CHROM. 6306

THE EFFECT OF COLUMN LENGTH AND COLUMN PACKING MESH SIZE ON THE ANALYSIS OF VOLATILE FATTY ACIDS BY GAS CHROMATOGRAPHY USING CHROMOSORB 101

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(First received May 23rd, 1972; revised manuscript received August 14th, 1972)

SUMMARY

The effect of column length on resolution and retention time has been studied for the analysis of volatile fatty acids on Chromosorb 101. Using 80–100 mesh packing an optimum column length of 0.5 m was found. A comparison was then made between two 0.5-m columns, one packed with 80–100 mesh and the other with 100–120 mesh Chromosorb 101. The smaller mesh size resulted in considerably better resolution of isobutyric and *n*-butyric acids and gave over twice as many plates per foot as the larger mesh size under optimum operating conditions. However, retention times were increased by about 40 % (4.8 to 6.7 min for *n*-valeric acid).

Several methods for isolating free volatile acids from aqueous solutions and plasma samples were tested and are discussed briefly.

INTRODUCTION

The analysis of free fatty acids in aqueous solution is one of the more difficult tasks in gas chromatography (GC) because of the severe tailing which results using conventional column packings.

Two approaches have been used to try to overcome this problem. The first is the use of modified stationary phases, the most common of these being Carbowax 20M or diethylene glycol succinate modified with 1–5 % phosphoric or terephthalic acids. The main disadvantage of modified stationary phases is that they tend to degenerate rapidly when aqueous samples are injected. The second, more recent, approach has been the use of adsorbents such as polyaromatic resins. These have the advantage of being hydrophobic and so water is eluted very rapidly and does not cause degeneration of the column packing. The use of Porapaks N[1], QS[2], and Q[3] has been reported, the latter being treated with phosphoric acid.

Both Porapak Q and Porapak Q + 1–5 % phosphoric acid were tried in this laboratory but severe tailing of the acid peaks was found on all columns packed with these. As Chromosorb 101 is claimed by the manufacturers (Johns-Mannville Co.) to be suitable for free fatty acid analysis, this was tested and the results are reported in this paper.

This investigation was prompted by an interest in metabolic deficiencies which result in abnormal amounts of volatile acids being present in the blood, *e.g.* isovaleric acidaemia. Several clean-up procedures for plasma samples were tried and these are discussed briefly.

EXPERIMENTAL

Materials

Chromosorb 101 (80–100, 100–120 mesh) and Porapak Q were obtained from Supelco, Inc. Other chemicals were obtained from the sources shown and were of the highest purity available: orthophosphoric acid (Ajax Chemicals Ltd., Australia), acetic acid (B.D.H.), propionic acid (B.D.H.), *n*-butyric acid (B.D.H.), isobutyric acid (Hopkin and Williams Ltd.), *n*-valeric acid (Hopkin and Williams Ltd.), caproic acid (Sigma), heptanoic acid (Sigma) and caprylic acid (Sigma).

Equipment

A Shimadzu GC-4APTF equipped with glass columns was used. The 2-m, 1-m and 0.5-m × 3-mm columns were obtained from Shimadzu Seisakusho Ltd. The 0.1-m and 0.01-m × 3-mm columns were made by Townson and Mercer (N.Z.) Ltd., Auckland.

Nitrogen was used as carrier gas. The flame ionization detectors were operated at optimum hydrogen and air flow-rates for each column. Injector and detector temperatures were kept at 200°.

The recorder (Shimadzu R-201, dual-pen) was operated at speeds of 5, 10 or 80 mm/min, depending on the column being used.

SGE syringes (Scientific Glass Engineering, Melbourne, Australia) were used to inject samples into the chromatograph. It was found to be essential that the syringes be thoroughly cleaned between injections. This was done by rinsing them in distilled water and then placing them in a Hamilton syringe cleaner.

An MSE super minor centrifuge was used for centrifugal ultrafiltration, the Centriflo membrane ultrafilters being obtained from Amicon Corporation (Mass., U.S.A.)

Packing of columns

Columns were washed with acetone and dried with dry compressed air. They were packed in the normal manner using vacuum and gentle tapping. Non-silanized glass wool was used to plug the column at either end.

The columns were found to be fully conditioned for use up to 180° by heating at 180° for 1 h with a slow stream of nitrogen through the column.

