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SEPARATION OF COMPLEX LONG CHAIN FATTY ACID MIXTURES BY HIGH-PERFORMANCE GLASS CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

Detailed descriptions are given for the preparation of high resolution open tubular glass capillary columns using SP-1000, Silar-5 CP and Silar-10 C as stationary phases. A comparison has been made between HCl etched soda lime glass capillaries and HCl leached soda lime or borosilicate glass capillaries with $BaCO_3$ deposition. The best results were obtained with Silar-5 CP coated on HCl leached borosilicate capillaries with $BaCO_3$ deposition. These types of columns have been used for the separation of complex long chain fatty acid mixtures including positional and geometrical isomers. The technique recommended has been used for 3 years and can easily be reproduced. A modification of the all-glass solvent-less injection system is also described. The qualitative and quantitative features of the glass capillary gas chromatography system are discussed.

INTRODUCTION

The development of thermostable open glass tubular capillary columns suitable for high resolution of fatty acids of biological origin has long been an aim of our laboratory in connection with studies on the biological effects of very long chain monounsaturated fatty acids[1]. We required a reproducible technique in order to make capillary columns suitable for our specific biological problems.

It is well known that not every polymer used as stationary phase for gas–liquid chromatography spreads uniformly over the glass surface. This fact is specially relevant to polar liquid phases. Therefore in recent years much effort has been directed towards finding a way of pretreating the glass surface of the capillary columns to meet, as far as possible, Giddings' condition[2] and decrease the contact angle of the stationary liquid phase with the glass[3]. One method is to chemically bind reactive polysiloxane polymers to the capillary glass surface[4]. Another is to increase the wettability of the wall by producing crystals of NaCl[3,5] or $BaCO_3$[6,7] to provide an appropriate degree of irregularity, since it is possible to prepare suitable films on surfaces having a high degree of microirregularity. Grob *et al.*[8,9] have shown the possibilities of the persilylation procedure. Godefroot *et al.*[10] have proposed some modifications

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of the persilylation procedure. Both methods include a leaching process which dissolves and eliminates the metal oxides of the glass surface and leaves the maximum concentration of free silanol groups. The different techniques for preparation of glass capillary columns have recently been reviewed by Lee and Wright[11].

Since 1958 when Lipsky and co-workers[12,13] demonstrated the use of high resolution open tubular capillary columns for the separation of long chain fatty acid esters including geometrical and positional isomers, many papers have dealt with the application of this technique to the lipid field[14–19]. Heckers *et al.*[17] showed the possibilities of the cyanopropyl siloxane type of stationary liquid phases in glass capillary gas chromatographic analysis of long chain fatty acids. These columns usually have different characteristics depending on the batch of stationary liquid phase used for their preparation[20]. The authors[12–20] mentioned above used either "home-made" columns without giving any detailed description of the preparation of the glass capillary columns, or commercially available columns.

It is not always possible to obtain commercial capillary columns with separation features which allow a solution to specific separation problems. Our experience with the separation and quantitation of complex long chain fatty acid mixtures including geometrical and positional isomers clearly indicates that it is important to be able to prepare one's own glass capillary columns. In the present work we give detailed descriptions for the preparation of high-performance glass capillary columns. The technique that we recommend can easily be reproduced and has been used in this laboratory for 3 years for quantitation and qualitation of fatty acids from biological origins.

EXPERIMENTAL

Capillary tubing

Prior to drawing, the glass tubes were treated with chromosulphuric acid (E. Merck, Darmstadt, G.F.R.) overnight. The glass tubes were washed with distilled water followed by acetone and finally air-dried[5]. A Hupe and Bush 1045 A capillary drawing machine was used. The coiling tube was lubricated with talcum powder instead of graphite.

