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Note

SP 2340 in the glass capillary chromatography of fatty acid methyl esters

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The development of the cyanopropylsiloxane stationary phases Silar 10C, Silar 9CP, SP 2340 and OV-275, which have high polarity and temperature resistance, made it possible to separate *cis/trans* isomeric fatty acids on packed columns[1]. Of course, a comparison of the retention data[2] indicated that some fatty acids which commonly occur in biological material show considerable overlapping in complex acid mixtures. However, gas–liquid chromatographic (GLC) analysis of these highly polar phases is useful when the complex sample is additionally analyzed on an EGA or DEGA packed column. Further, the cyanopropylsiloxanes gave small but constant differences in retention data[2] for many positional isomeric fatty acids on packed columns, suggesting that SP 2340 is as suitable as FFAP for the separation of fatty acid mixtures in glass capillary chromatography[4]. In this paper, we report on some GLC characteristics of a 30-m glass capillary column coated with SP 2340 used to re-evaluate the fatty acid composition of red cell phospholipids in young and old people[5].

EXPERIMENTAL

All of the standards of fatty acid methyl esters tested were more than 99% pure and were purchased from Nu-Check-Prep (Elysian, Minn. 56028, U.S.A.) and from Analabs (North Haven, Conn., U.S.A.). Octadecadienoate and octadecatrienoate mixtures containing the *cis/trans* isomers were prepared from pure linoleic and linolenic standards according to Litchfield and co-workers[6,7]. Pure standards of 9-*trans*-12-*cis*- and 9-*cis*-12-*trans*-octadecadienoic fatty acids were a generous gift from Th. Wieske, Union Deutsche Lebensmittelwerke (Hamburg, G.F.R.).

The quantitative extraction of lipids from human red cell ghosts, the separation of the pure phospholipid fraction[8] and the preparation of the fatty acid methyl esters derived from this lipid fraction were performed by a modification of the method of Dodge and Phillips[5], details of which are reported elsewhere[9]. The results of the fatty acid composition of the human red cell phospholipids are given in Table I as means ± standard deviation from 25 individuals of both sexes and of two age groups (twelve between 15 and 30 years of age and thirteen older than 70 years). All subjects were on a normal diet. The blood donors were free from haematological and liver diseases, had red cell counts and haematocrits in the normal range, and were not taking any drugs.

GC analysis

A Hewlett-Packard Model 5830 A gas chromatograph, equipped with a dual flame-ionization detector and an integrator, was used for all analyses. The chromatograph was fitted with a 30-m glass capillary column of 0.3 mm I.D., coated with the cyanopropylsiloxane SP 2340 of average molecular weight 2800. The oven temperature was programmed from 100° to 190°, heating for 5 min at the rate of 5°/min, followed by 0.5°/min up to 35.5 min and finally 1°/min up to the maximum temperature of 190°, which was maintained for a further 10 min before cooling. The recorded analysis time for the fatty acid mixture from red cell phospholipids was 95 min; longer runs could not rule out later peaks. The splitting ratio was 1:70, the column was operated with nitrogen as the carrier gas at a flow-rate of 2.5 ml/min and the injection and detector temperatures were 240°. The sample size was 0.1–0.2 μl (solutions in chloroform) and the peaks were identified by means of known standards. Additionally, samples were run a second time after catalytic hydrogenation over PtO_2 for identification purposes.

RESULTS AND DISCUSSION

In order to characterize the SP 2340 column performance, the following numerical values were established. Using *n*-hexadecanoic and *n*-heptadecanoic fatty acid methyl esters (recorder chart speed 3 cm/min), the separation number of the column was calculated with $n_{sep}{}^{*} = 7.76$ (mean value of three recordings) and $n_{sep}/m = 0.26$. The measurements were performed under the abovementioned temperature-programmed conditions. The separation number expressed as the number of theoretical plates gave $n^{*} = 7674$ or $n/m = 256$. The capacity ratio for decanoic acid methyl ester, measured under isothermal conditions at 90° with the methyl esters of (a) octanoic, (b) nonanoic and (c) decanoic acid (mean value of three runs) was $K^{*} = 4.39$. The loadability of the column for decanoic acid methyl ester could be calculated with $l^{*} = 1.80 \cdot 10^{-6}$ g.

