in fatty acid ester analysis is considered in connection with the problems mentioned above. The aim of the work was to examine the possibility of pre-fractionating the fatty acid ester mixture by HPLC in such a manner that the two esters of a critical pair would be isolated in different fractions. To that end, the utility of reversed-phase (RP-) and argentation (Ag-) HPLC is compared.

Argentation chromatography has been applied extensively on TLC plates[10]. It is based on the formation of a reversible charge-transfer complex involving the silver ion and an (olefinic) double bond[11,12]. Dudley and Anderson[13] showed that Ag-TLC can be used to fractionate fatty acid ester mixtures according to the number of double bonds. However, in order to obtain reproducible results the relative humidity in the TLC chamber must be kept within the narrow limits of 42–44%. The recovery of highly unsaturated esters ($n_{C=C} = 5$ and 6) was about 80%, presumably as a result of oxidation. This recovery can be improved by means of HPLC, as will be shown.

Heath *et al.*[14] showed that the geometrical isomers of 3,13-octadecadiene-1-ol acetate can be separated by Ag-HPLC. Similar results were obtained by Lam and Grushka[15] for some *p*-bromophenacyl fatty acid esters. The *trans*-isomers are eluted before the corresponding *cis*-isomers, owing the less pronounced influence of steric effects and to the release of intramolecular strain on the formation of the *cis* double bond–silver (I) complex[11]. Hence, a good resolution of *cis*- and *trans*-fatty acid methyl esters can be expected.

Positional isomers will be resolved in only a few instances, as follows from Ag-TLC work[8,16–18].

Reversed-phase chromatographic separations are largely based on the sorption of hydrophobic moieties of the solute molecules from a polar solvent to an apolar sorbent. Therefore, the order of elution of fatty acid esters will be in accord with their alkyl chain length, *i.e.*, be analogous to that in GLC columns. However, because of (polar) eluent–double bond interactions, the solute retention decreases with increasing number of double bonds[19] at constant chain length, *i.e.*, contrary to that on polar GLC columns. Therefore, *ECL* values of fatty acid methyl esters on RP columns can be presented by the equation

$$ECL = n - yn_{C=C}$$

From unresolved ester pairs in RP chromatograms given by Jordi[20], it can be deduced

that y decreases as $n_{C=C}$ increases. *Cis*- and *trans*-isomers have been separated successfully by RP-HPLC[20,21]. Owing to the influence of steric effects on the eluent–double bond interaction, the *cis*-isomer is eluted first. Although the C18:3ω3 and C18:3ω6 *p*-bromophenacyl esters have been separated by RP-HPLC[22], RP systems would hardly be expected to show good selectivity towards positional isomers in general because of the similar hydrophobicities of these isomers.

Summarizing, it can be concluded that critical pairs will occur in (capillary) GLC and Ag- and RP-HPLC, but that (apart from some positional isomers) the ester pairs involved are generally not the same.

Some investigators have combined the selectivities of both HPLC modes by using an RP system with a dilute aqueous silver salt solution as the eluent[23–27]. Excellent separations of the *cis–trans* isomers of 1,5,9-cyclododecatriene[26,27] and of the positional isomers of cyclooctadiene have been obtained in this way[27].

At present, the most suitable HPLC detectors for fatty acid methyl esters are the refractive index and the moving wire detector[28]. UV detection can be applied in the 203–214 nm range[29]. Unfortunately, the detection limits for these esters, in the microgram range, leave much to be desired. Therefore, in this work quantitation was performed in a final GLC analysis step by flame-ionization detection, the sensitivity and ease of which are unsurpassed.

The aim of this work was to examine the extent to which an HPLC pre-fractionation step can eliminate potential critical pair problems in GLC analysis. In order to compare the merits of Ag- and RP-HPLC in this respect, fatty acid ester mixtures from fish oils (*e.g.*, cod liver oil) were chosen because of their complex fatty acid profiles[9]. Further, some consideration is given to the mechanism of retention of unsaturated fatty acid esters in Ag-HPLC.