HCl etching of capillary tubes

Capillaries drawn from soda lime (soft) glass tubes were etched with dry HCl gas to produce NaCl crystals on the glass surface. Prior to etching the capillary tubes were dried at 360°C under a stream of He (250 kPa) for 3 h. The "static" HCl etching method was used[3,5]. The capillary tubes were purged with HCl gas (200 kPa, purity 99%) for 10 min. After sealing both ends with a microflame, the capillary tubes were heated to 360°C for 8 h. The etching procedure was repeated once as the amount of HCl gas in the tube is the limiting factor for production of the NaCl crystals. The double etched columns were then purged with dry He at room temperature and finally sealed at both ends to protect the NaCl crystal layer from moisture.

$BaCO_3$ deposition in capillary tubes

Capillary columns drawn from either soda lime or borosilicate glass were used. The capillary wall surface was roughened by deposition of $BaCO_3$ crystals. The $BaCO_3$ procedure described by Grob *et al.*[7] was used with some modifications.

Prior to $BaCO_3$ deposition the capillary tubes were acid leached to remove soluble components from the glass surface in order to produce a silica-rich layer. The capillary tubes were partially filled with a 20% HCl solution (80% of the length of columns with 0.25 mm I.D., 60% for columns with 0.30 mm I.D.). To avoid contact with metal parts, the HCl solution was introduced by means of a coating reservoir (Scientific Glass, North Melbourne, Australia). One end of the capillary column was straightened and inserted into the HCl solution in the bottom of the reservoir via a septum-type seal at the top of the unit. The reservoir was connected via a gas pressure regulator to a gas bottle, and the plug of HCl solution was forced through the column. When the first drops appeared at the end of the column, the whole interior surface was wet and essentially free from particles and dirt. The other end was transferred to a vacuum pump and the plug was moved backwards by about 10% of the column length. The free end was wiped and sealed with a microflame. After further movement of the plug the remaining end was sealed under vacuum to avoid any gas expansion during the heating period. After leaching the sealed columns overnight at 150°C (soda lime glass columns) or 210°C (borosilicate glass columns), they were rinsed by forcing a plug of distilled water corresponding to 20% of the column length through the column. Finally the column was dried at 240°C under He for 4 h. The reason for leaching the borosilicate glass used at a temperature of 210°C is to avoid agglomeration of the $BaCO_3$ crystals when using a saturated solution of $Ba(OH)_2$. On leaching at even higher temperatures (230°C) the glass became more active and the ends impossible to straighten with a microflame without whisker or bubble formation.

To the leached and dried capillary column, buffer columns (about 60 coils) were connected at both ends. The buffer column at the outlet was connected to a vacuum pump and that at the inlet was filled by suction with 0.5% HCl solution corresponding to 10% of the column length, one coil of air, saturated $Ba(OH)_2$ solution containing 0.01% Marlazin L 10 (Chemische Werke Huls, G.F.R.) corresponding to 20% of the column length and finally one and a half coils of air. The buffer column at the inlet was connected to a carbon dioxide bottle and the speed of the plug was adjusted to 1 cm/sec with a pressure regulator. To obtain a stable pressure, the regulator was set at a high flow range. The relatively high gas flow through the regulator was then reduced in a splitter controlled by a needle restrictor. When all the liquid had entered the buffer column at the outlet it was removed and the CO_2 gas was replaced with Ar. After purging the column with Ar for 1 h, the column was transferred to a gas chromatograph and purged with He at 90°C for 1 h. The column was then rinsed twice by forcing a plug of acetone (about 10 coils) through the column at a speed of 1 cm/sec. Finally the column was dried at room temperature under Ar for 1 h.

Deactivation of capillary tubes

The roughened capillary tubes were deactivated with Carbowax 1000. A 1% solution of Carbowax 1000 in dichloromethane was forced through the column at a speed of 1 cm/sec. The remaining solvent was evaporated under Ar. Both ends of the capillary were sealed with a microflame, still under slight argon pressure to exclude air, and the column was heated to 280°C for 8 h. Finally the treated column was washed twice with 1 ml acetone and dried under Ar.

