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EQUIVALENT CHAIN-LENGTHS OF METHYL ESTER DERIVATIVES OF FATTY ACIDS ON GAS CHROMATOGRAPHY

A REAPPRAISAL

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SUMMARY

The equivalent chain-lengths of the methyl ester derivatives of synthetic isomeric unsaturated fatty acids in addition to many from natural sources have been determined by gas chromatography (GC) with modern wall-coated open-tubular columns of fused silica coated with Carbowax 20M™, Silar 5CP™, CP-Sil 84™ and a 5% phenyl–methyl silicone. The complete series of *cis*-octadecenoates (3- to 17-, but not the 2-), the methylene-interrupted *cis,cis*-octadecadienoates (3,6- to 14,17- but not the 2,5-isomer) and some *cis,cis*-octadecadienoates with more than one methylene group between the double bonds (5,12-, 6,12-, 7,12-, 8,12-, 6,10- and 6,11-) were used, together with natural fatty acids from pig testis, rich in the $(n-6)$ family, and from cod liver oil, rich in the $(n-3)$ family. There were 79 fatty acids in total. The availability of all these data from a single laboratory at one time allows a reassessment of the value of GC equivalent chain-lengths for the identification of unknown fatty acids, and of stationary phases of particular types for fatty acid analysis in general.

INTRODUCTION

The carbon number[1] or equivalent chain-length (ECL) concepts[2] for the identification of fatty acids separated in the form of the methyl ester derivatives by gas chromatography (GC) have proved of great value to analysts. An enormous amount of data has been produced, and this has been reviewed comprehensively by Jamieson[3] and by Ackman[4,5]. The nature of the stationary phase is the primary factor determining retention values, but nominally identical phases from different manufacturers and even from different batches can vary somewhat. In packed column work especially, it is well established that ECL values are rather susceptible to such factors as the age and conditioning of the column, column dimensions, the loading of the phase on the inert support, and the nature of the support[3]. Operational factors such as column temperature and the nature and flow-rate of the carrier gas are also important. Much smaller but real differences are seen with wall-coated open-tubular (WCOT) columns made from fused silica, as opposed to stainless steel[5]. It is not

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surprising, therefore, that appreciable variation in ECL values are reported between laboratories. Some of the more useful studies of the GC retention properties of fatty acids have been made with synthetic isomeric compounds. For example, data are available for the complete series of *cis*- and *trans*-octadecenoates, all the methylene-interrupted octadecadienoates, and for some octadecadienoates with more than one methylene group between the double bonds[3–5]. Unfortunately, many of the relevant measurements were performed at different times in different laboratories around the world. In addition, a high proportion of the determinations were made twenty years or so ago, and there has been a dramatic change in the nature of the stationary phases in common use and in the physical nature of the columns during the intervening period. For example, fused-silica WCOT columns are now widely available and are likely to become the standard, and the inherent resolution of these is such that there has been a trend towards liquid phases of much lower polarity than is the common practise with packed columns.

The author recently had access to a number of different isomeric C_{18} fatty acids, including most of the isomeric *cis*-octadecenoates and methylene-interrupted *cis,cis*-octadecadienoates, and some *cis,cis*-octadecenoates with more than one methylene group between the double bonds for a study of their mass spectrometric (MS) properties[6,7]. In addition, two natural samples, namely cod liver oil and pig testis lipids, containing a wide range of different unsaturated fatty acids, were characterised by related GC–MS procedures and were available to extend the scope of this study[8,9]. ECL values have now been obtained for all of these fatty acids as the methyl esters over a short period in one gas chromatograph with modern WCOT columns of fused silica and coated with liquid phases in current use. Strict comparisons are, therefore, possible.

EXPERIMENTAL

Isomeric *cis*-octadecenoates (3- to 17-isomers)[10], methylene-interrupted octadecadienoates (3,6- to 14,17-isomers)[11] and 5,12-, 6,12-, 7,12-, 8,12-, 6,10- and 6,11-octadecadienoates[12] had been prepared earlier by total synthesis. Pig testis lipids and cod liver oil samples were from a previous study[8]; these were methylated and then were fractionated according to degree of unsaturation by high-performance liquid chromatography with a silver ion column as described elsewhere[13].