After several cycles of heating and cooling breaks appeared in the column packing. They did not appear to greatly affect column performance and efforts to eliminate them by using different packing methods or heating the Chromosorb 101 to 200° for several hours before packing the column were unsuccessful.

RESULTS

Van Deemter plots of HETP (for isobutyric acid) against nitrogen pressure were constructed for each column at a series of temperatures to determine the optimum operating conditions for that column (as indicated by the minimum HETP

value for isobutyric acid on that column). The optimum conditions are summarised in Table I.

The following equations were used for the calculations:

$$N = 16\,(t^1/W)^2$$
$$H = L/N$$
$$R = 2\,(t_2 - t_1)/(W_2 + W_1)$$

where:

N = number of theoretical plates
t^1 = corrected retention time
W = peak width at base
H = height equivalent to a theoretical plate (HETP)
L = column length (cm)
R = resolution
t_1, t_2 = retention times of two components whose resolution is being calculated
W_1, W_2 = base widths of the two components.

Two criteria were selected as the basis for the comparison under their optimum operating conditions of the columns studied. The first was the absolute retention time of the last component to be eluted (*n*-valeric acid) and the second was the resolution between isobutyric and *n*-butyric acids. The values for these factors are given in Table I.

Of the columns packed with 80–100 mesh Chromosorb 101 only two gave a resolution greater than $R = 1.0$ (98% separation) although the 0.5-m column gave $R = 0.96$. The retention times for the 2-m and 1-m columns were considered excessive for the analysis being performed and the slight sacrifice in resolution in using the 0.5-m column was more than balanced by the large decrease in analysis time.

This 0.5-m column was then compared with a column of similar length packed with 100–120 mesh Chromosorb 101. There was a 40% gain in resolution accompanied by a 40% increase in retention time on the 100–120 mesh column over the 80–100 mesh column. As the retention time of *n*-valeric acid on the finer mesh column (6–7 min) was not much greater than on the 80–100 mesh column and the resolution was significantly better, the 100–120 mesh column was chosen for analysis of C_2 to C_5 acids. Chromatograms of the C_2 to C_5 acids on the two columns are illustrated in Fig. 1.

Analysis of C_2 to C_8 acids

Several of the columns were tested for analysis of a mixture of the C_2 to C_8 *n*-acids under isothermal conditions. Sample chromatograms are illustrated in Fig. 2 (0.5 m, 80–100 mesh), Fig. 3 (0.5 m, 100–120 mesh) and Fig. 4 (0.1 m, 80–100 mesh). If separation of isomeric acids is not important then the 0.1-m column gives excellent results in only 2 min (note the high chart speed of 80 mm/min). The 0.5-m, 100–120 mesh column, on the other hand, while giving excellent resolution, has a rather long retention time and there is some peak tailing evident. The 0.5-m, 80–100 mesh column appears to be a good compromise if some resolution of isomeric acids is required.

The retention times of the C_2 to C_8 *n*-acids on the above columns are summarised in Table II.

TABLE I

OPTIMUM OPERATING CONDITIONS FOR EACH COLUMN

Length (m)	*Diameter (I.D., mm)*	*Chromosorb 101 (mesh)*	*Column temp. (°C)*	*N_2 inlet pressure (kg/cm^2)*	*N_2 flow (ml/min)*	*N (n-valeric)*	*H (cm)*	*Resolution (isobutyric/ n-butyric)*	*Retention time (n-valeric, min)*
2.0	3	80–100	160	0.40	10	625[d]	0.32	1.5	25
1.0	3	80–100	120	0.59	12	1271.4	0.08	1.48	15.6
0.5	3	80–100	140	0.50	20	380.7	0.13	0.96	4.8
0.5	3	100–120	160	0.67	25	886.7	0.06	1.38	6.7
0.1	3	80–100	140	0.26	33	120.8	0.08	[e]	2.5
0.01[a]	3	80–100	50[b]	0.10	N.D.[c]	32.5	0.03	0.9	11.4

[a] Column conditions not optimised by Van Deemter plot.
[b] Temperature programmed 50° to 120° at 4°/min.
[c] Not determined.
[d] For isobutyric acid.
[e] Not resolved, slight peak broadening.