Dynamic coating of capillary tubes

The mercury drop dynamic method[21] was used with some modifications. The column to be coated was provided with buffer columns (about 60 coils) at both ends to ensure a constant speed through the column of a plug of stationary phase dissolved in dichloromethane. The coating reservoir described in the leaching procedure and equipped with the needle restrictor controlled splitter was used. From the reservoir, 40 coils of the buffer column were filled with 15% SP-1000 or Silar-10 C in dichloromethane followed by one coil ($\pm$1 cm) of mercury. The speed of the plug through the column was adjusted to 1 cm/sec. This arrangement made it possible to keep the column hanging with the coils in a vertical position without any drastic changes in the "up" and "down" speed. After the mercury plug had entered the second buffer column, the coated capillary column was disconnected and sealed at one end with a microflame. The open end was connected to a vacuum pump and the column was evacuated for 24 h. The remaining solvent was thus evaporated without the appearance of any solvent lenses. Finally the column was opened and purged with Ar for 1 h.

Static coating of capillary tubes

The static coating method described by Rutten and Rijks[22] was used with some minor practical modifications. A freshly prepared coating solution containing 0.2% (v/v) Silar-5 CP in dichloromethane was degassed by application of a slight vacuum in an ultrasonic bath. It was then forced into the column by means of a 10-ml gas tight syringe. The connection to the column was made by shrinking PTFE tube (Chrompack, Middelburg, The Netherlands) by heating. To overcome the great back pressure built up in the column, a laboratory elevator was applied to the syringe plunger. The free end of the column was inserted into a coloured water glass solution, previously degassed in a vacuum desiccator. When the first drops appeared at the column end, the direction of the syringe plunger was reversed in order to suck some of the water glass into the column. Any air bubbles between the two solutions must be avoided. The water glass was allowed to dry overnight. The column was placed in a water tank and the open end was connected to a vacuum pump. The vacuum was increased until the solvent began to evaporate. The water tank was not temperature regulated but covered with aluminium foil. After the solvent had been completely evaporated, two coils from each end of the column were removed and the column was ready for conditioning.

Column conditioning

Freshly coated capillary columns were conditioned at normal flow-rates. The temperature was raised from 70°C to 240°C at 3°C/min, then kept at this temperature for 8 h.

Injection system

In connection with quantitative analysis of fatty acids using glass capillary columns, the theory of different injection systems has been much debated[23]. In early work with packed columns we found that it was of great advantage to use a simple solid injection technique for separation of high boiling components[24]. Kuppens and De Jong[25] described the first all-glass solvent-less injection system for glass capillary gas chromatography which was later modified by Van den Berg and Cox[26]. We have found their system very fragile in practical work and made further modifications.

By cutting the glass supporting tube of a commercially available solvent-less injector into two parts and modifying the lower part as shown in Fig. 1, the system became more easy to handle. This part was more accessible for cleaning and exchangeable when broken. Both parts were fixed with nuts and graphite ferrules into the injection tube of a Hewlett-Packard 5710 A or Packard 427 gas chromatograph. However, to make the system gas tight, the end of the injection tubes had to be widened to 1/4 in. I.D., but the standard carrier gas inlet could be used, so that the carrier gas entered the injection system between the upper and lower parts of the modified injector. The capillary column was connected to the injection system by means of a shrinking polytetrafluoroethylene tube (Chrompack, Middelburg, The Netherlands). This material was easy to handle and could be used up to 260°C without leaks.