A Carlo Erba Model 4130 capillary gas chromatograph (Erba Science, Swindon, U.K.), fitted with split/splitless injection and equipped with capillary columns of fused silica coated with various stationary phases, was used. Columns coated with Carbowax 20M™, Silar 5CP™ and CP-Sil 84™ (25 m × 0.22 mm I.D.) were obtained from Chrompak (London, U.K.), and one coated with a 5% phenyl–methyl silicone (25 m × 0.2 mm I.D.) was obtained from Hewlett-Packard (Wokingham, U.K.). The Silar 5CP column had been in use for some months, but the remainder were new and were conditioned in the gas chromatograph for 48 h before use. Hydrogen was the carrier gas in each instance at a flow-rate of 1 ml/min. For ECL measurements, isothermal conditions were used, selected so that the longest running component [usually the 22:6 ($n-3$) fatty acid] emerged in about 1 h, *i.e.*, silicone at 175°C, Carbowax 20M at 175°C, Silar 5CP at 170°C, and CP-Sil 84 at 160°C. Samples were injected in hexane, and a small amount of a standard solution of 16:0, 18:0 and

20:0 fatty acid methyl esters was introduced simultaneously. Retention times were measured from the time of elution of the solvent, considered as an unretained solute. Each ECL value was determined in duplicate, but they were rarely more than 0.01 of a unit apart.

For purely analytical runs with the natural lipid materials and the Carbowax 20M column, the temperature was held at 165°C for 3 min, it was temperature programmed at 4°C/min to 195°C, then was held at this point for a further 23 min. With the CP-Sil 84 column, the temperature was maintained at 150°C for 2 min, it was temperature progrtammed at 2°C/min to 180°C, then was held at this point for a further 10 min.

RESULTS AND DISCUSSION

The selection of the most appropriate phases for this study was not easy, because of the range available, even for WCOT columns of fused silica. It was certainly essential to have a column of Carbowax 20M, since Ackman[5] has proposed that phases of the Carbowax 20M™ type should be utilised in the "'standard' reference WCOT column for interlaboratory studies as well as for application in its own right". Phases such as FFAP™, Supelcowax-10™, and SP-1000™, for example, are very similar to this. Silar 5CP was selected as a common phase, slightly more polar than Carbowax 20M, and with somewhat different selectivity. CP-Sil 84 is a much more polar phase, similar in its properties to the widely used polymer of diethyleneglycol-succinate (DEGS). A non-polar silicone phase was selected as some analysts favour its use for fatty acid analysis[14], and its low bleed characteristics render it particularly suitable for GC–MS purposes.

The ECL values of each of the isomeric C_{18} fatty acids were obtained and the results are listed in Table I. While the absolute values differ for each stationary phase, increasing with the polarity of the phase, the elution patterns are broadly similar. Thus the ECL values are lowest when the double bonds are approximately central, *i.e.* in positions 8 or 9. They increase relatively rapidly as the double bonds near the terminal (methyl) end of the molecule, reaching a maximum with 16–18:1, before falling slightly for the 17-isomer. Similarly, the ECL values increase, although rather more slowly, as the double bonds near the carboxyl group and reach a second lower maximum at 3-18:1. They would then be expected to drop for the 2-isomer[15,16]. In addition, the ECL value of the 6-isomer is sometimes slightly out of line, being higher than those of adjacent isomers. The results are similar in nature to those obtained earlier with very different columns[15,16]. A theoretical explanation of the phenomenon in terms of the shapes of molecules and the opportunities for interaction between the double bonds and the walls of a WCOT column has been published[17].

As isomers differing in ECL value by about 0.04 should be separable on most WCOT column, it would be expected that those fatty acids with central double bonds (about 4-18:1 to 9-18:1) will not be easily resolved. Petroselinic (6-18:1) and oleic occur together in some seed oils and are not readily resolved by GC[18]. In the monoenoic fatty acids from animal tissues, there tend to be isomers in which the double bond positions are two carbon atoms apart, because they are formed biosynthetically from homologous fatty acids by chain-elongation or by β-oxidation, in each instance the difference being two carbon atoms. Thus 16:1($n-9$) and 16:1($n-7$), 18:1($n-9$)

