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IMPROVED COLUMNS FOR THE SEPARATION OF C_{14}–C_{20} FATTY ACIDS IN THE FREE FORM

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SUMMARY

A study was made of two highly polar polyester stationary phases for the separation of fatty acids in the C_{14}–C_{20} range. These stationary phases were also compared to moderately polar stationary phases currently in use. The most complete separation was obtained using DEGS-PS. The fastest separation was obtained with SP-216-PS, a new polyester, in the absence of C_{20}.

INTRODUCTION

Fatty acids in the range of C_{14}–C_{20} are difficult compounds to analyze by gas chromatography because the acid peaks show severe tailing. To avoid this problem the fatty acids are usually converted to the methyl ester, which eliminates the tailing problem. Nevertheless, some technology has been developed on the separation of free acids.

James and Martin[1] studied the separation of the C_1–C_{12} free acids using DC-550 silicone fluid as the stationary phase, but found it necessary to add stearic acid and phosphoric acid to the phase to reduce tailing of the peaks. They attributed the tailing phenomenon to both the association of the acid in the stationary phase and to the interaction of the acid with the diatomaceous earth support. Much of the subsequent work on free acids has been on methods to eliminate the tailing of the peaks.

Beerthius *et al.*[2] studied the separation of the higher fatty acids using DC-550, a silicone fluid, and also Apiezon L as stationary phases. β-Anthraquinone was added to the silicone oil stationary phase as a tail reducer, but this was lost when the column was operated at a higher temperature. The work of Beerthius *et al.* shows considerable tailing of the chromatographic peaks.

Metcalfe[3,4] studied the use of polyesters for the separation of the higher free fatty acids and incorporated H_3PO_4 into them to both increase the thermal stability of the stationary phase and to eliminate the tailing of the peaks. He shows that the separation obtained is in the order of the degree of unsaturation as is the case in the separation of the fatty acid methyl esters. Diethylene glycol adipate (DEGA) was found to be the most stable polyester of a series tested.

Byars and Jordan[5,6] modified Carbowax 20M, a polyethylene glycol (mol. wt. 20,000), with terephthalic acid (TPA), and later with a modified TPA in order to

incorporate an acid into the stationary phase. It is assumed that the TPA reacts with a terminal hydroxyl group, forming a half ester, and the remaining carboxyl group is free to deactivate the support. The second modification has been sold by Varian Associates under the name FFAP.

For many years it has been assumed that the tailing of the acid peaks is caused by dimerization of the acid in the stationary phase as proposed by James and Martin. They also attributed the tailing of the peaks to the interaction of the acid solution and the support. Kirkland[7], in studying the chromatographic properties of fluorocarbon supports, showed that no tailing of peaks was encountered with such a support. This was confirmed elsewhere[8]. In studying the separation of the volatile free acids, it was found that the tailing of the acid peaks appeared to be caused by active sites on the surface of the diatomaceous earth support, and not by a dimerization of the acid in the stationary phase.

In evaluation of polyester stationary phases for the separation of fatty acid methyl esters, Supina[9] has shown that the degree of separation between saturated and unsaturated esters increases as the polarity of the stationary phase increases, as measured by the Rohrschneider and McReynolds values for *X*. A McReynolds *X* value of 340 is necessary for a stationary phase to obtain a separation factor for stearate/oleate of 1.10. EGA and DEGA have *X* values in this range; DEGS, with an *X* value of 502, gives a stearate/oleate separation factor of 1.18. At the same time, it is very evident that the more polar DEGS column gives a faster elution of the sample than the less polar EGA and DEGA ones at the same column temperature.

The purpose of this paper is to introduce a new, more polar, polyester stationary phase and to evaluate some of the other polyester stationary phases used for the separation of free fatty acids in the C_{14}–C_{20} range, and to compare the separation to that obtained with less polar stationary phases. This paper will show that as the polarity of the stationary phase increases, the elution time is reduced, the separation factor for the stearic/oleic acid pair is increased, and the position of $C_{20:0}$ relative to $C_{18:3}$ will change.