EXPERIMENTAL

Chemicals and sorbent specification

Even-carbon-numbered saturated fatty acid methyl esters (C14:0 up to C22:0), palmitoleate, palmitelaidate, oleate, elaidate, linoleate and linolenate were purchased from Applied Science Labs. (State College, Pa., U.S.A.). Their purities were at least 99%. Purified cod liver oil was a gift from the Institute for Fishery Products TNO (IJmuiden, The Netherlands). The oil was *trans*-esterified by the boron trifluoride–methanol method according to Morrison and Smith[30]. All ester samples were stored under nitrogen in a refrigerator.

LiChrosorb 10 RP-18 (E. Merck, Darmstadt, G.F.R.; particle diameter about 10 μm) was used as the RP sorbent. Elemental analysis gave 20.3% (w/w) of carbon.

Silver nitrate-impregnated silicas were prepared as follows. Partisil-10 (Reeve Angel Scientific, London, Great Britain; particle diameter 10 μm, specific surface area 400 m^2/g) was suspended in 15 ml of aqueous silver nitrate solutions, the concentrations of which corresponded to amounts of 5.0 and 7.5% (w/w) of silver (I) on to Partisil, respectively. The water was removed in a rotary vacuum evaporator. The residue was kept under purified nitrogen at 110° for 2 h. During the preparation, the adsorbent was protected from light.

The GLC packing was 12% (w/w) OV-275 on Chromosorb P AW DMCS (100–120 mesh).

Apparatus and procedure

The apparatus was a Pye LCM 2 chromatograph, equipped with a moving wire detector. The wire was coated with the sample components in the eluent by means of a stainless-steel spray nozzle (type V) as described by Van Dijk[31]. It was slightly modified by heating the spray nozzle and the spray nitrogen in order to promote evaporation of the eluent. The vapour was sucked away by pumping. Optimal detector performance was achieved with 0.5 atm of spray nitrogen at a nozzle tip-wire distance of 10 mm and maximum wire speed (13 cm/sec). The temperatures of the cleaner, evaporator, oxidizer oven, reduction chamber and flame-ionization detector were 900°, 150°, 700°, 390° and 175°, respectively. The nitrogen, air and hydrogen flow-rates were as recommended by the supplier. The samples were injected by means of a six-port Valco sample valve (7000 p.s.i., sample loop 50 μl). The detection limit for oleate was about 0.2 μg, *i.e.*, about a factor 25 better than that obtained with the conventional dip-coating device.

The GLC analyses were carried out with a Packard-Becker (Delft, The Netherlands) M417 chromatograph, equipped with a dual flame-ionization detector with quartz flame tips. The samples were injected directly on to the column packing.

The HPLC columns (polished precision-bore stainless steel, length 25 cm, I.D. 4.6 mm) were packed by the viscous slurry method[32]. The slurry [10% (w/w) of sorbent in *n*-hexane–squalane (1:3)] was degassed and homogenized by ultrasonic treatment and forced into the column at 300 atm. Finally, 200 ml of *n*-hexane were flushed through the column in order to settle the packed bed. The HETP of linoleate [$k' = 2.19$ on the 5% Ag(I) column, eluent 0.4% acetonitrile in *n*-hexane, flow-rate 0.20 cm/sec] was 0.14 mm. The HETP of stearate ($k' = 6.24$ on the LiChrosorb RP-18 column, eluent acetonitrile, flow-rate 0.16 cm/sec) was 0.09 mm.

The GLC columns (coiled glass, length 2 m, I.D. 2.7 mm) were filled according to the method described by Kuksis and Breckenridge[33]. The column packings were equilibrated at 250° for 24 h by flushing purified nitrogen through the columns. GLC analyses were carried out at 215°.

As eluents 0.3, 0.4 and 0.5% (v/v) of acetonitrile in *n*-hexane (Ag-HPLC) were used at ambient temperature. Both solvents were dried with molecular sieve 5A and carefully degassed by sonication. The eluent flow-rates were about 1.25 and 1.5 ml/min. In GLC, purified water- and oxygen-free nitrogen was used as the carrier gas (flow-rate 10 ml/min).

Retention times were corrected for the eluent hold-up in the column and the dead time of the transport detector by means of the retention time of squalane (Ag-HPLC) or approximately by that of sucrose (RP-HPLC). The measurements of the capacity ratios (k') were made in triplicate and were reproducible within 4%. In the GLC k' measurements, methane was used as the dead volume marker (precision better than 1%).