TABLE I

EQUIVALENT CHAIN-LENGTHS OF THE METHYL ESTER DERIVATIVES OF ISOMERIC C_{18} MONO- AND DIENOIC FATTY ACIDS

Fatty acid	*Stationary phase*			
	Silicone	*Carbowax*	*Silar 5CP*	*CP-Sil 84*
3-18:1	17.91	18.44	18.47	18.64
4-18:1	17.80	18.19	18.21	18.30
5-18:1	17.72	18.09	18.17	18.29
6-18:1	17.75	18.18	18.25	18.43
7-18:1	17.72	18.14	18.24	18.40
8-18:1	17.72	18.14	18.29	18.43
9-18:1	17.73	18.16	18.30	18.47
10-18:1	17.75	18.19	18.32	18.49
11-18:1	17.78	18.23	18.36	18.54
12-18:1	17.83	18.30	18.46	18.62
13-18:1	17.89	18.37	18.52	18.67
14-18:1	17.95	18.46	18.57	18.76
15-18:1	18.00	18.56	18.62	18.83
16-18:1	18.19	18.84	18.91	19.14
17-18:1	17.94	18.54	18.61	18.82
3,6-18:2	17.73	18.69	18.74	19.14
4,7-18:2	17.58	18.43	18.48	18.82
5,8-18:2	17.53	18.36	18.48	18.85
6,9-18:2	17.59	18.47	18.63	19.04
7,10-18:2	17.57	18.45	18.70	19.02
8,11-18:2	17.61	18.50	18.75	19.12
9,12-18:2	17.65	18.58	18.80	19.20
10,13-18:2	17.73	18.67	18.88	19.30
11,14-18:2	17.81	18.79	18.98	19.41
12,15-18:2	17.89	18.91	19.08	19.51
13,16-18:2	18.12	19.28	19.41	19.90
14,17-18:2	17.90	18.98	19.06	19.52
5,12-18:2	17.56	18.40	18.60	18.93
6,12-18:2	17.60	18.48	18.69	19.06
7,12-18:2	17.56	18.40	18.64	18.99
8,12-18:2	17.63	18.49	18.70	19.07
6,10-18:2	17.58	18.42	18.61	18.94
6,11-18:2	17.52	18.38	18.62	18.93

and 18:1($n-7$), and 20:1($n-11$), 20:1($n-9$) and 20:1($n-7$) are frequently found together, as in the cod liver oil sample, and they are usually separable.

With the C_{18} dienes and each of the phases examined (Table I) also, the ECL values tended to increase with the distance of the double bonds from the carboxyl group, though there are discontinuities for the 3,6- and 13,16-isomers, where the ECL values are higher than those of adjacent compounds (*c.f.* the data for the 3- and 16-monoenoic isomers). The 2,5-isomer was not available, but its ECL values would be expected to be lower than those of the adjacent (3,6-isomer). Again the pattern is qualitatively similar to that obtained in older work with very different columns[19].

As the first natural substrate for analysis, pig testis lipids were selected, as the fatty acids have been well-characterised and have been used as an external standard in the Hormel Institute for some years[20,21]; they contain a wide range of fatty acids of the $(n-6)$ series, encountered typically in animal tissues. The second substrate is cod liver oil, which has also been well-characterised and recommended as an external standard in the analysis of lipids of marine origin[22]; it contains a wide range of fatty acids, and especially those of the $(n-3)$ family. Both of these materials were used by the author in studies of the efficacy of picolinyl ester derivatives of fatty acids for identification by GC–MS[8,9]. Together, they contain most of the fatty acids likely to be encountered in the common animal tissues. The ECL values were determined on fractions obtained by silver ion chromatography in order to simplify identifications and as the retention times of esters are influenced to some extent by components eluting immediately adjacent to them. The data is listed in Table II and is entirely consistent with that for the synthetic standards. Data such as these from WCOT columns of fused silica probably have greater relevance to other laboratories than those from packed columns. Absolute values may vary somewhat among laboratories, but the order of elution should not change.

One major advantage of determining all these ECL values at the same time in a single laboratory is that strict comparisons can be made. Thus, if the fractional chain-length (FCL) values (*i.e.* ECL minus 18) for the complete series of C_{18} monoenes is used to predict the ECL values for the dienes, the calculated value is generally somewhat lower than that actually found. This probably means that there is some interaction (possibly homo-conjugation) between the double bonds or with the diallyl methylene group, that increases the dipole moment of the unsaturated system. Comparable results were obtained in other studies, and the same principle held whether the double bonds were of the *cis*- or the *trans*-configuration[3–5]. The differences between the actual and predicted ECL values for each of the dienes is listed in Table III. With the methylene-interrupted isomers, the discrepancy tends to vary with the position of the double bonds as well as with the stationary phase, and is highest for double bonds in positions 5–11 (0.10–0.15) and diminishes towards either end of the molecule. Similarly, when the FCL values from the monoene data are used to calculate ECL values for the non-methylene-interrupted dienes, the difference between the actual and predicted results was found to be small, *i.e.* of the order of 0.05–0.08, when there are two methylene groups between the double bonds with the Carbowax and silicone liquid phases; it becomes negligible, *i.e.* 0.00–0.02 or even negative, when there are more than two methylene groups with all the phases. Again, these data are similar in kind to those reported earlier.