EXPERIMENTAL

The apparatus used was a Hewlett-Packard Model 7610 gas chromatograph (Hewlett-Packard, Avondale, Pa., U.S.A.) equipped with flame ionization detectors. The columns were 3 ft. × 2 mm I.D. glass U tubes, and samples were injected directly into the columns. The nitrogen used as the carrier gas was dried with a 2 ft. × 1 in. diameter tube filled with activated molecular sieve 4A. All of the stationary phases, except FFAP, which was obtained from Varian (Palo Alto, Calif., U.S.A.), were prepared in this laboratory. Stationary phases used were: SP-216-PS, polyethylene glycol adipate (EGA-PS), polydiethylene glycol adipate (DEGA-PS) and Carbowax 20M–terephthalic acid (20M–TPA). The PS designation denotes the incorporation of orthophosphoric acid (H_3PO_4) into the stationary phase. All were coated on 80–100 mesh Supelcoport, an acid-washed and DMCS-treated diatomaceous earth support (Supelco, Bellefonte, Pa., U.S.A.).

All of the columns were evaluated by analysis of Supelco Qual Mix FA consisting of the following free acids: $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:2}$, $C_{18:3}$, and $C_{20:0}$. Additional evaluation was carried out with the Supelco RM-1 mixture of methyl

esters which had been converted to free acids. In each case the total concentration of acid was 10 mg/ml in chloroform.

The stationary phases studied are highly susceptible to deterioration by moisture in the carrier gas. A 2 ft. × 1 in. diameter metal tube filled with activated molecular sieve 4A or 5A has been found effective in removing this moisture. The drying tube should be flushed with carrier gas for 4 h to remove air before it is connected to the chromatograph. If a drier is not used, the SP-216-PS will deteriorate in a week, while DEGS-PS will deteriorate in about three weeks. The adsorbent must be replaced regularly to be effective; the frequency of change depends upon the amount of moisture in the gas. A second cause of deterioration is oxygen in the carrier gas or oxygen which enters from leaks in the chromatographic system. A good quality carrier gas free of oxygen should be used, or an oxygen scrubber should be incorporated into the carrier gas stream; then a careful check should be made to be sure no leaks are present.

RESULTS

The various stationary phases were compared under the same conditions with a standard test mixture and results are presented in Table I. Here the corrected retention time for stearic acid is listed for each column along with the relative retention time for each of the components relative to stearic acid.

TABLE I

RELATIVE RETENTION DATA FOR C_{14}–C_{20} FATTY ACIDS

Column: 3 ft. × 2 mm I.D., 10% stationary phase on 80–100 mesh Supelcoport; temperature, 200°; flow-rate, 20 ml/min.

	SP-216-PS	*DEGS-PS*	*DEGA-PS*	*FFAP*	*20M-TPA*
$C_{14:0}$	0.41	0.34	0.29	0.27	0.26
$C_{16:0}$	0.64	0.59	0.54	0.52	0.51
$C_{16:1}$	0.80	0.71	0.62	0.58	0.57
$C_{18:0}$	1.00	1.00	1.00	1.00	1.00
$C_{18:1}$	1.23	1.20	1.12	1.09	1.07
$C_{18:2}$	1.60	1.49	1.35	1.27	1.25
$C_{18:3}$	2.22	1.96	1.74	1.58	1.54
$C_{20:0}$	1.60	1.71	1.87	1.95	1.92
$C_{18:0}$ (minutes)	5.3	11.5	22.8	44.3	49.5
McReynolds factor (X)	632	496	378	340	321

The most complete separation was obtained with DEGS-PS, but it required 23 min to obtain a complete base line separation of all of the components (Fig. 1). The most rapid separation was obtained with SP-216-PS in 12 min with complete separation of the stearic/oleic acid pair, but with no separation of $C_{20:0}$ and $C_{18:2}$ (Fig. 2). In the case of DEGA-PS, FFAP and 20M–TPA, the analysis was considerably longer, and the separation was quite poor for the 18:0/18:1 pair. With these last three columns the $C_{20:0}$ was eluted last after the 18:3 without any problem of interference.