RESULTS AND DISCUSSION

Reversed-phase HPLC

Log k' values of some fatty acid ester standards (indicated in Table I) and those of palmitelaidate and elaidate are plotted against n in Fig. 1. It was assumed that the data points of homologous fatty acid esters lay on lines parallel to that for the

TABLE I

FATTY ACID ESTER CONTENTS OF THE RP-HPLC FRACTIONS OF COD LIVER OIL, THEIR LOG *k'* AND *ECL* VALUES ON OV-275, *ECL* VALUES ON SILAR 10C GIVEN BY HECKERS *et al.*[4] AND QUANTITATIVE RESULTS FROM THIS WORK AND THOSE BY WORKERS AT THE TNO[35]

Fatty ester	*RP fraction*			*OV-275*		*Silar 10C: ECL*[4]	*Concentration (%, w/w)*	
	I	*II*	*III*	*Log [k'/k' (C18:0)]***	*ECL*		*This work*	*TNO*[35]
14:0*		+		−0.343	14.01		4.6	4.2
15:0 (+ iso)			+	−0.265	14.92	—	0.5	0.7***
15:1 (ω5)	+			−0.174	15.98	16.04	<0.2	—
16:0*			+	−0.173	15.99		11.6	10.4
?		+		−0.130	16.49		<0.2	—
16:1ω7*		+		−0.086	17.00	16.89	10.4	11.7
17:1 (ω7)?		+		−0.012	17.87	17.89	<0.2	0.1
16:2	+			−0.002	17.98	—	0.7	1.0‡
18:0*			+	0.000	18.01		2.3	2.5‡‡
18:1ω9*			+	0.075	18.88	18.77	25.5	26.0
16:3	+			0.098	19.15	—	1.0	—
18:2ω6*		+		0.165	19.93	19.71	1.7	1.5
20:0*				0.170	19.99		—	—
18:3 (ω6)	+			0.207	20.42	20.51	<0.2	0.5
18:3ω3*	+			0.274	21.20	20.91	0.7	
20:1 (ω7)			+	0.240	20.81	20.84	11.6	12.9
20:2 (ω6)			+	0.334	21.90	21.71	<0.2	—
22:0*				0.343	22.01		—	—
18:4	+			0.353	22.12	—	2.7	2.5
22:1 (ω7)			+	0.414	22.83	22.77	5.7	4.9
20:4 (ω6)	+			0.512	23.98	(22.92?)	0.6	—
20:5 (ω3)	+			0.571	24.67	(24.08?)	9.4	9.8
21:5	+			0.665	25.76	—	<0.2	—
22:5	+			0.741	26.65	—	0.6	0.5
22:6 (ω3)	+			0.786	27.17	27.16	10.4	10.8

* Also applied as standards.
** Log k' (C18:0) = 0.622.
*** C15:0 (0.3%) + iso-C15:0 (0.4%).
‡ C16:2 + C17:0 (1.0%).
‡‡ C18:0 (2.3%) + iso-C18:0 (0.2%).

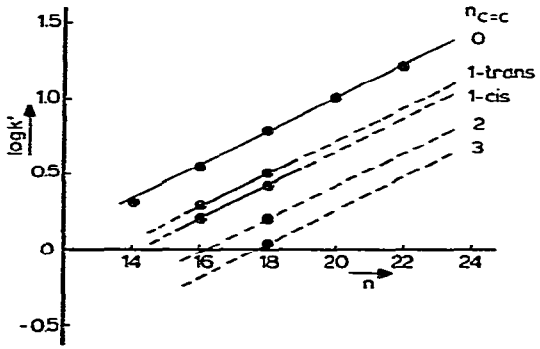

Fig. 1. Log *k'* values of some standard fatty acid methyl esters on LiChrosorb RP-18 *versus* the number of (chain) carbon atoms (*n*). Eluent: acetonitrile.

saturated esters. The *trans*-isomers are eluted after the corresponding *cis*-esters, as expected.