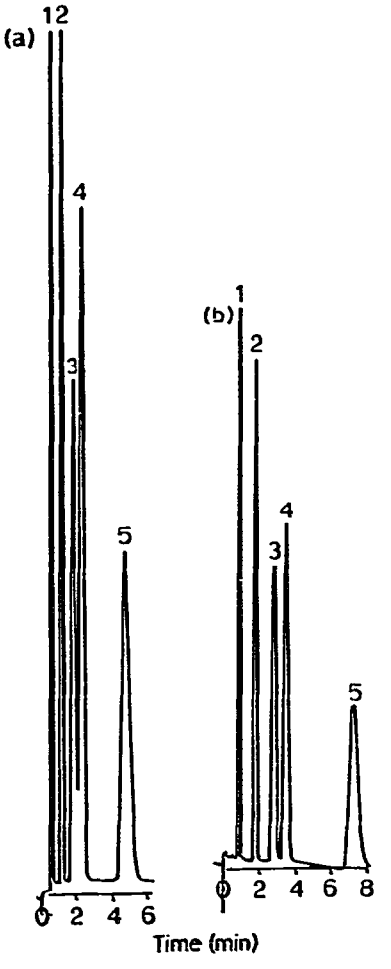

Fig. 1. Separation of fatty acids of low molecular weight. (a) 0.5-m × 3-mm glass column packed with 80–100 mesh Chromosorb 101. Resolution of isobutyric/*n*-butyric acids = 0.96. (b) 0.5-m × 3-mm glass column packed with 100–120 mesh Chromosorb 101. Resolution of isobutyric/*n*-butyric acids = 1.38. Peak identification: 1 = acetic acid; 2 = propionic acid; 3 = isobutyric acid; 4 = *n*-butyric acid; 5 = *n*-valeric acid.

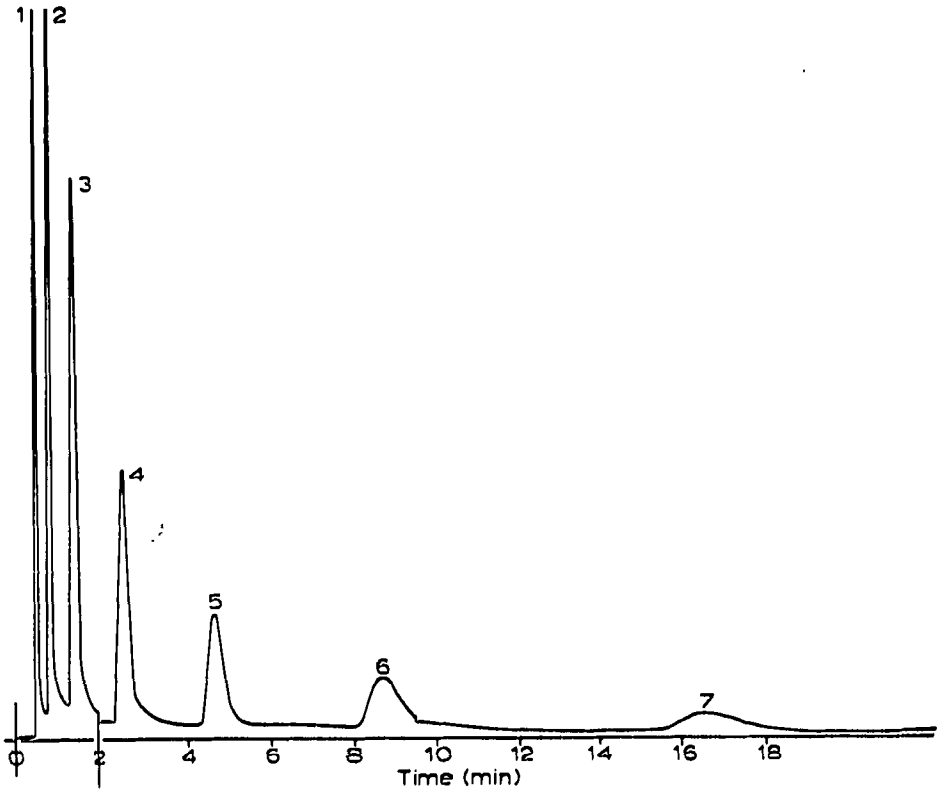

Fig. 2. Separation of C_2 to C_8 free fatty acids on a 0.5-m × 3-mm glass column packed with 80–100 mesh Chromosorb 101. Peak identification: 1 = acetic acid; 2 = propionic acid; 3 = *n*-butyric acid; 4 = *n*-valeric acid; 5 = *n*-caproic acid; 6 = *n*-heptanoic acid; 7 = *n*-caprylic acid.

