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GAS CHROMATOGRAPHIC SEPARATION AND DETERMINATION OF MICRO QUANTITIES OF C_1–C_7 BRANCHED AND STRAIGHT-CHAIN SATURATED FATTY ACIDS

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SUMMARY

A survey of thirteen different columns was carried out to find a suitable combination for the separation and determination of micro quantities of formic, acetic, propionic, isobutyric, *n*-butyric, isovaleric, *n*-valeric, isocaproic, *n*-caproic and heptanoic acid. A 4 ft. × 1/4 in. O.D. stainless steel column with 3.125 % PEGA on acid-washed Chromosorb W (60/80 mesh) gave the best results in separation and sensitivity under isothermal and temperature-programming conditions. Using the dual hydrogen flame ionization detector on a Beckman GC-M, a helium flow rate of 100 ml/min and temperature programming between 100–150° at a rate of 5°/min, all acids could be separated and 0.5 μg of each acid with the exception of formic acid was standardizable. 0.1 μg of acid could be estimated. Isothermally with the thermoconductivity cell, 10 μg was the lowest amount detectable, for all investigated acids.

INTRODUCTION

The quantitative determination of lower fatty acids as intermediate and end products in bacterial fermentation is at present still based upon the methods developed by NEISH[1]. Column chromatography[2] as well as paper[3] and thin-layer chromatography[4] are the main determination materials. The quantitative determination of the C_1–C_7 straight- and branched-chain saturated fatty acids are of the greatest importance to obtain an as complete as possible carbon balance sheet for microbial fermentation[5]. Qualitative attempts in this direction are increasing over the past years[6–10], which brought out the re-investigation of the most promising looking conditions with a view towards quantitation.

In this report, a method of high sensitivity for the determination of the straight- and branched-chain saturated C_1–C_7 fatty acids is reported.

MATERIALS AND METHODS

Gas chromatograph

For isothermal investigations a Beckman GC-2 was used with the same arrangements as described for alcohol determinations[11,12].

For temperature-programming investigations the instrument employed was a Beckman GC-M linear-programmed gas chromatograph equipped with thermoconductivity cell and dual flame ionization detectors. The recorder was a 0–100 mV Honeywell recorder.

Columns

The following columns, which were all made of stainless steel and 1/4 in. O.D., were used: 6 ft. 10% Carbowax 20M; 4 ft. 21% PEG-600; 6 ft. 20% Carbowax 20M; 4 ft. 2.0% PEGA; 6 ft. 15% Apiezon L coated with 0.1% PEG-9,000; 8 ft. 15% DEGS; 4 ft. 3.125% PEGA; 6 ft. 3.125% PEGA coated with 2% orthophosphate; 6 ft. 20% LAC-269 coated with 2% orthophosphate; 6 ft. 25% Carbowax 20M coated with 4% orthophosphate; 4 ft. 20% DEGA; 6 ft. 2% PEGA coated with 2% orthophosphate and 6 ft. 20% E-301.

All liquid phases were supported on acid-washed Chromosorb W (60/80 mesh).

In order to keep the sample small, the Beckman 22,400 liquid sampler was used with its minimum volume size of 0.005 ml (5 μl) or a microsyringe. All quotations of sensitivities therefore refer to the number of micrograms per 5 μl sample except where specially stated.

Reagents

The fatty acids formic acid, acetic acid, propionic acid, isobutyric acid and *n*-butyric acid were of the highest purity obtainable from Fluka AG, Buchs, Switzerland. Isovaleric acid, *n*-valeric, isocaproic acid and heptanoic acid were obtained from K & K Laboratories, Inc., Plainview, N.Y., U.S.A. Hexanoic acid (*n*-caproic acid) was purchased from Koch & Light Lab., England, petroleum and ethyl ether (A.R.) from British Drug House Comp., London, England.

Column material

Chromosorb W (60/80 mesh), LAC-269 (polydiethylene glycol adipate) and DEGA (diethylene glycol adipate) were obtained from Analabs Anal. Eng. Lab. Inc., Hampden, Conn., U.S.A.; Carbowax 20M, DEGS (diethylene glycol succinate) from Appl. Sci. Lab. Inc., State College, Pa., U.S.A.; E-301 (silicone elastomer) from Griffin & George (Sales) Ltd., London, England; PEG-600 (polyethylene glycol) from Koch & Light Lab., England and Apiezon L from Associated Electrical Inc. Ltd., Shell Comp., England; PEGA (polyethylene glycol adipate) was prepared in our laboratory according to JAMES[13].