Fig. 2 shows the chromatogram of the cod liver oil esters. Geometrical isomers can be separated, provided that their contents are not too large. Otherwise, a longer column or a 5-μm sorbent has to be used. A 25-μl sample containing about 0.6 mg of fatty acid esters was injected and collected in three fractions as indicated in Fig. 2. The collected eluate was concentrated nearly to dryness by vacuum evaporation. The residue was dissolved in 100 μl of *n*-heptane and 0.5–2-μl samples were analysed by GLC. The chromatograms are given in Fig. 3. The small shaded peaks are traces

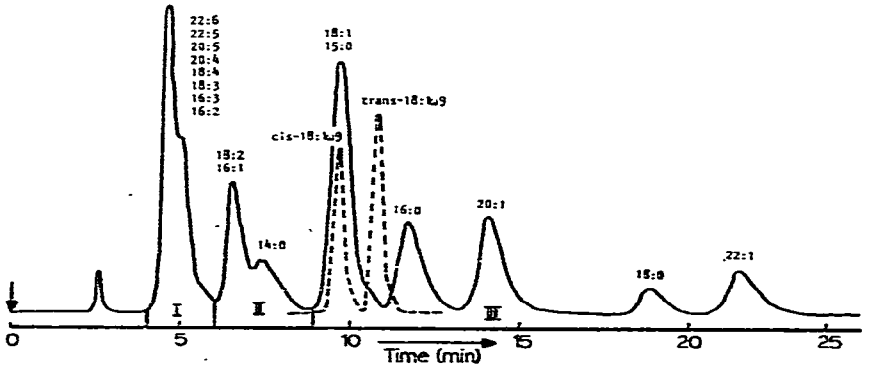

Fig. 2. Fractionation of cod liver oil fatty acid methyl esters on LiChrosorb 10 RP-18. Eluent: acetonitrile. Flow-rate: 1.27 ml/min. Sample size: 25 μl containing 0.6 mg of fatty acid esters. Broken lines: methyl oleate and elaidate standards.

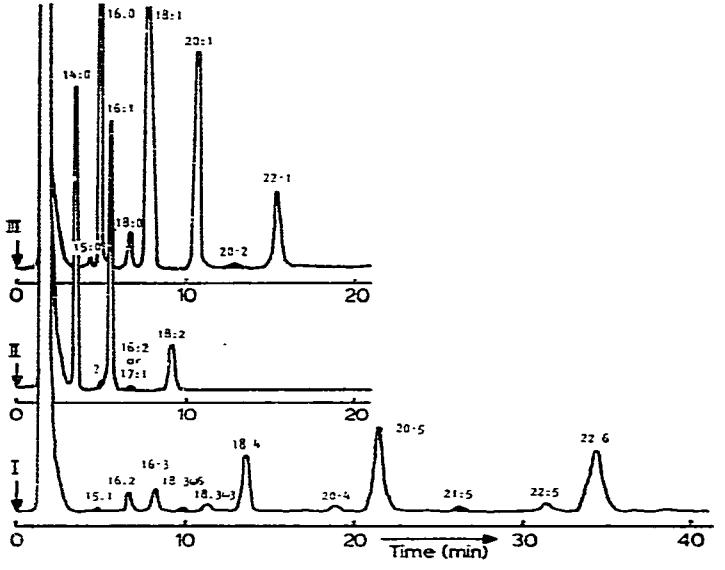

Fig. 3. Gas chromatograms of reversed-phase HPLC fractions I–III (see Fig. 2). Column: 2 m × 2.7 mm I.D. (glass), 12% (w/w) of OV-275 on Chromosorb P AW DMCS. Carrier gas: nitrogen (10 ml/min). Temperature: 215°.

(amounts < 0.2%). The small peak in II, ascribed to C17:1, can be a trace of C16:2 from fraction I.

The log k' values (relative to that of C18:0) are given in Table I. The corresponding esters were identified from log[k'/k'(C18:0)] *versus n* plots. Both the predictability of the esters potentially present in a certain fraction on the basis of Figs. 1 and 2, and the fact that esters with similar log k' values belong to different fractions (see Table I), greatly facilitates this procedure.

Using saturated fatty acid esters as standards, *ECL* values were calculated from the log k' values of all traced fatty esters by linear regression. As OV-275 and Silar 10C have similar chemical constitutions[4], the *ECL* values on the former can be compared with those on the latter given by Heckers *et al.*[4]. The agreement is good, except for C20:4 and C20:5. The large discrepancies in this instance can hardly be due to positional isomerism or different polarities of the two stationary phases (compare the *ECL* values for C22:6). The *ECL* values of these fatty acid esters on OV-275 are consistent with a contribution of 1 *ECL* unit per methylene group (compare the *ECL* values of C20:4 and C20:5 with those of C18:4 and C22:5, respectively).