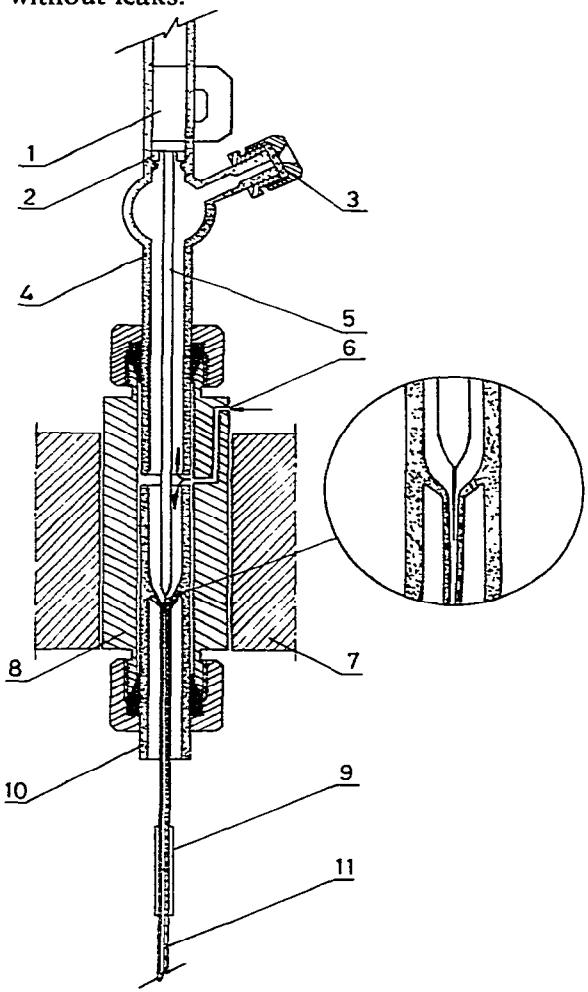

Fig. 1. Diagram of a modified all-glass solvent-less injector originally given by Van der Berg and Cox[20]. 1 = Magnet; 2 = polytetrafluoroethylene washer; 3 = septum; 4 = upper part of injector; 5 = glass needle; 6 = carrier gas inlet; 7 = oven wall; 8 = injection port; 9 = polytetrafluoroethylene shrinking connection; 10 = lower part of injector; 11 = glass capillary column.

Testing of the glass capillary gas chromatography system

The described system was evaluated using reference mixtures of fatty acid methyl esters. Most of the fatty acids were obtained from Nu Check Prep. (Elysian, MN, U.S.A.), Larodan Fine Chemicals (Malmö, Sweden) and Supelco Inc. (Bellefonte, PA, U.S.A.). Geometrical and positional isomers of octadecenoic acid were a generous gift from Prof. F. D. Gunstone (University of St. Andrews, St. Andrews, Great Britain). For evaluation of column separation efficiency a mixture of about 70 fatty acid methyl esters of different chain lengths and different degrees of unsaturation, including some geometrical and positional isomers, was used (Table I). The real plate number, n_{real}, and separation number, SN, were calculated according to Kaiser and Rieder[27]. To evaluate the quantitative features of the system used, four different mixtures of known amounts of fatty acid methyl esters of different chain

TABLE I

FATTY ACIDS USED IN THE EVALUATION OF THE GLASS CAPILLARY GAS CHROMATOGRAPHY SYSTEM

*Peak no.**	*Fatty acid***	*Peak no.*	*Fatty acid*	*Peak no.*	*Fatty acid*
1	6:0	2	8:0	3	9:0
4	10:0	5	11:0	6	12:0
7	12:1 ω1	8	13:0 *anteiso*	9	BHT
10	13:0	11	14:0 *iso*	12	14:0
13	14:1 ω5c	14	15:0 *anteiso*	15	15:0
16	16:0 *iso*	17	16:0 *anteiso*	18	16:0
19	16:1***	20	16:1 ω7c	21	17:0 *anteiso*
22	17:0	23	18:0 *iso*	24	18:0 *anteiso*
25	18:0	26	18:1***	27	18:1 ω13c
28	18:1 ω12c	29	18:1 ω9c	30	18:1 ω8c
31	18:1 ω7c	32	18:1 ω6c	33	18:1 ω5c
34	18:1 ω15c	35	18:1 ω4c	36	18:1 ω3c
37	18:1 ω1	38	19:0 *anteiso*	39	18:2 ω6c
40	18:1 ω2c	41	19:0	42	18:3 ω6c
43	18:3 ω3c	44	20:0 *iso*	45	20:0
46	20:1 ω15c	47	20:1 ω12c	48	20:1 ω9c
49	20:1 ω3c	50	20:1 ω7c	51	20:2 ω6c
52	21:0	53	20:3 ω9c***	54	20:3 ω6c
55	20:4 ω6c	56	20:3 ω3c	57	20:5 ω3c***
58	22:0	59	22:1 ω11c	60	22:1 ω9t
61	22:1 ω9c	62	22:1 ω7c	63	22:2 ω9c, ω17c
64	22:1 ω3c	65	22:2 ω6c	66	23:0
67	22:4 ω6c	68	22:3 ω3c	69	22:5 ω6c***
70	22:5 ω3c***	71	22:6 ω3c	72	24:0
73	24:1 ω9c	74	26:0		

* The peak numbers correspond to the peaks in Figs. 2–5.