The FCL values for monoenes together with factors for the interaction with the appropriate methylene groups (the difference between the actual and predicted results for the dienes) have been used by Ackman *et al.*[23] especially for the prediction of ECL values, *e.g.* for the identification of an 18:5$(n-3)$ in a marine alga. If the ECL values obtained here are used in this way, using the data from Tables I and III, it would be predicted that an ECL value for an 18:4$(n-3)$ fatty acid on Carbowax 20M, for example, would be equal to 18 plus the FCL values (0.18 + 0.16 + 0.30 + 0.56 = 1.20) plus the methylene group factors (0.13 + 0.12 + 0.05 = 0.30), *i.e.* ECL = 19.50; the actual value found by direct measurement (Table II) is 19.45. With the silicone phase for this acid, the calculated and actual values were only 0.02 units

TABLE II

EQUIVALENT CHAIN-LENGTHS OF THE METHYL ESTER DERIVATIVES OF SOME NATURAL FATTY ACIDS

No.	*Fatty acid*	*Stationary phase*			
		Silicone	*Carbowax*	*Silar 5CP*	*CP-Sil 84*
1	14:0	14.00	14.00	14.00	14.00
2	14-*isobr*	14.64	14.52	14.52	14.51
3	14-*anteiso*	14.71	14.68	14.68	14.70
4	14:1($n-5$)	13.88	14.37	14.49	14.72
5	15:0	15.00	15.00	15.00	15.00
6	16:0	16.00	16.00	16.00	16.00
7	16-*isobr*	16.65	16.51	16.51	16.50
8	16-*anteiso*	16.73	16.68	16.68	16.69
9	16:1($n-9$)	15.76	16.18	16.30	16.48
10	16:1($n-7$)	15.83	16.25	16.38	16.60
11	16:1($n-5$)	15.92	16.37	16.48	16.70
12	16:2($n-4$)	15.83	16.78	16.98	17.47
13	16:3($n-3$)	15.69	17.09	17.31	18.06
14	16:4($n-3$)	15.64	17.62	17.77	18.82
15	17:0	17.00	17.00	17.00	17.00
16	17:1($n-9$)	16.76	17.20	17.33	17.50
17	17:1($n-8$)	16.75	17.19	17.33	17.51
18	18:0	18.00	18.00	18.00	18.00
19	18:1($n-11$)	17.72	18.14	18.24	18.40
20	18:1($n-9$)	17.73	18.16	18.30	18.47
21	18:1($n-7$)	17.78	18.23	18.36	18.54
22	18:2($n-6$)	17.65	18.58	18.80	19.20
23	18:2($n-4$)	17.81	18.79	18.98	19.41
24	18:3($n-6$)	17.49	18.85	19.30	19.72
25	18:3($n-3$)	17.72	19.18	19.41	20.07
26	18:4($n-3$)	17.55	19.45	19.68	20.59
27	19:1($n-8$)	18.74	19.18	19.32	19.47
28	20:1($n-11$)	19.67	20.08	20.22	20.35
29	20:1($n-9$)	19.71	20.14	20.27	20.41
30	20:1($n-7$)	19.77	20.22	20.36	20.50
31	20:2($n-9$)	19.51	20.38	20.59	20.92
32	20:2($n-6$)	19.64	20.56	20.78	21.12
33	20:3($n-9$)	19.24	20.66	20.92	21.43
34	20:3($n-6$)	19.43	20.78	21.05	21.61
35	20:3($n-3$)	19.71	20.95	21.22	21.97
36	20:4($n-6$)	19.23	20.96	21.19	21.94
37	20:4($n-3$)	19.-47	21.37	21.64	22.45
38	20:5($n-3$)	19.27	21.55	21.80	22.80
39	22:1($n-11$)	21.61	22.04	22.16	22.30
40	22:1($n-9$)	21.66	22.11	22.23	22.36
41	22:3($n-9$)	21.20	22.52	22.78	23.25
42	22:3($n-6$)	21.40	22.71	22.99	23.47
43	22:4($n-6$)	21.14	22.90	23.21	23.90
44	22:5($n-6$)	20.99	23.15	23.25	24.19
45	22:5($n-3$)	21.18	23.50	23.92	24.75
46	22:6($n-3$)	21.04	23.74	24.07	25.07