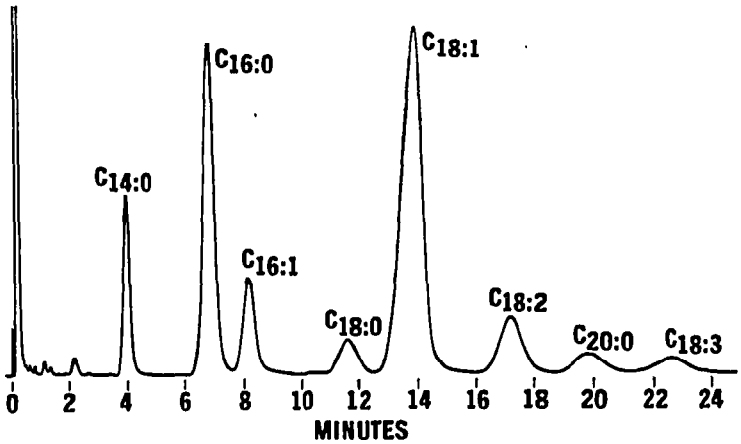

Fig. 1. Separation of C_{14}–C_{20} fatty acids on a 3 ft. × 2 mm I.D. glass column loaded with 10% DEGS-PS on 80–100 mesh Supelcoport. Column temperature, 200°; flow-rate, 20 ml/min.

In order to obtain separation with DEGA-PS, FFAP and 20M–TPA in a time roughly comparable to DEGS-PS, it was necessary to increase the column temperature by approximately 25° to 225°. Under these conditions, the retention time was considerably reduced, as seen in Table II; however, the 18:0/18:1 separation remained inadequate. The separation of the 18:3/20:0 pair remained good for both FFAP and 20M–TPA. In the case of DEGA-PS, these two components were no longer separated at 225°.

A study was made to determine the effect of reducing the amount of stationary phase for both SP-216-PS and DEGS-PS while maintaining a column temperature of 200°. In the case of DEGS-PS, stationary phase loadings of 2.5, 5.0, and 10% were studied (retention data are summarized in Table III). Only with the 2.5% DEGS-PS was there a problem of peak tailing, and this concentration does not appear to be a

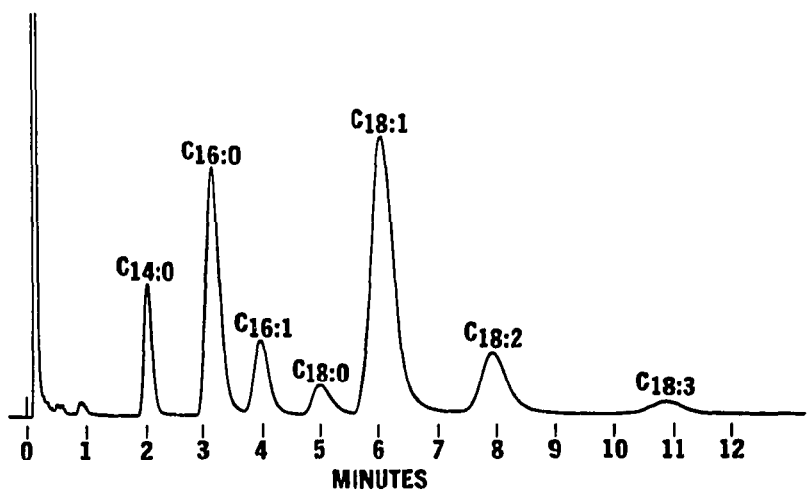

Fig. 2. Separation of C_{14}–C_{18} fatty acids on a 3 ft. × 2 mm I.D. glass column loaded with 10% SP-216-PS on 80–100 mesh Supelcoport. Column temperature, 200°; flow-rate, 20 ml/min.

TABLE II

COMPARISON OF RELATIVE RETENTION DATA AT 200° AND 225° FOR C_{14}–C_{20} FATTY ACIDS

Column: 3 ft. × 2 mm I.D., 10 % stationary phase on 80–100 mesh Supelcoport; flow-rate, 20 ml/min.