RESULTS AND DISCUSSION

Preliminary investigations were carried out under isothermal conditions, varying temperatures, type of carrier gas and carrier gas flow rate, in order to find out which one of the thirteen columns used would give the best separation and sensitivity. Most of these columns were chosen from reports, using the methyl-, ethyl or propyl esters of the lower fatty acids[14–20] or the free acids[21–31], but none of them gave the separation of all the fatty acids under investigation. This survey is summarized in Table I.

The acids formic and acetic acid were not separable under isothermal conditions

TABLE I

A SURVEY OF ELEVEN COLUMNS OF DIFFERENT SIZES AND WITH DIFFERENT LIQUID PHASES FOR THE SEPARATION AND HIGHEST SENSITIVITY OF TEN LINEAR FATTY ACIDS UNDER ISOTHERMAL CONDITIONS
Details are given in the text.

Substance	*20% Carbowax 20M*	*21% PEG-600*	*2% PEGA*	*Apiezon L*
Formic acid	No separation	No separation	Good separation	Separation not satisfactory
Acetic acid				
Propionic acid	No separation	Low sensitivity, broad peaks	Low sensitivity	Separation not satisfactory
Isobutyric acid				
n-Butyric acid	Low sensitivity	Low sensitivity		Very low sensitivity
Isovaleric acid				
n-Valeric acid				
Isocaproic acid				
n-Caproic acid				
n-Heptanoic acid				

using the columns with the liquid phases 10% Carbowax 20M, 20% Carbowax 20M; 25% Carbowax 20M coated with 4% orthophosphate, 21% PEG-600, 15% DEGS, and 20% LAC-269 coated with 2% orthophosphate. An unsatisfactory separation was obtained with 15% Apiezon L and 20% E-301. This left all three columns with PEGA as the only liquid phases, which separated these two acids. The other problem encountered was the separation of propionic acid from isobutyric acid. These two acids could not be separated on any of the Carbowax phases. The column with 21% PEG-600 gave broad peaks, which made a quantitation extremely difficult, as it also showed a very low sensitivity (200 μg). The columns involving the liquid phases Apiezon L, DEGS, DEGA and LAC-269 with 2% orthophosphate gave some separation, but the retention times were too close (0.2–0.6 min) to be of any use in quantitation. Again, all the PEGA columns proved to be far superior in separation. These PEGA columns, however, showed a significant difference in their sensitivity. The two columns with 2% orthophosphate trailed and were therefore excluded, as neither the 2% nor the 3.125% PEGA column gave this phenomenon. Although the 2% PEGA column gave a slightly better separation (4.8 and 5.6 min with He at 600 ml/min; 16.3 and 18.3 min with He at 400 ml/min, temperature at 114°), the 3.125% PEGA column was chosen for further investigations, as it gave an almost ten-fold higher sensitivity. With regard to the higher fatty acids, isovaleric to heptanoic acid, the separation was generally good, but the sensitivity was highest with the 3.125% PEGA column. As far as the type of carrier gas was concerned, helium proved to be

15% DEGS	*3.1% PEGA*	*20% LAC-269 2% H_3PO_4*	*20% DEGA*	*25% Carbowax 20M*	*20% E-310*	*10% Carbowax 20M*
No separation	Good separation	No separation	Retention times very close	No separation	As Apiezon L	No separation
No separation	High sensitivity	Close retention times	Retention times very close	No separation	As Apiezon L	No separation
As Carbowax		Good separation Good sensitivity	Good sensitivity	Low sensitivity	As Apiezon L	Low sensitivity

far superior to nitrogen by using both the conductivity cell and hydrogen flame ionization detector in the case of the PEGA columns.

The results of these preliminary investigations are demonstrated in Fig. 1, which gives the separation of formic, acetic, propionic, isobutyric and *n*-butyric acid with the 3.125 % PEGA column.