** The shorthand notation used for the fatty acids indicates chain length: number of double bonds. ω = First double bond position beginning from the hydrocarbon end; methylene interruption is assumed if not otherwise specified. *c* = *cis*; t = *trans*. BHT = 2,6-Di-*tert.*-butyl-*p*-cresol.

*** Only occurred in biological samples and not included in the reference mixture.

TABLE II

ACCURACY AND IMPRECISION OF THE GLASS CAPILLARY GAS CHROMATOGRAPHY SYSTEM CALCULATED FROM KNOWN AMOUNTS OF FATTY ACID METHYL ESTERS INJECTED ON A SP-1000 COLUMN

The shorthand notation used for the fatty acid esters indicates chain length: number of double bonds. Each value of analyzed composition represents the mean of five analyses. C.V. = Coefficient of variation.

Fatty acid methyl ester	*Known composition (wt. %)*	*Analyzed composition (area %)*	*C.V. (%)*	*Fatty acid methyl ester*	*Known composition (wt. %)*	*Analyzed composition (area %)*	*C.V. (%)*
16:0	20.0	20.1	2.9	16:1	20.0	19.9	2.2
18:0	20.0	20.4	1.3	18:1	20.0	20.4	1.0
20:0	20.0	20.1	0.5	20:1	20.0	20.2	0.8
22:0	20.0	20.1	2.5	22:1	20.0	19.2	1.1
24:0	20.0	19.3	0.6	24:1	20.0	20.3	3.0
18:0	25.0	25.1	0.5	20:0	20.0	20.7	0.5
18:1	25.0	24.5	0.6	20:1	20.0	20.0	0.6
18:2	25.0	25.5	0.3	20:2	20.0	20.9	0.3
18:3	25.0	24.9	0.5	20:3	20.0	19.2	0.6
				20:4	20.0	19.2	0.5

lengths and degree of unsaturation (Table II) were analyzed using a CDS-111 chromatography data system (Varian, Palo Alto, CA, U.S.A.). The results were expressed as area %. The geometrical and positional isomers of octadecenoic acid were used to study in more detail the elution behaviour of different isomers.

RESULTS AND DISCUSSION

Fig. 2 shows the separation of a standard mixture of fatty acid methyl esters used for evaluation of column efficiency on a HCl etched soda lime glass capillary column coated with SP-1000 according to the dynamic procedure. The different fatty acids were well separated but there was a tendency for tailing. SP-1000, a polyether with substituted terephthalic acid, is a moderately polar stationary phase. Thus there was a low degree of chain length overlap and in most cases *trans* monounsaturated fatty acids were eluted after the corresponding *cis* fatty acids. Polysiloxane stationary phases substituted with various combinations of phenyl and cyanoalkyl functional groups could not be coated on HCl etched soda lime capillary columns under the conditions used. Probably the soda lime glass has a high catalytic activity on this type of stationary phase.