	DEGA-PS		*FFAP*		*20M-TPA*	
	200°	*225°*	*200°*	*225°*	*200°*	*225°*
$C_{14:0}$	0.29	0.34	0.27	0.32	0.26	0.30
$C_{16:0}$	0.54	0.58	0.52	0.56	0.51	0.55
$C_{16:1}$	0.62	0.66	0.58	0.63	0.57	0.61
$C_{18:0}$	1.00	1.00	1.00	1.00	1.00	1.00
$C_{18:1}$	1.12	1.14	1.09	1.10	1.07	1.08
$C_{18:2}$	1.35	1.35	1.27	1.26	1.25	1.23
$C_{18:3}$	1.74	1.68	1.58	1.53	1.54	1.49
$C_{20:0}$	1.87	1.71	1.94	1.78	1.92	1.79
$C_{18:0}$ (minutes)	22.8	7.75	44.3	15.2	47.5	17.2

TABLE III

COMPARISON OF RETENTION DATA WITH VARYING AMOUNTS OF DEGS-PS AT 200°

Column: 3 ft. × 2 mm; temperature, 200°; flow-rate, 20 ml/min.

	5%	*10%*
$C_{14:0}$	0.34	0.34
$C_{16:0}$	0.59	0.59
$C_{16:1}$	0.70	0.71
$C_{18:0}$	1.00	1.00
$C_{18:1}$	1.19	1.20
$C_{18:2}$	1.49	1.49
$C_{18:3}$	1.96	1.96
$C_{20:0}$	1.70	1.71
$C_{18:0}$ (minutes)	7.0	11.5

TABLE IV

COMPARISON OF RETENTION DATA WITH VARYING AMOUNTS OF SP-216-PS AT 200°

Column: 3 ft. × 2 mm; temperature, 200°; flow-rate, 20 ml/min.

	5%	*10%*
$C_{14:0}$	0.41	0.41
$C_{16:0}$	0.63	0.64
$C_{16:1}$	0.77	0.80
$C_{18:0}$	1.00	1.00
$C_{18:1}$	1.18	1.23
$C_{18:2}$	1.52	1.60
$C_{18:3}$	2.04	2.22
$C_{20:0}$	1.52	1.60
$C_{18:0}$ (minutes)	2.63	5.3

useful one. The retention values are reduced considerably as the loading of stationary phase is reduced. The 18:0/18:1 separation factor is diminished as the loading is reduced. At 5% and 10% loading, 18:3 is eluted after 20:0.

SP-216-PS was studied with stationary phase loadings of 2.5, 5.0 and 10.0% (data are summarized in Table IV). At 2.5% SP-216-PS tailing is a problem and it does not appear to be a useful column. The 5% and 10% loadings gave good peak symmetry. Both the 5% and 10% SP-216-PS are considerably faster than DEGS-PS. While the separation of 18:0 and 18:1 is good, no separation was obtained for the 18:2/20:0 pair.

DISCUSSION

The development of columns for the separation of the C_{14}–C_{20} fatty acids requires both a high degree of deactivation and a stationary phase which will both separate the components completely as well as elute the sample in a reasonable time. Metcalfe's techniques of incorporating H_3PO_4 into a polyester stationary phase provide a good means of support deactivation as well as stabilization of the stationary phase.

The uses of low or moderately polar stationary phases favors the separation of the $C_{20:0}/C_{18:3}$ pair; but these phases do not adequately separate the important 18:0/18:1 pair and analysis time is lengthy. By using a more polar stationary phase, the 18:0/18:1 separation is enhanced and the time of analysis is greatly reduced, but the more polar stationary phases elute 20:0 more rapidly with a tendency for overlap of 18:2 and 18:3. At the temperatures used DEGS-PS has the proper polarity to avoid this situation, eluting 20:0 between 18:2 and 18:3. SP-216-PS does not resolve this pair, limiting its use to situations where 20:0 is absent. Where 20:0 is absent, it can be used to obtain a more rapid analysis than in the case of DEGS-PS.