Although the separation number and the specific separation number are low, probably indicating insufficient impregnation with stationary liquid, the separation efficiency for fatty acid methyl esters, especially for geometric isomeric acids and for positional isomers of acids, was much better than expected with the tested material. This aspect is demonstrated by the capillary gas–liquid chromatograms depicted in Figs. 1–3. All five pairs of geometric isomeric monoethylenic fatty acids and the all-*cis versus* all-*trans* components of octadecadiethylenic and octadecatriethylenic acids

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* The following equations were used[10]:

$$n_{sep} = \frac{\Delta T}{b_{0.5} + b_{0.5}} - 1$$

$$n = 100\,(n_{sep} + 1)^2$$

$$t_d = \frac{t_{dr(a)} \cdot t_{dr(c)} - t_{dr(b)}^2}{t_{dr(a)} + t_{dr(c)} - 2t_{dr(b)}}$$

$$K = \frac{t_s}{t_d}$$

$$l = 0.05\,M\,d^3(1 + K) \cdot 10^{-6}\ \text{g}$$

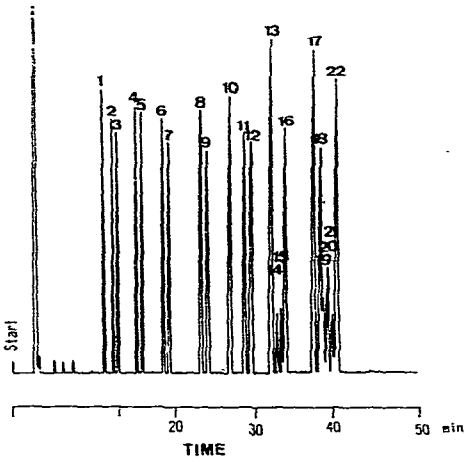

Fig. 1. Chromatogram of a mixture of five pairs of *cis/trans* isomeric monounsaturated fatty acids completed by the methyl esters derived from nitrous acid isomerized linoleic and linolenic acids. Peaks: 1 = C_{14}; 2 = $C_{14:1tr\omega5}$; 3 = $C_{14:1c\omega5}$; 4 = $C_{15:1tr\omega5}$; 5 = $C_{15:1c\omega5}$; 6 = $C_{16:1tr\omega7}$; 7 = $C_{16:1c\omega7}$; 8 = $C_{17:1tr\omega7}$; 9 = $C_{17:1c\omega7}$; 10 = C_{18}; 11 = $C_{18:1tr\omega9}$; 12 = $C_{18:1c\omega9}$; 13 = $C_{18:2tr,tr\omega6}$; 14 = $C_{18:2c,tr\omega6}$; 15 = $C_{18:2tr,c\omega6}$; 16 = $C_{18:2c,c\omega6}$; 17 = $C_{18:3tr,tr,tr\omega3}$; 18, 19, 20, 21 = mixed geometric isomers of $C_{18:3\omega3}$ (individual peaks were not definitely identified); 22 = $C_{18:3c,c,c\omega3}$. The composition of the diethylenic and triethylenic acid mixtures corresponds to the final products resulting from the nitrous acid isomerization, with the exception of the individual all-*cis* components, which were added, because they occurred only in minimal amounts. Operating conditions were identical for all presented fractograms. For details see *GC analysis*.