TABLE III

DIFFERENCES BETWEEN THE CALCULATED AND ACTUAL EQUIVALENT CHAIN-LENGTH VALUES FOR THE METHYL OCTADECADIENOATES

Fatty acid	*Stationary phase*			
	Silicone	*Carbowax*	*Silar 5CP*	*CP-Sil 84*
3,6-18:2	0.07	0.07	0.02	0.07
4,7-18:2	0.06	0.10	0.03	0.12
5,8-18:2	0.09	0.13	0.02	0.13
6,9-18:2	0.11	0.13	0.08	0.14
7,10-18:2	0.10	0.12	0.14	0.13
8,11-18:2	0.11	0.13	0.10	0.15
9,12-18:2	0.09	0.12	0.04	0.11
10,13-18:2	0.09	0.11	0.04	0.14
11,14-18:2	0.08	0.10	0.05	0.11
12,15-18:2	0.06	0.05	0.00	0.06
13,16-18:2	0.04	0.07	−0.02	0.09
14,17-18:2	0.01	−0.02	−0.12	−0.06
5,12-18:2	0.01	0.01	−0.03	0.02
6,12-18:2	0.02	0.00	−0.02	0.01
7,12-18:2	0.01	−0.04	−0.06	−0.03
8,12-18:2	0.08	0.05	−0.05	0.02
6,10-18:2	0.08	0.05	0.04	0.02
6,11-18:2	0.01	0.03	0.01	0.04

apart, while with the more polar phases the difference was 0.07 in each instance. The results, therefore, confirm the value of this approach to the identification of unknowns provided that the primary data are of sufficient accuracy.

The real value of various phases in WCOT columns for fatty acid analysis can only be judged when they are applied to authentic samples of natural origin. The separation of the methyl esters of the fatty acids of cod liver oil on the Carbowax 20M column in a temperature-programmed run is illustrated in Fig. 1. Each of the main chain length groups is reasonably well resolved. For example, two 16:1 isomers are seen and they are distinct from the C_{17} branched and unsaturated fatty acids. Similarly the important C_{18} components are well separated from each of the C_{20} unsaturated constituents. With the last, the only serious overlap problem is with 20:3($n-3$), which co-chromatographs with 20:4($n-6$); these are, however, just separable on a slightly more polar Silar 5CP column. Others did not find this specific separation to be a problem[24]. Finally, all the biologically-important C_{22} fatty acids are cleanly separated. An equally good results was seen with the pig testis fatty acids.

With the more polar CP-Sil 84 column, there is again excellent resolution of the pig testis fatty acids (Fig. 2). Individual unsaturated esters are particularly well resolved, and for example there is near base-line separation of 18:1($n-9$) and 18:1($n-7$). On the other hand, 18:3($n-6$) emerges after the minor C_{19} fatty acid. The C_{20} group are all well separated from each other but are beginning to run into an area occupied by C_{22} fatty acids. This last effect can be more troublesome with fish oils, which contain appreciable amounts of 22:1 isomers as in the cod liver oil