Both the DEGS-PS and SP-216-PS give more than adequate separation of the test mixture with 10% loading in 3-ft. columns. By reducing the stationary phase loading to 5%, approximately comparable separation is obtained in a shorter period of time. In the case of both SP-216-PS and DEGS-PS a column temperature of 200° is the recommended maximum for reasonable column life. Of the two, DEGS-PS is somewhat more stable.

Concentration of stationary phase

Metcalfe used 20% stationary phase in his evaluation of DEGA-H_3PO_4 and orthophosphoric acid-stabilized polyesters. These high loadings, which were normal at the time, required analysis at high column temperatures to elute the sample within a reasonable time. Metcalfe also used low liquid loading on glass bead columns, but the efficiency of these columns was poor with minimal separation of 18:0 and 18:1. The use of lower loading of polyester stationary phase on conventional supports allows the sample to be eluted in shorter periods of time. From Tables III and IV, it can be seen that the separation factors for 18:0 and 18:1 are more favorable at 5% with DEGS-PS and SP-216-PS than at 10% with DEGA-PS, FFAP, and 20M-TPA.

It was noted earlier that Supina[9] had observed separation factors for 18:1/18:0 methyl esters of 1.18 and 1.10 for DEGS and DEGA, respectively. With free acids,

separation factors for 18:1/18:0 of 1.20 and 1.12 are found. The even more polar SP-216-PS provides an 18:1/18:0 separation factor of 1.23. Again, from Table I one sees a marked increase in the retention of 20:0 and an increase in the retention of successive saturated free acids as the polarity of the stationary phase diminishes. Novák[10] and Littlewood[11] have discussed in great detail the relationship between relative retention and polarity of stationary phases. For members of a homologous series, the relative retention time decreases with increases in column polarity. Therefore, the stationary phases with the highest polarity will provide the best separation between the saturated and monounsaturated components and will accomplish this in the shortest period of time. Conversely, analysis on a less polar phase would result in less complete separation and would require longer analysis time. It is possible to predict much of the performance of the stationary phase from the McReynolds constants for these stationary phases. The McReynolds constant, X value (benzene), is listed in Table I for each of the stationary phases studied.

Stability of polyesters

Metcalfe has found the DEGA-H_3PO_4 polyester to be the most stable with operating temperature to 250°. The DEGS and NGS were found to be erratic while EGS could be used up to 180° for separation of the acids up to C_{18}. Metcalfe suggested that phosphoric acid might form a phosphate ester with the terminal groups of the polyester, thereby stabilizing it. In our experience with the various polyesters, it was found that those polyesters made from ethylene glycol were less stable than those made from diethylene glycol. Ethylene glycol succinate with H_3PO_4 was less stable than ethylene glycol succinate without H_3PO_4. Some exploratory work[12] on this subject shows that the manner in which the H_3PO_4 is incorporated with the polyester is important in terms of the stability of the stationary phase. The stability is expressed in terms of the ability of the stationary phase to elute free acids with good peak symmetry. In most instances when a column showed tailing of the free acid peaks, it would still elute methyl esters with good peak symmetry. This indicated that H_3PO_4 was lost from this column and that the column was now adsorptive.

Sample size

It was found that the DEGS-PS and SP-216-PS columns tended to show overloading more readily than the lower polarity columns. The overloading was seen as a tailing of the peaks. The samples were generally at a concentration of 10 mg/ml and a 1.0-μl sample size was used. By reducing the sample size to 0.5 μl peak symmetry was improved. The tendency to overload was increased as the amount of stationary phase was decreased.

A similar overloading situation was observed in the separation of the volatile free acids, C_2–C_5, using a low-polarity stationary phase. Too large a sample caused overloading and poor peak shape, particularly for acetic and propionic acid[13].

CONCLUSIONS

Polar polyesters stabilized with H_3PO_4 can be used to separate the common free acids C_{14}–C_{20} in a relatively short period of time. The DEGS-PS column will separate all of the C_{14}–C_{20} free acids, while SP-216-PS will separate all of these acids

except $C_{20:0}$ and $C_{18:2}$ in half the time of DEGS-PS. The time of analysis with these stationary phases is substantially less than that obtained with moderately polar stationary phases.

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