However, somewhat better results were obtained when HCl leached soda lime glass capillaries with $BaCO_3$ deposition were coated with these types of polysiloxane stationary phases. Fig. 3 shows the results after dynamic coating of Silar-10 C on these types of pretreated columns. The separation efficiency was relatively poor and a high degree of chain length overlap was obtained with this very polar stationary phase. *Trans* monounsaturated fatty acids were eluted before the corresponding *cis*

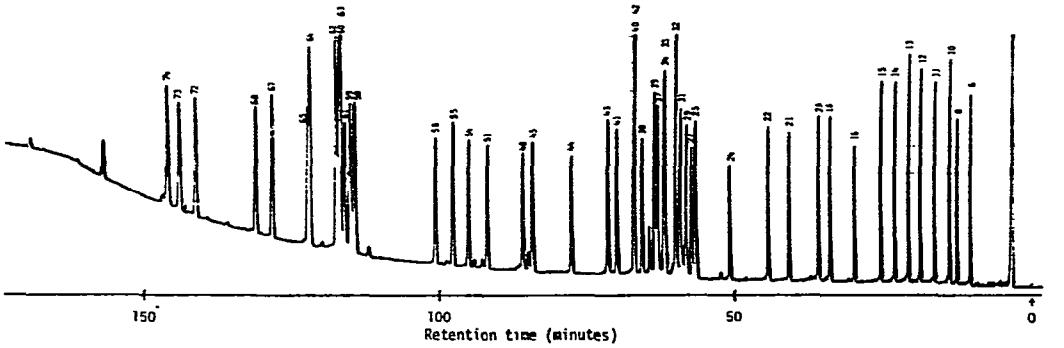

Fig. 2. Chromatogram of a standard mixture of fatty acid methyl esters separated on a HCl etched soda lime glass capillary column coated with SP-1000. Column: 60 m × 0.25 mm I.D.; temperature 150°C isothermal for 4 min, then programmed at 0.5°C/min to 230°C, finally isothermal for 16 min. Helium flow-rate: 0.4 ml/min. Peak numbers as in Table I.

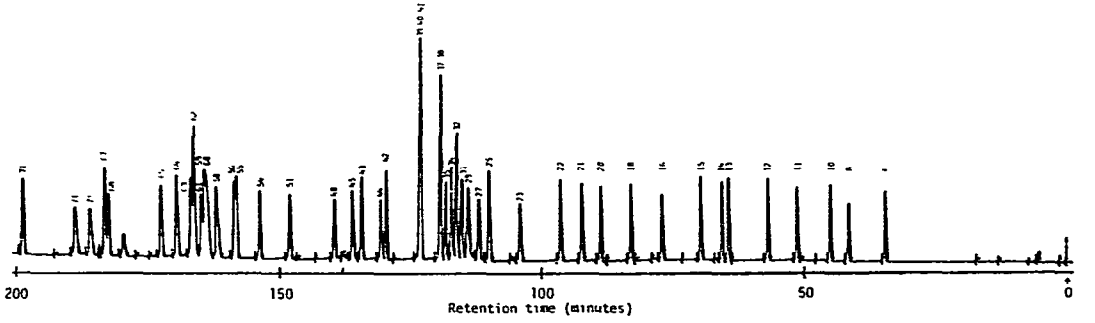

Fig. 3. Chromatogram of a standard mixture of fatty acid methyl esters separated on a HCl leached soda lime glass capillary column with $BaCO_3$ deposition coated with Silar-10 C. Column: 50 m × 0.25 mm I.D.; temperature 140°C isothermal for 8 min, then programmed at 0.5°C/min to 220°C, finally isothermal for 16 min. Helium flow-rate: 0.6 ml/min. Peak numbers as in Table I.

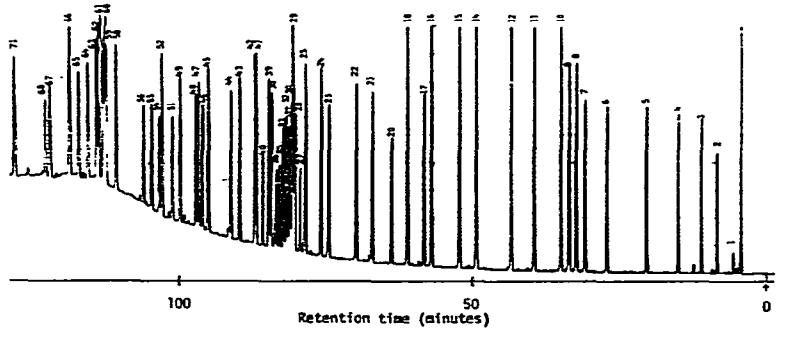

Fig. 4. Chromatogram of a standard mixture of fatty acid methyl esters separated on a HCl leached soda lime glass capillary column with $BaCO_3$ deposition coated with Silar-5 CP. Column: 50 m × 0.25 mm I.D.; temperature 120°C isothermal for 4 min, then programmed at 1°C/min to 220°C, finally isothermal for 16 min. Helium flow-rate: 0.5 ml/min. Peak numbers as in Table I.