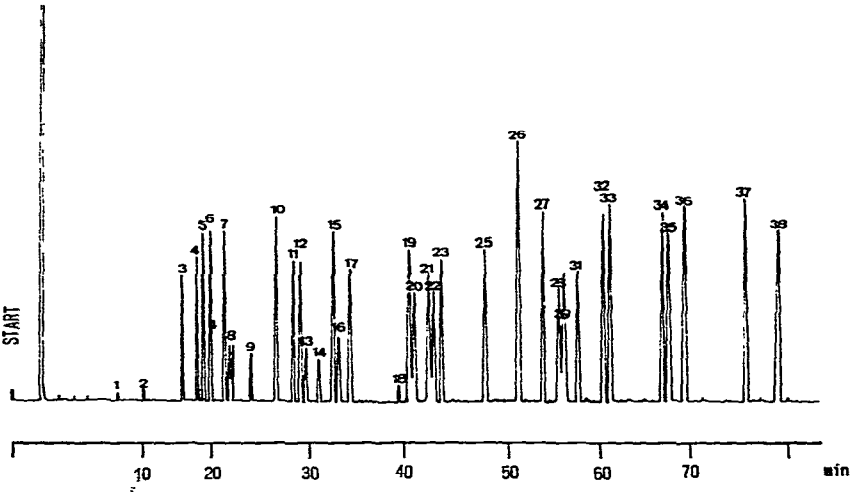

Fig. 2. Capillary gas chromatogram of a standard mixture of fatty acid methyl esters on an SP 2340 capillary column. Peaks: 1 = C_{12}; 2 = C_{14}; 3 = C_{16}; 4 = $C_{16:1tr\omega7}$; 5 = $C_{16:1c\omega7}$; 6 = anteiso-C_{17}; 7 = C_{17}; 8 = phytanic acid; 9 = iso-C_{18}; 10 = C_{18}; 11 = $C_{18:1tr\omega9}$; 12 = $C_{18:1c\omega9}$; 13 = $C_{18:1c\omega7}$; 14 = anteiso-C_{19}; 15 = $C_{18:2tr,tr\omega6}$; 16 = C_{19}; 17 = $C_{18:2c,c\omega6}$; 18 = $C_{18:3tr,tr,tr\omega3}$; 19 = C_{20}; 20 = $C_{18:3c,c,c\omega3}$; 21 = $C_{20:1c\omega12}$; 22 = $C_{20:1c\omega9}$; 23 = $C_{20:1c\omega7}$; 25 = $C_{20:2c,c\omega6}$; 26 = $C_{20:3c,c,c\omega6}$; 27 = $C_{20:4c,c,c,c\omega6}$ + C_{22} + $C_{20:3c,c,c\omega3}$; 28 = $C_{22:1tr\omega9}$; 29 = $C_{22:1c\omega9}$; 31 = $C_{22:2c,c\omega9}$; 32 = $C_{20:5all\text{-}cis\omega3}$; 33 = $C_{22:2c,c\omega6}$; 34 = $C_{22:3c,c,c\omega3}$ + C_{24}; 35 = $C_{22:4c,c,c,c\omega6}$; 36 = $C_{24:1c\omega9}$; 37 = $C_{22:6all\text{-}cis\omega3}$; 38 = C_{26}. For operating conditions see *GC analysis*.

TABLE I

FATTY ACID COMPOSITION OF TOTAL PHOSPHOLIPID FROM RED CELL GHOSTS OF YOUNG (LESS THAN 30 YEARS) AND OLD (MORE THAN 70 YEARS) SUBJECTS

Values (area%) are given as means ± standard deviation. Numbers in parentheses correspond to the numbers in Fig. 3.