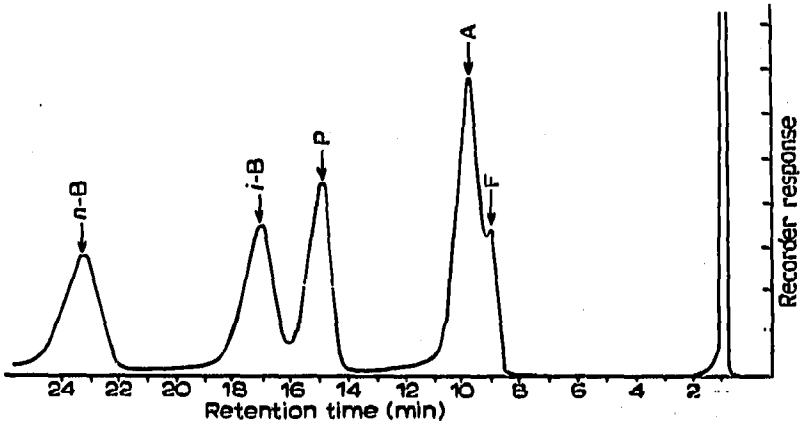

Fig. 1. Separation of formic (F), acetic (A), propionic (P), isobutyric (*i*-B) and *n*-butyric (*n*-B) acid. Conditions: GC-2 gas chromatograph; temperature, 114°; thermoconductivity cell; currency, 250 mA; carrier gas, helium; flow rate, 400 ml/min or 20 p.s.i.; injection size, 500 μg of each acid in diethyl ether; column, 3.125% PEGA on Chromosorb W, acid-washed (60/80 mesh) 4 ft. × 1/4 in. O.D. stainless steel; attenuation, × 1.

The peak heights were determined from the point of rise towards the peak of the particular compound according to CRIPPEN AND SMITH[32], which proved to be extremely satisfactory and far superior to peak area measurements. It was also confirmed that formic acid was not detectable on the hydrogen flame ionization detector. The standardization of these acids was carried out using a number of different concentrations with at least five replicates for each concentration. In using the correction factors of PACKETT AND MCCUNE[33] for the peaks of equimolar mixtures of organic acids, the values as presented in Table II were obtained. This table also indicated that the lowest sensitivity quantitated was 100 μg of either of the acids. This was only slightly above the sensitivity reported by FAICHNEY[34], who used a 6 ft. × 1/8 in. O.D. stainless steel column with 20% Tween 80 coated with 2% orthophosphate on Embacel (60/80 mesh) and nitrogen as carrier gas under temperature-programming conditions.

In the case of isovaleric, *n*-valeric, isocaproic, *n*-caproic and heptanoic acid, a higher temperature was necessary, as the peaks at 114° broadened too much and the sensitivity dropped too low. Fig. 2 gives the separation of these acids with the exception of *n*-caproic acid.

TABLE II

RETENTION TIME AND PEAK CORRECTION FACTORS (*n*-BUTYRIC ACID = 1.0) FOR ISOTHERMAL GAS CHROMATOGRAPHY CONDITIONS AND THERMOCONDUCTIVITY CELL

Carrier gas flow rate: 400 ml/min (20 p.s.i.); temperature: 114°; column: 4 ft. × 1/4 in. O.D. stainless steel with 3.12% PEGA on acid-washed Chromosorb W (60/80 mesh).

Substance	*Retention time (min)*	*100–500 µg*
Formic acid	8.8	1.3939 ± 0.134
Acetic acid	9.7	1.7574 ± 0.252
Propionic acid	14.8	1.4744 ± 0.032
Isobutyric acid	17.0	0.9771 ± 0.102
n-Butyric acid	23.2	1.0

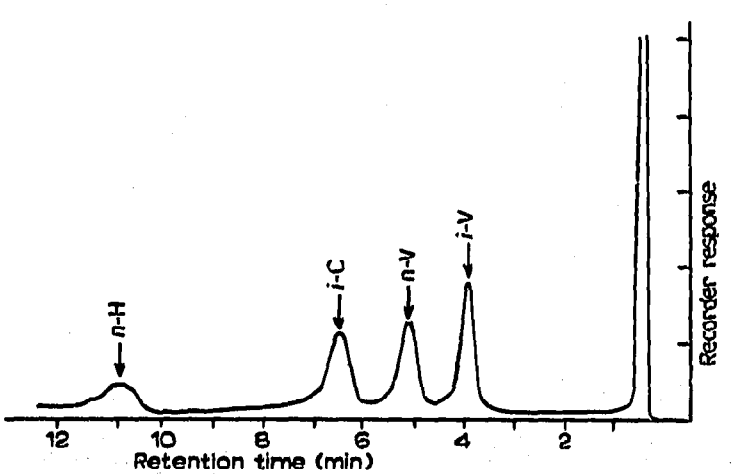

Fig. 2. Separation of isovaleric (*i*-V), *n*-valeric (*n*-V), isocaproic (*i*-C) and *n*-heptanoic (*n*-H) acid. Conditions: GC-2 gas chromatograph; temperature, 130°; thermoconductivity cell; currency, 250 mA; carrier gas, helium; flow rate, 600 ml/min or 30 p.s.i.; injection size, 100 μg of each acid in diethyl ether; column, as in Fig. 1.