The indication of double bond positions (ω notation in Table I) refers to *ECL* values given by Heckers *et al.*[4], and does not exclude the occurrence of other isomers in cod liver oil. Generally, they are hard to distinguish on packed GLC columns. Inspection of the *ECL* values in Table I shows that neighbouring esters which belong to the same fraction have *ECL* values, the difference of which is about 1 *ECL* unit in most instances. Hence a pre-fractionation by RP-HPLC can facilitate the search for positional isomers in complex mixtures by capillary GLC. *Cis–trans* separations of monoenoic esters by this HPLC method can also be useful in this respect, as follows from Fig. 2.

Quantitative results were obtained by triangulation of the peaks and normalization of the peak areas to constant sample size by means of resolved peaks in the chromatogram of the unfractionated cod liver oil esters. Response factors were estimated from the weight fractions of carbon in the esters (the carbonyl carbon atom was excluded because its contribution to the response of the flame-ionization detector can be neglected[34]). The weight percentages are given in Table I, together with GLC data* provided by TNO[35] for the same sample. The former are precise to about 0.5% and the latter (obtained with a digital integrator) to about 0.2%. The agreement is satisfactory in most instances.

Argentation HPLC

The log k' values of the fatty acid esters with 0–6 *cis* double bonds on both silver (I) columns are plotted *versus* $n_{C=C}$ in Fig. 4A. The data points for $n_{C=C} = 0$, 1, 2 and 3 belong to standard C18 esters and those for $n_{C=C} = 4$, 5 and 6 to C18:4, C20:5 and C22:6, respectively. The latter were obtained from the chromatograms of the cod liver oil fatty acid esters. The (smoothed) chromatogram on the 5% Ag(I)–0.4% acetonitrile–*n*-hexane column is given in Fig. 5.

---

* Analytical procedure according to the Dutch NEN-norm 3428 entitled "Gas chromatographic determination of fatty acid composition of vegetable and animal fats and oils, and of technical fatty acids". The hydrogenation step was excluded and replaced with analyses on EGA and Apiezon L columns.

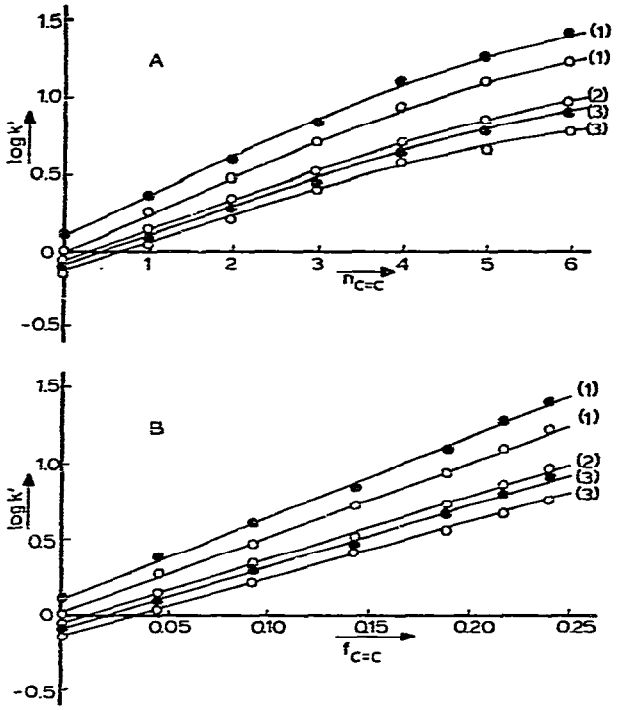

Fig. 4. Log k' values of fatty acid esters *versus* (A) the number of double bonds ($n_{C=C}$) and (B) their double bond fraction ($f_{C=C}$) on 5 and 7.5% (w/w) Ag(I) on Partisil 10 (data points indicated by open and full circles, respectively). Eluents: (1) 0.3, (2) 0.4 and (3) 0.5% (v/v) acetonitrile in *n*-hexane.