Component	*Young subjects*	*Old subjects*	*Component*	*Young subjects*	*Old subjects*
C_8	(1) tr*	tr		0.02 ± 0.01	0.02 ± 0.02
C_{10}	(2) 0.03 ± 0.03	0.06 ± 0.04		0.02 ± 0.01	0.03 ± 0.01
C_{12}	(3) 0.36 ± 0.43	0.34 ± 0.53		(33) 0.07 ± 0.04	0.11 ± 0.15
C_{14}	(4) 1.33 ± 1.14	1.31 ± 0.92	C_{20}	(34) 0.48 ± 0.11	0.50 ± 0.14
	0.22 ± 0.33	0.22 ± 0.25	$C_{18:3c,c,c\omega3}$	(35) 0.26 ± 0.38	0.25 ± 0.29
	0.14 ± 0.13	0.35 ± 0.24		(36) 0.11 ± 0.11	0.10 ± 0.09
	(5) 0.07 ± 0.05	0.08 ± 0.06	$C_{20:1c\omega9}$	(37) 0.35 ± 0.16	0.39 ± 0.26
$C_{14:1tr\omega5}$	(6) 0.19 ± 0.23	0.14 ± 0.10	$C_{20:1c\omega7}$	(38) 0.02 ± 0.01	0.03 ± 0.03
$C_{14:1c\omega5}$	(7) 0.13 ± 0.15	0.12 ± 0.08		(39) 0.14 ± 0.23	0.34 ± 0.48
C_{15}	(8) 0.43 ± 0.35	0.39 ± 0.18		(40) 0.19 ± 0.22	0.16 ± 0.10
iso-C_{16} + $C_{15:1tr\omega5}$	(9) 0.16 ± 0.20	0.14 ± 0.13	$C_{20:2c,c\omega6}$	(41) 0.15 ± 0.07	0.11 + 0.05
anteiso-C_{16}	(10) 0.06 ± 0.04	0.12 ± 0.12		(42) 0.03 ± 0.03	0.04 ± 0.04
C_{16}	(11) 23.16 ± 2.44	22.56 ± 3.53		(43) 0.03 ± 0.02	0.04 ± 0.05
	(12) 0.04 ± 0.03	0.19 ± 0.27	$C_{20:3c,c,c\omega6}$	(44) 0.95 ± 0.35	0.85 ± 0.39
	(13) 0.07 ± 0.08	0.16 ± 0.16		(45) 0.06 ± 0.08	0.16 ± 0.15
$C_{16:1tr\omega7}$	(14) 1.04 ± 1.32	0.67 ± 0.48	$C_{20:4\omega6}$*** + C_{22} + $C_{20:3\omega3}$***	(46) 10.53 ± 3.51	9.08 ± 2.95
$C_{16:1c\omega7}$**	(15) 0.47 ± 0.20	0.68 ± 0.29		(47) 0.03 ± 0.02	0.03 ± 0.02
anteiso-C_{17}	(16) 0.12 ± 0.10	0.16 ± 0.10		(48) 0.03 ± 0.01	0.03 ± 0.02
	0.02 ± 0.02	0.02 ± 0.01	$C_{22:1c\omega9}$	(49) 0.06 ± 0.04	0.12 ± 0.10
C_{17}	(17) 0.56 ± 0.06	0.62 ± 0.13		(50) 0.06 ± 0.09	0.04 ± 0.04
Phytanic acid	tr	tr	$C_{20:5\omega3}$***	(51) 0.57 ± 0.26	0.58 ± 0.32
	(18) 0.07 ± 0.10	0.09 ± 0.18		(52) 0.04 ± 0.02	0.11 ± 0.25
$C_{17:1tr\omega7}$	(19) Internal standard			(53) 0.07 ± 0.03	0.96 ± 1.33
$C_{17:1c\omega7}$	(20) tr	tr		(54) 0.08 ± 0.11	0.28 ± 0.32
iso-C_{18}	tr	tr		(55) 0.04 ± 0.06	0.12 ± 0.21
	(21) 0.06 ± 0.05	0.11 ± 0.11		(56) 0.08 ± 0.12	0.22 ± 0.19
	(22) 0.07 ± 0.06	0.08 ± 0.04	$C_{22:3\omega3}$*** + C_{24}	(57) 5.15 ± 1.65	5.15 ± 1.81
C_{18}	(23) 15.16 ± 1.96	14.23 ± 1.18	$C_{22:4\omega6}$***	(58) 2.20 ± 0.60	1.65 ± 0.77
$C_{18:1tr\omega6-12}$	(24) 0.38 ± 0.28	0.75 ± 0.42	$C_{24:1\omega9}$***	(59) 5.55 ± 1.90	6.40 ± 2.29
$C_{18:1c\omega9}$	(25) 12.73 ± 2.79	14.61 ± 2.86		(60) 0.39 ± 0.19	0.37 ± 0.20
$C_{18:1c\omega7}$	(26) 0.92 ± 0.32	1.12 ± 0.26		(61) 0.03 ± 0.01	0.04 ± 0.04
	(27) 0.10 ± 0.04	0.13 ± 0.03		(62) 0.04 ± 0.02	0.09 ± 0.21
	(28) 0.07 ± 0.08	0.06 ± 0.05	$C_{22:5\omega3}$***	(63) 1.80 ± 0.82	1.65 ± 0.77
$C_{18:2tr,tr\omega6}$	tr	tr	$C_{22:6\omega3}$***	(64) 2.30 ± 1.31	1.99 ± 1.01
C_{19}	(29) 0.13 ± 0.21	0.21 ± 0.38		(65) 0.23 ± 0.11	0.28 ± 0.12
$C_{18:2c,c\omega6}$	(30) 9.13 ± 2.87	7.75 ± 4.41		(66) 0.14 ± 0.05	0.17 ± 0.07
	(31) 0.40 ± 0.31	0.25 ± 0.15		(67) 0.03 ± 0.02	0.15 ± 0.17
	(32) 0.04 ± 0.07	0.09 ± 0.09			