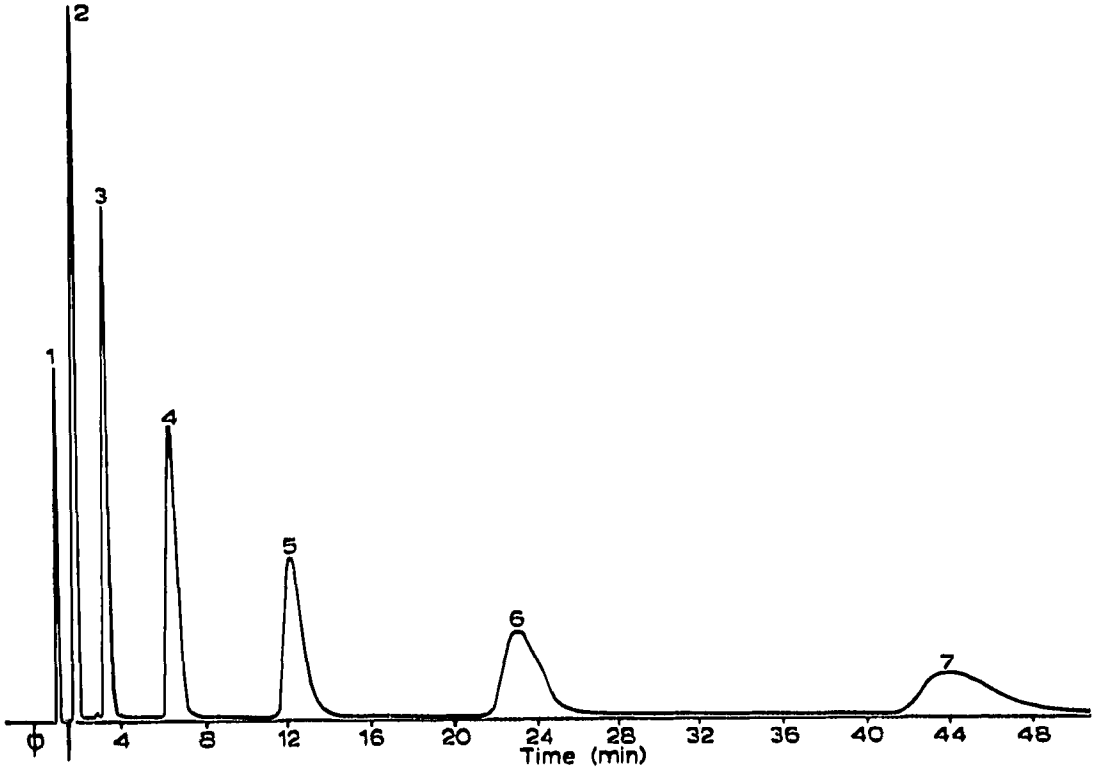

Fig. 3. Separation of C_2 to C_8 free fatty acids on a 0.5-m × 3-mm glass column packed with 100–120 mesh Chromosorb 101. For peak identification see the legend to Fig. 2.