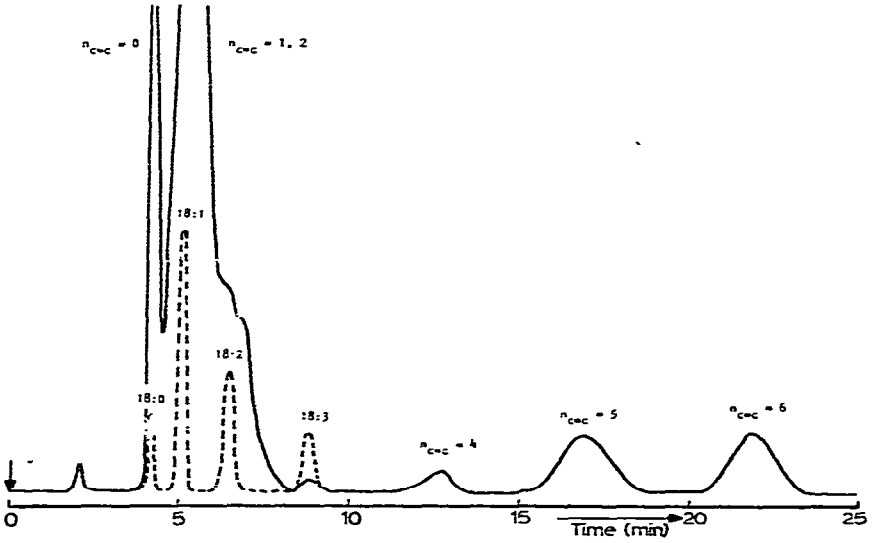

Fig. 5. Fractionation of cod liver oil fatty esters on 5% (w/w) Ag(I) on Partisil 10. Eluent: 0.4% (v/v) acetonitrile in *n*-hexane. Flow-rate: 1.54 ml/min. Sample size: 25 μl containing 0.6 mg of fatty esters. Broken lines: C18 fatty acid ester standards.

The log k' values increase with increasing silver(I) content, as expected. Further, the log k' values increase rapidly with decreasing acetonitrile content of the eluent. Finally, the log k' values are not linearly related to $n_{C=C}$ and hence solute retention is not merely related to the number of double bonds. As the retention mechanism in Ag-HPLC has not yet been described in detail, a few comments will be made.

It is assumed that the contribution of the adsorption of the ester group to log k' is similar for all fatty acid esters under examination. Hence, it cancels in the log α_t $\{\equiv \log[k'_i/k'(C18:0)]\}$ values. As the adsorption of an olefinic double bond to silanol sites will be weak because of the strong adsorption of the acetonitrile molecules to the silica surface[39], the log α_t values represent the contribution of double bond–silver(I) interactions. This contribution increases as the probability of double bond–silver(I) complexation increases, *i.e.*, with increasing silver(I) content.

The ratios of the α_t values obtained at 0.3 and 0.5% of acetonitrile in the eluent are equal, within the limits of experimental error, on both columns [5 and 7.5% Ag(I)]. This result would be expected if the silver(I) sites on both columns are roughly equivalent, *i.e.*, act as single sites towards all fatty esters under discussion and merely form 1:1 complexes. These sites are surrounded by acetonitrile molecules, the number of which is merely dependent on the acetonitrile concentration in the eluent. It can be estimated[36] that the influence of a 0.2% change in the acetonitrile concentration on log α_t would be negligible on silica. Hence, the relatively large eluent effect on silver nitrate-impregnated silica must be related to competitive complex formation between acetonitrile and unsaturated fatty acid ester molecules and silver(I) sites. This phenomenon has recently been described in detail[37].

The equivalence of the complexing sites can also be expected from a consideration of the site distribution. At the low surface concentrations of silver(I) in our columns (about 1.2 and 1.7 μmole/m^2), the mean distance between adjacent sites will be at least 13.5 and 11.4 Å, respectively. If part of the silver nitrate molecules form small clusters (which seems plausible), these distances are even larger and simultaneous complexation with two sites becomes even less probable. Hence, the most plausible explanation for the curved plots in Fig. 4A is that 1:1 complexes are formed to an extent that is simply proportional to the chance of complexation. A measure of this chance is the ratio of $n_{C=C}$ and the total number of carbon and oxygen atoms in the fatty acid ester, $f_{C=C}$. Indeed, plots of log k' against $f_{C=C}$ are linear (Fig. 4B). Obviously, the double bonds are equivalent with respect to complex formation. It is noteworthy that the influence of the chain length on the contribution of complexation to log k' values is also evident on 4% Ag aluminosilicate[15], where C16:1Δ^9 elutes *after* C18:1Δ^9 in 0.011% acetonitrile–chloroform+*n*-hexane (1:12).