* tr (trace) means less than 0.01% of total acids.

** This peak contains an artifact of BHT, *ca.* 20–30% (see Fig. 4).

*** All double bonds are in a *cis* configuration.

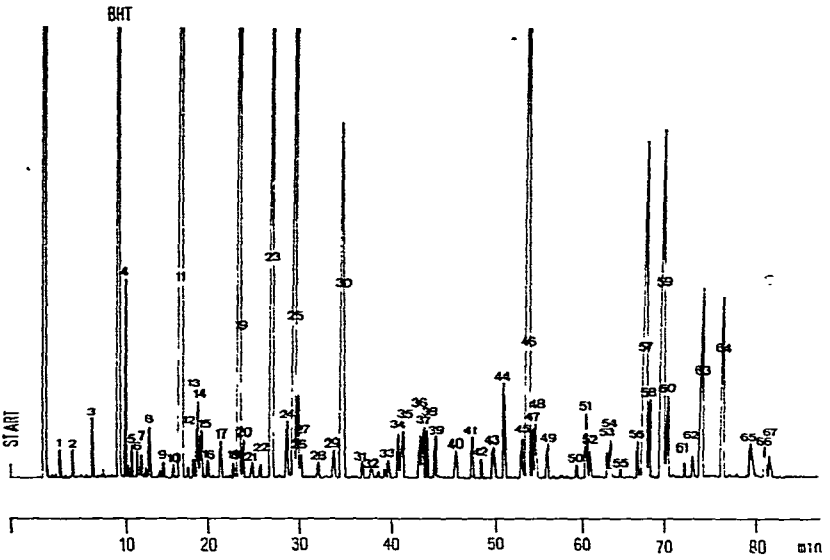

Fig. 3. Typical GLC pattern of human red cell total phospholipid (glycolipids separated) fatty acid methyl esters including the antioxidant BHT and $C_{17:1tr\omega7}$ as internal standard. Peak numbers correspond to the numbers in Table I.

show a separation of more than 4.6 σ, thus being very suitable for correct automatic integration (Fig. 1). Whereas $C_{18:2c,tr\omega6}$ and $C_{18:2tr,c\omega6}$ show more than baseline separation, the latter peak overlapped somewhat with that of elaidolinoleic acid. The GLC elution pattern of the genuine nitrous acid isomerization mixture of linolenic acid showed seven peaks out of the eight theoretically possible isomerization components. After addition of a pure linolenic acid standard, this substance occurred in the true isomerization mixture in only spurious amounts, the latest eluted combined *cis/trans* isomeric compound becoming apparent only as a slight shoulder on the all-*cis*-octadecatriethylenic acid peak (Fig. 1).

As was assumed from the characteristics of the cyanopropylsiloxane stationary phase in GLC on packed columns[2], the SP 2340 capillary column made it possible to separate even positional isomeric acids. Consequently, $C_{18:1}$ fatty acid originating from isolated human red cell phospholipids gave three peaks (Fig. 3), which could be identified by means of known standards as elaidic (or isoelaidic), oleic and *cis*-vaccenic acids (Fig. 2) when enumerated in their elution order. A further *cis*-isooleic acid peak (see peak 27 in Fig. 3) possibly occurs in the biological material, but we had no standard ($C_{18:1c\omega5}$) for definite identification. Figs. 2 and 3 further outline sufficient or baseline separations for the all-*cis* isomers of $C_{18:3}$ ($\omega6$; $\omega3$), $C_{20:1}$ ($\omega12$; $\omega9$; $\omega7$), $C_{20:3}$ ($\omega6$; $\omega3$) and $C_{22:2}$ ($\omega9$; $\omega6$). Unfortunately, arachidonic acid eluted together with behenic and Δ17-eicosatrienoic acids, and Δ19-docosatrienoic acid had almost the same retention time as lignoceric acid. As individual components were recognizable by small shoulders in all of these combined peaks, we believe that it will be possible in future to separate these individual compounds on a 50-m Silar 10C or SP 2340 coated capillary column with a normal, *i.e.*, higher separation number than on our column. It is worth mentioning that the commercially available phytanic acid standard