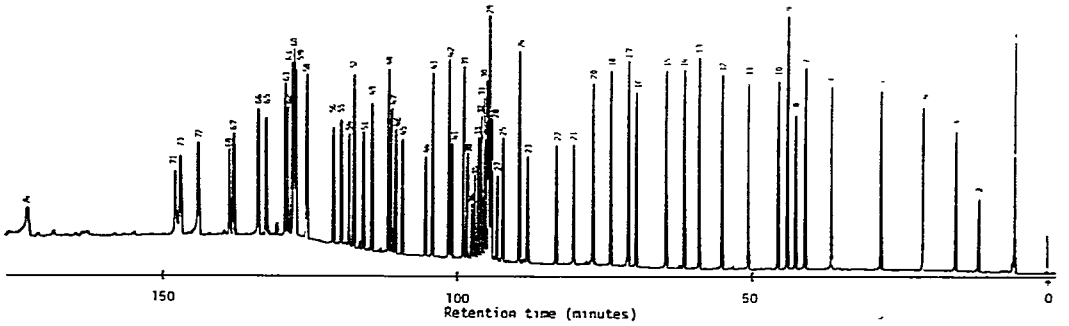

Fig. 5. Chromatogram of a standard mixture of fatty acid methyl esters separated on a HCl leached borosilicate glass capillary column with $BaCO_3$ deposition coated with Silar-5 CP. Column: 58 m × 0.30 mm I.D.; temperature 100°C isothermal for 4 min, then programmed at 1°C/min to 220°C, finally isothermal for 60 min. Helium flow-rate: 0.6 ml/min. n_{real} = 131.000; SN = 24. Peak numbers as in Table I.

fatty acids. Columns coated with Silar-5 CP on these capillaries showed a high separation efficiency, but had a very short lifetime (Fig. 4).

The best results were obtained by coating Silar-5 CP on HCl leached borosilicate glass capillaries with $BaCO_3$ deposition, as shown in Fig. 5. These columns generally had a high separation efficiency, with a real plate number of more than 130,000 and a separation number of about 24. As shown in Fig. 6, the relative bleeding from these types of borosilicate glass capillaries was low compared to columns prepared from soda lime glass capillaries. Consequently, columns coated with Silar-5 CP on borosilicate glass capillaries with $BaCO_3$ deposition were thermostable and had a long lifetime. The columns were slightly more polar than SP-1000 columns.

The accuracy and imprecision of the glass capillary gas chromatography system was investigated by injection of known amounts of fatty acid mixtures. Different mixtures containing equal amounts of four or five fatty acids with various chain lengths or degrees of unsaturation were analyzed. In Table II, results obtained after analysis on an SP-1000 column are shown. The results indicate that the influence of chain length on quantitation was negligible and that there was only a very small discrimination tendency of polyunsaturated fatty acids. With a few exceptions, the coefficient of variation was lower than 2%. Similar results were obtained after analysis on Silar-5 CP coated on borosilicate glass capillaries with $BaCO_3$ deposition.