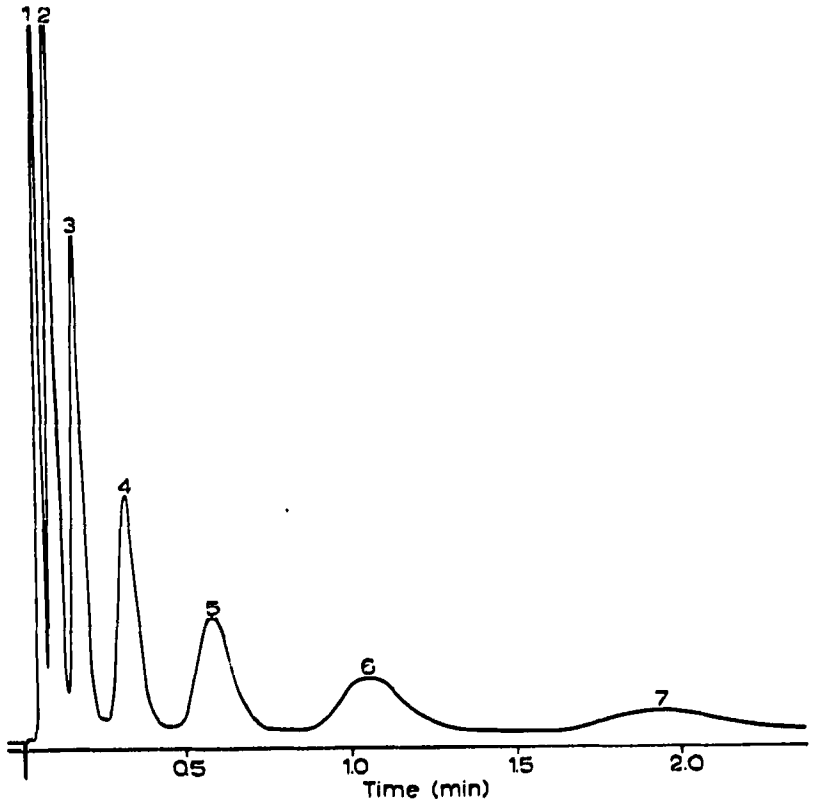

Fig. 4. Separation of C_2 to C_8 free fatty acids on a 0.1-m × 3-mm glass column packed with 80–100 mesh Chromosorb 101. For peak identification see the legend to Fig. 2.

TABLE II

RETENTION TIMES OF FREE *n*-ACIDS

Column[a]	*Retention time (min)*						
	C_2	C_3	C_4	C_5	C_6	C_7	C_8
0.5 m, 100–120 mesh	1.08	1.92	3.4	6.5	12.2	22.9	43.8
0.5 m, 80–100 mesh	0.5	0.82	1.40	2.56	4.7	8.8	16.5
0.1 m, 80–100 mesh	0.06	0.10	0.17	0.30	0.75	1.05	1.9

[a] Column conditions as in Table I.

Clean-up of plasma

The first clean-up method tried was precipitation of the protein using either zinc sulphate/sodium hydroxide or trichloroacetic acid (TCA). The first of these involves a very basic solution and no free acid was found in the supernatant liquid after centrifugation. The second precipitation worked well but the TCA eluted from the column between propionic and isobutyric acids and was present in such a large excess that it masked the peaks of the fatty acids.

The second clean-up procedure attempted was using ultrafiltration to remove all high-molecular-weight protein, etc., followed by direct injection of the filtrate into the column. The method used was similar to that of HENKEL[1] except that centrif-

ugal ultrafiltration instead of pressure filtration was used. The method was as follows:

A blood sample (as little as 0.3 ml has been used) is allowed to clot (about 10 min) and is then centrifuged at 2000 r.p.m. (1300 × *g*) for 5 min to remove the cells. The filtrate is transferred to a Centriflo ultrafiltration filter in a centrifuge (as little as 0.1 ml has been used) and centrifuged at 2000 r.p.m. for 2 min. The sample is then injected directly into the gas–liquid chromatograph. The total time required for an analysis is approximately 30 min.

The ultrafiltration membranes were tested for recovery of the acids from aqueous solution and the results are given in Table III (the concentration of each

TABLE III

PERCENT RECOVERY OF FREE ACIDS FROM AQUEOUS SOLUTION USING ULTRAFILTRATION

Acid	*Recovery* (%)
Acetic	57
Propionic	75
Isobutyric	89
n-Butyric	90
n-Valeric	100

acid was 200 μg/ml water). As yet no study of membrane performance over a large number of uses has been carried out although there has been no noticeable degeneration of membrane activity over about fifty uses.

No deterioration in column performance has been noticed after injection of about thirty treated plasma samples although a reddish ring has formed around the inside of the column where the sample is injected. Proper cleaning of the syringe as already discussed is essential for a good column performance. Lack of proper cleaning leads to "ghost" peaks and non-reproducible results.

Further work is being carried out on other types of ultrafilter and screening of blood samples from a number of subjects to establish normal concentration ranges is continuing.

ACKNOWLEDGEMENTS

The author is indebted to Professor R. B. ELLIOTT for advice and suggestions, and to the Medical Research Council of New Zealand for financial support.

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