TABLE III

RETENTION TIME AND PEAK CORRECTION FACTORS (*n*-VALERIC ACID = 1.0) FOR ISOTHERMAL GAS CHROMATOGRAPHY CONDITIONS AND THERMOCONDUCTIVITY CELL

Carrier gas flow rate: 600 ml/min (30 p.s.i.); temperature: 160°; columns, as in Table I.

Substance	*Retention time (min)*	*100–500 μg*
Isovaleric acid	4.0	1.4988 ± 0.018
n-Valeric acid	5.2	1.0
Isocaproic acid	6.6	0.6535 ± 0.044
n-Heptanoic acid	11.0	0.2547 ± 0.020

For determining the peak heights, retention time and peak correction factors, Table III indicated that the lowest amounts usable for quantitation were 100 μg of each acid and the variation in peak heights was very small and smaller than in the case of the lower fatty acids. The isothermal conditions allowed therefore the detection of 100 μg of each acid or 0.2 % in a culture fluid, if the ether extracts are concentrated to a final volume of 10 ml.

The differences in sensitivity obtained by using isothermal[35] and temperature-programming conditions[34] with the same ratio of liquid phase to solid support but different column dimensions were investigated with the 3.125 % PEGA column. In our case the same column was used. Until recently temperature programming was

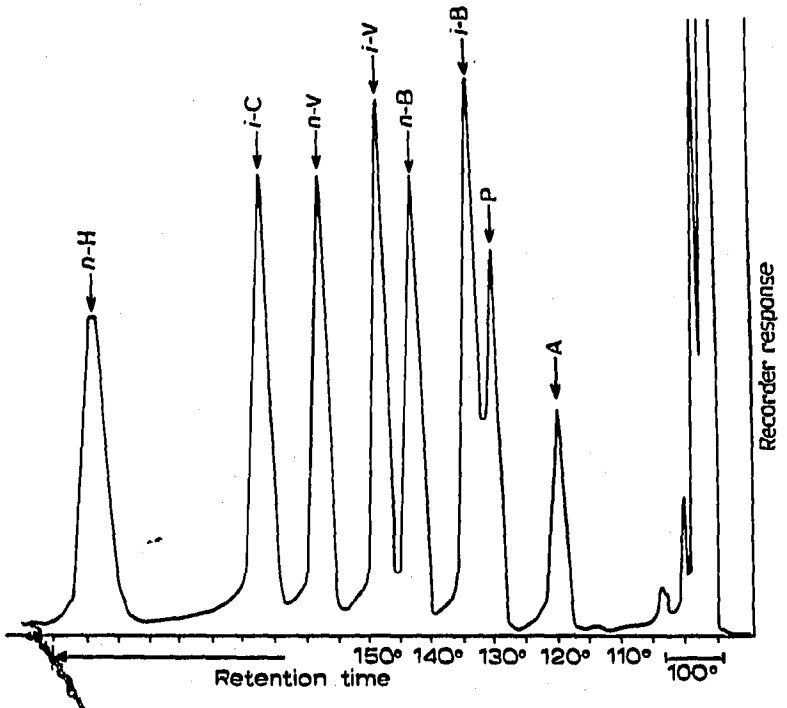

Fig. 3. Separation of acetic (A), propionic (P), isobutyric (*i*-B), isovaleric (*i*-V), *n*-valeric (*n*-V), isocaproic (*i*-C) and *n*-heptanoic (*n*-H) acid by temperature programming using Beckman GC-M under the following conditions: injection port temperature, 150°; detector room temperature, 200°; carrier gas, helium; flow rate, 100 ml/min; dual hydrogen flame ionization detector; attenuation, 1×10^3; inoculation size, 10 μg; column, as in Figs. 1 and 2; temperature programming rate, 100°–150°, 5°/min starting 2 min after injection.

only used for the detection of the methyl esters of the lower fatty acids[30]. The conditions employed are listed in the legend to Fig. 3.