gave two peaks with poor resolution, providing evidence of an unidentified phytanic acid impurity[3]. Identical behaviour of phytanic acid was observed on a 50-m FFAP capillary column[4] which, although characterized by a high separation number and consequently many more theoretical plates than ours, did not give a better overall column performance for the resolution of isomeric fatty acids in complex mixtures.

The complexity that can occur in biologically derived fatty acids is best demonstrated for crude human red cell phospholipids in Fig. 3. The numbers on top of the peaks are repeated and as far as possible identified in Table I, which gives the quantitative results derived from young people in comparison with those from old people. Further identification requires a combination of GLC with mass spectrometry. To avoid autoxidation during the analytical procedure, the antioxidant BHT (butylated hydroxytoluene; 2,6-di-*tert.*-butyl-*p*-cresol) was added. The capillary chromatogram in Fig. 4 demonstrates an artifact[5] produced by the permethylation procedure with a retention time identical with that of $C_{16:1c\omega7}$. As the artifact comprises 1.6 area-% of the BHT standard, we were able to calculate that the mean *cis*-hexadecenoic acid concentration was increased by 20–30%. Re-evaluating the work of Dodge and Phillips[5], we found 67 different peaks of greater quantitative importance, plus many additional acids in smaller amounts. This is more than double the number of peaks known hitherto and demonstrates the efficiency of our SP 2340 column.

Provided that the technical problems encountered in coating capillaries with SP 2340 or Silar 10C, even by very experienced manufacturers, can be satisfactorily

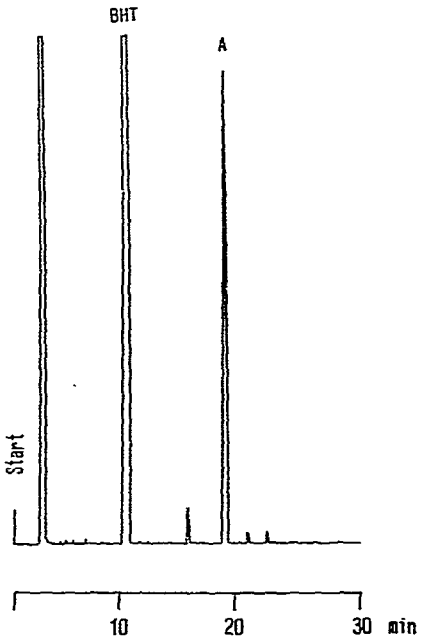

Fig. 4. Chromatogram of the antioxidant BHT following the boron trifluoride methylation procedure[11]. The additional peak A demonstrates an artifact produced by the methylation procedure. The artifact comprises 1.6 area% of the chromatogram. The remaining minute peaks were present in commercial BHT (Merck, Darmstadt, G.F.R.). The retention time for peak A was found to be identical with $C_{16:1c\omega7}$.

solved in the future, we believe that such columns will be highly selective for the separation of many types of isomeric fatty acids. Such high-performance capillary columns will probably obviate the laborious and time-consuming ozonization technique for the evaluation of the positions of double bonds in fatty acid analysis.

ACKNOWLEDGEMENT

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LIST OF SYMBOLS

n_{sep} = separation number, *i.e.* separated peaks within the one carbon number range of C_{16} and C_{17}
ΔT = total retention time of C_{17} − total retention time of C_{16}
$b_{0.5}$ = peak width at half height
t_d = (gas) hold-up time
t_s = adjusted retention time
K = capacity ratio
l = loadability
M = molecular weight of decanoic acid methyl ester
d = inner diameter of capillary column

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