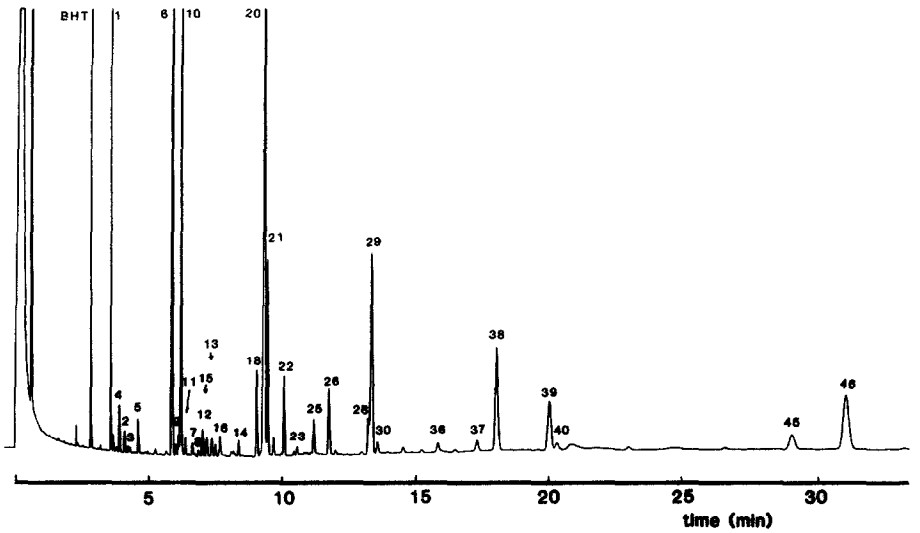

Fig. 1. GC separation of the methyl ester derivatives of the fatty acids of cod liver oil on a WCOT column of fused silica coated with Carbowax 20M. Chromatographic conditions are given in the Experimental section. The number above each peak for identification refer to the listing in Table II.

sample (not shown). The latter compounds emerge just before the C_{20} polyene, 20:4($n-3$). Similarly the C_{16} polyenes elute among the C_{18} fatty acids, and the C_{18} polyenes run into the C_{20} fatty acids. The C_{16} branched and monoenoic constituents tend to co-chromatograph. Nonetheless, with tissue lipids from plants and terrestrial animals especially, the polar column gives excellent results provided that care is taken in identifying the fatty acids.

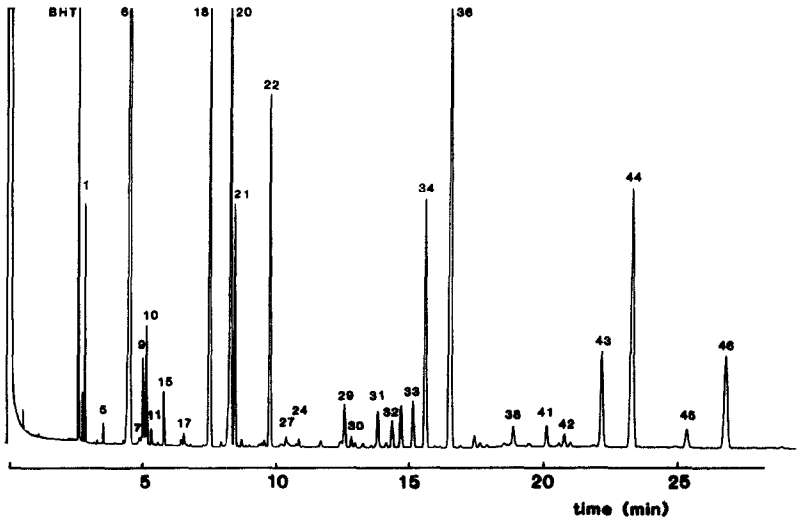

Fig. 2. GC separation of the methyl ester derivatives of the fatty acids of pig testis on a WCOT column of fused silica coated with CP-Sil 84. Chromatographic conditions are given in the Experimental section. The numbers above each peak for identification refer to the listing in Table II.

The nature of the separation attained on non-polar columns is rather different from that with polar columns. Unsaturated components emerge ahead of saturated fatty acids of the same chain-length. Isomeric fatty acids differing in the positions of double bonds are usually clearly resolved, thus 18:1($n-9$) and 18:1($n-7$) are separated as are many of the polyenes. Indeed, the C_{22} fatty acids are possibly almost as well separated as on a polar column of the same length. Unfortunately, there are substantial overlaps among the C_{18} fatty acids, and 18:2($n-6$) is not fully resolved from 18:1($n-9$); 18:2($n-6$) and 18:3($n-3$) merge completely, and this is also true of the corresponding C_{20} and C_{22} compounds. As linoleate and linolenate are essential fatty acids with major nutritional importance, the deficiency in this aspect of the separation is likely to mitigate against a wider use of non-polar column. The order of elution of the C_{22} components is not that which might expected intuitively, *i.e.* 22:5($n-6$), 22:6($n-3$), 22:4($n-6$) and 22:5($n-3$). In this instance, it appears that the position of the double bonds has a greater effect on retention time than does the number of double bonds.

Nearly all analysts then are going to make use of polar stationary phases for the major proportion of their work. The chromatographic traces illustrated here lend support to Ackman's view that Carbowax 20M is the best general purpose stationary phase in WCOT columns for fatty acid analysis. Non-polar phases do have advantages in specific applications, *e.g.* with fatty acids of high molecular weight or containing thermally-labile functional groups and perhaps for certain *cis/trans* separations, where their stability at high temperatures and their considerable degree of inertness are virtues.

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