This simple picture of the adsorption process does not apply to the R_F values of series of C18 positional isomers[8,16–18], which are a shallow sinusoidal function of the double bond position. For these isomers the strongest complexation is invariably found for the Δ^5 or Δ^6 isomer, irrespective of the silver content (10–30% of silver nitrate on silica gel G). Presumably, the surface concentration of silver(I) is so large here that the mean spacing between the ester group and the sixth carbon atom fits to the average distance between a silver site and its surrounding silanol groups[8]. Similar "localization" phenomena have been described by Snyder[38] for a series of dithiaalkanes on alumina.

Fig. 5 shows the chromatogram of the cod liver oil fatty acid esters together

with that of a synthetic mixture of C18 standards. The bad resolution of the saturated, mono- and dienoic esters can be expected for most vegetable and fish oils, in which C16:0, C18:0, C18:1, C18:2, C20:1 and C22:1 are generally abundantly present. The resolution can be improved by using a larger amount of silver(I) and/or a lower acetonitrile content. However, the analysis time will then be unacceptably long. Gradient elution is not suitable because the regeneration of these columns to their original condition with *n*-hexane is time consuming. Analysis of the combined fraction ($n_{C=C} = 0$, 1 and 2) is not advantageous in view of the large number of esters involved. Therefore, it is concluded that this type of argentation HPLC is not suitable for fractionation in total fatty acid analysis.

On the other hand, the resolution of the highly unsaturated esters is good and can be achieved within 25 min. Hence, Ag-HPLC is a promising fractionation technique (milligram scale) for highly unsaturated esters under favourable chromatographic conditions. The recovery of the esters from the column was examined by comparing the peak heights of unsaturated esters (relative to that of C22:1) in gas chromatograms of a sample that passed the silver(I) column and of a sample that was injected directly into the gas chromatograph. The recoveries of C22:6, C20:5 and C18:4 were 96 ± 4, 101 ± 2 and $101 \pm 2\%$, respectively. Previously, De Vries[39] reported a complete recovery of linolenate (C18:3). Obviously, oxidation of the esters by silver nitrate does not occur. A disadvantage of columns with silver nitrate-impregnated silicas is their restricted lifetime. Under the chromatographic conditions used here, the column properties were constant for only 2 months with daily use. This is probably due to the slight solubility of silver nitrate in the eluents used. This problem may be overcome by saturating the eluent with silver nitrate by means of a pre-column.

CONCLUSIONS

Reversed-phase HPLC on LiChrosorb RP-18 with acetonitrile as the eluent is a promising pre-fractionation method for complex fatty acid methyl ester mixtures. Apart from positional isomers, the collected fractions are free of most of the "critical pairs" which can occur in GLC analysis on polar columns. The isolation of *cis*- and *trans*-isomers in complex ester mixtures in different RP fractions (although restricted to monoenoic esters with chain lengths of at least 15 carbon atoms) further facilitates the analysis of the RP fractions by means of (capillary) GLC, including positional isomers.

Argentation HPLC appears to be less suitable as a pre-fractionation method in total fatty acid ester analysis. However, it seems to be a good method for the fractionation of unsaturated ester species with 3–6 double bonds (milligram scale). The mechanism of retention of fatty acid methyl esters on low-loaded silver nitrate-impregnated silicas [surface coverages up to 1.7 μmol/m^2 of Ag(I)] is controlled by their degree of unsaturation, *i.e.*, the ratio of the number of double bonds to the total number of carbon and oxygen atoms in the ester. The large influence of the content of acetonitrile in *n*-hexane on the retention of the unsaturated esters is due to competitive 1:1 complex formation with silver(I) sites.

ACKNOWLEDGEMENT

The authors are indebted to Dr. J. B. Luten for kindly providing the fish oil samples and data on their fatty acid contents.