The influence of the location and geometrical configuration of the double bond on the elution behaviour of octadecenoic acid on SP-1000 and Silar-5 CP columns is shown in Fig. 7. Generally, octadecenoic acids with the double bond located near the middle of the molecule were difficult to separate. It was easier on both columns to separate positional *cis*-isomers than *trans*-isomers. When analyzed on SP-1000, *trans*-isomers of octadecenoic acid with the double bond located at high ω-positions (8–13) were eluted after the corresponding *cis*-isomers. Reversed elution orders were obtained for isomers with the double bond located at low ω-positions (3–7). Columns coated with Silar 5-CP showed better separation capacity for geometrical isomers than SP-1000 columns. Our experiments with the more polar stationary phase Silar-10 C showed an even better separation of geometrical isomers, but not sufficient to produce a complete group separation of *cis* and*trans* monounsaturated fatty acids

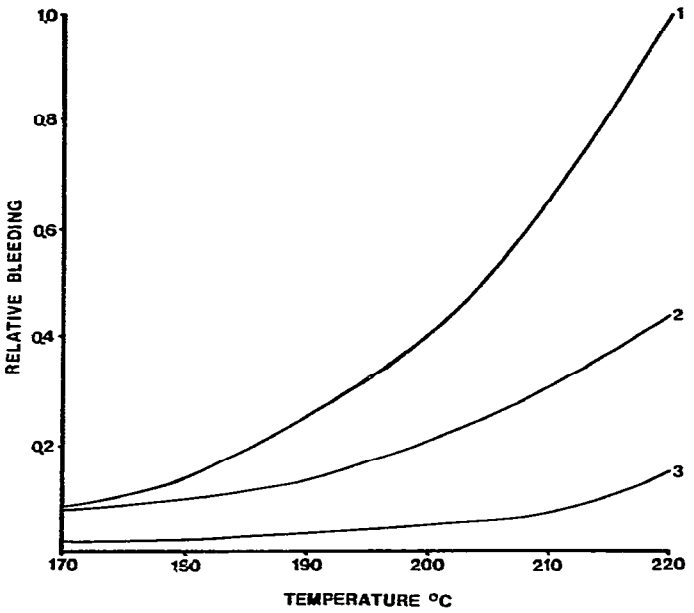

Fig. 6. Relative bleeding rates of different capillary columns under a temperature programme of 1°C/min. 1, HCl etched soda lime glass column coated with SP-1000; 2, HCl leached soda lime glass column with $BaCO_3$ deposition coated with Silar-5 CP; 3, HCl leached borosilicate glass column with $BaCO_3$ deposition coated with Silar-5 CP.

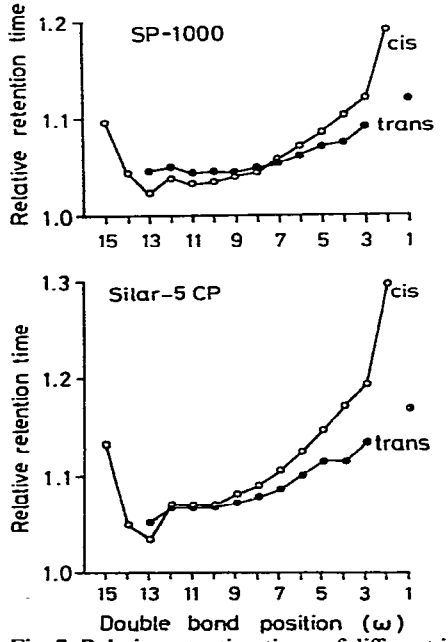

Fig. 7. Relative retention times of different isomers of octadecenoic acid methyl esters as a function of the position and geometry of the double bond. The retention times are related to the retention time of stearic acid methyl ester (18:0). The double bond position (ω) is stated from the hydrocarbon end. For further details, see Figs. 2 and 5.

from partially hydrogenated vegetable or marine oils. *Trans* monounsaturated fatty acids from this type of oils often have double bonds distributed over a great number of positions, including the low ω-positions. Consequently these *trans*-isomers interfered in the analysis of *cis*-isomers having the double bond located at high ω-positions.

To overcome this problem we have developed a combined technique based on high-performance liquid chromatography (HPLC) and glass capillary gas chromatography[28]. After a group separation by HPLC according to geometry and chain length, the *cis* and *trans* monounsaturated fatty acids were collected and analyzed for their content of positional isomers using high efficiency Silar-5 CP capillary columns. These columns were also used to determine the distribution of naturally occurring fatty acids.

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