REFERENCES

1 R. G. Ackman and J. D. Castell, *Lipids*, 1 (1966) 341.
2 W. W. Christie, *J. Chromatogr.*, 37 (1968) 27.
3 C. H. Lam and M. S. F. Lie Ken Jie, *J. Chromatogr.*, 117 (1976) 365.
4 H. Heckers, K. Dittmar, F. W. Melcher and H. O. Kalinowski, *J. Chromatogr.*, 135 (1977) 93.
5 H. Heckers, F. W. Melcher and U. Schloeder, *J. Chromatogr.*, 136 (1977) 311.
6 H. B. S. Conacher, *J. Chromatogr. Sci.*, 14 (1976) 405.
7 E. S. van Vleet and J. G. Quinn, *J. Chromatogr.*, 151 (1978) 396.
8 F. D. Gunstone, I. A. Ismail and M. S. F. Lie Ken Jie, *Chem. Phys. Lipids*, 1 (1967) 376.
9 R. G. Ackman, J. C. Sipos and P. M. Jangaard, *Lipids*, 2 (1967) 251.
10 L. J. Morris, *J. Lipid Res.*, 7 (1966) 717.
11 F. R. Hartley, *Chem. Rev.*, 73 (1973) 163.
12 O. K. Guha and J. Janák, *J. Chromatogr.*, 68 (1972) 325.
13 P. A. Dudley and R. A. Anderson, *Lipids*, 10 (1975) 113.
14 R. R. Heath, J. H. Tumlinson and R. E. Doolittle, *J. Chromatogr. Sci.*, 15 (1977) 10.
15 S. Lam and E. Grushka, *J. Chromatogr. Sci.*, 15 (1977) 234.
16 W. W. Christie, *J. Chromatogr.*, 34 (1968) 405.
17 M. S. F. Lie Ken Jie and C. H. Lam, *J. Chromatogr.*, 124 (1976) 147.
18 L. J. Morris, D. M. Wharry and E. W. Hammond, *J. Chromatogr.*, 31 (1967) 69.
19 P. T.-S. Pei, R. S. Henly and S. Ramachandran, *Lipids*, 10 (1974) 152.
20 H. C. Jordi, *J. Liquid Chromatogr.*, 1 (1978) 215.
21 J. D. Warthen, Jr., *J. Amer. Oil Chem. Soc.*, 52 (1975) 151.
22 P. T.-S. Pei, W. C. Kossa, S. Ramachandran and R. S. Henly, *Lipids*, 11 (1976) 814.
23 A. G. Vereshchagin, *J. Chromatogr.*, 17 (1965) 382.
24 M. M. Paulose, *J. Chromatogr.*, 21 (1966) 141.
25 J. Janák, Z. Jagarić and M. Dressler, *J. Chromatogr.*, 53 (1970) 525.
26 G. Schomburg and K. Zegarski, *J. Chromatogr.*, 114 (1975) 174.
27 B. Vonach and G. Schomburg, *J. Chromatogr.*, 149 (1978) 417.
28 R. P. W. Scott and J. G. Lawrence, *J. Chromatogr. Sci.*, 8 (1970) 65.
29 W. S. M. Geurts van Kessel, W. M. A. Hax, R. A. Demel and J. de Gier, *Biochim. Biophys. Acta*, 486 (1977) 524.
30 W. R. Morrison and L. M. Smith, *J. Lipid Res.*, 5 (1964) 600.
31 J. H. van Dijk, *J. Chromatogr. Sci.*, 10 (1972) 31.
32 J. Asshauer and I. Halász, *J. Chromatogr. Sci.*, 12 (1974) 139.
33 A. Kuksis and W. C. Breckenridge, *J. Lipid Res.*, 7 (1966) 576.
34 R. G. Ackman and J. C. Sipos, *J. Amer. Oil Chem. Soc.*, 41 (1964) 377.
35 J. B. Luten, Institute for Fishery Products TNO, IJmuiden, The Netherlands, personal communication.
36 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968.
37 W. E. Hammers, M. C. Spanjer and C. L. de Ligny, *J. Chromatogr.*, 174 (1979) 291.
38 L. R. Snyder, *J. Chromatogr.*, 8 (1962) 319.
39 B. de Vries, *J. Amer. Oil Chem. Soc.*, 40 (1963) 184.