Under these conditions both resolution and sensitivity were greatly enhanced. Fig. 3 gives the resolution with the corresponding retention times and temperatures at which the individual acids appeared. *n*-Caproic acid was not added to the mixture, as injection of the acid alone revealed considerable impurities of the other acids, which would greatly influence quantitation. The main peak, however, appeared perfectly separable between isocaproic and heptanoic acid. The sensitivity could be greatly enhanced by using attenuations 1×10^3 and 1×10^2. The linearity of the peak heights against concentration at attenuation 1×10^3 for the range between 2.5 μg and 25 μg is given in Fig. 4. In using attenuation 1×10^2 a sample of 0.5 μg was easily detectable. The linearity between 0.5 and 2.5 μg was as good as for the range shown in Fig. 4. The variation in peak heights is presented in Table III in form of the peak correction factors, which were calculated for each attenuation separately. Compared with the isothermal conditions of analyses, the variation was far lower if temperature programming was used, even at as low a concentration as 0.5 μg. The lowest amount detectable was 0.1 μg, which, however, could only be estimated.

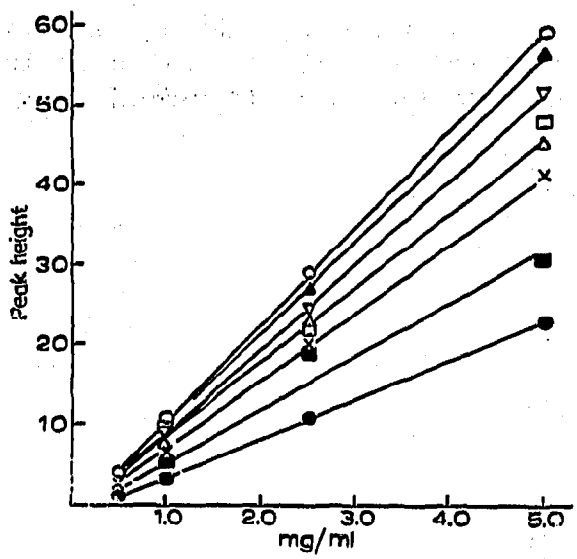

Fig. 4. Standard curves for acetic (●—●), propionic (×—×), isobutyric (○—○), *n*-butyric (△—△), isovaleric (▲—▲), *n*-valeric (▽—▽), isocaproic (□—□), and *n*-hexanoic acid (■—■) in the range of 2.5–25 μg as obtained with the dual hydrogen flame ionization detector at attenuation 1×10^3 and the conditions stated in Fig. 3. The values of the 5 μl injection size have been converted to mg/ml.

All the acids were injected into the gas chromatograph individually and in combination for this standardization, but no significant difference in peak heights for any particular compound was observed. The reproducibility was between 1–3 % and thus similar to the one obtained with alcohols[11]. It is therefore possible to determine 0.001 % or less of any of the nine acids investigated.

The column 4 ft. × 1/4 in. O.D. 3.125 % PEGA on Chromosorb W (60/80 mesh) column gave a very similar sensitivity to the Dow Corning Silicone 550 with stearic

TABLE IV

PEAK CORRECTION FACTORS (*n*-BUTYRIC ACID = 1.0) FOR TEMPERATURE PROGRAMMING CONDITION ON GC–M AND DUAL HYDROGEN FLAME IONIZATION DETECTORS

Carrier gas flow rate: 100 ml/min; starting temperature: 100° with 5°/min up to 160°; columns, as in Table I; retention times, see Fig. 3.

Substance	*0.5–5.0 μg* (1×10^2)	*2.5–25 μg* (1×10^3)	*25–250 μg* (1×10^4)
Acetic acid	0.2163 ± 0.054	0.4072 ± 0.105	0.4362 ± 0.085
Propionic acid	0.8125 ± 0.096	0.8277 ± 0.072	0.8449 ± 0.128
Isobutyric acid	0.6908 ± 0.097	1.2371 ± 0.064	0.9300 ± 0.122
n-Butyric acid	1.0	1.0	1.0
Isovaleric acid	1.0033 ± 0.102	1.2349 ± 0.039	0.9905 ± 0.127
n-Valeric acid	1.1437 ± 0.112	1.1072 ± 0.065	0.9568 ± 0.027
Isocaproic acid	1.2808 ± 0.167	1.1206 ± 0.122	0.7172 ± 0.074
n-Heptanoic acid	0.7813 ± 0.135	0.7751 ± 0.113	0.4486 ± 0.026

acid coated column on depolarized Chromosorb W[37]. It is in contrast, however, to the findings of CLARKE AND FREDERICKS[38], who reported an insufficient separation with PEGA and found the additional coating with 2 % orthophosphate necessary, although they still seem to encounter difficulties in quantitation of the acids.

REFERENCES

1 A. C. NEISH, *Analytical Methods for Bacterial Fermentations*, 2nd Rev., Natl. Res. Council of Canada, 1952.
2 I. P. TING AND W. M. DUGGER, JR., *Anal. Biochem.*, 12 (1965) 571.
3 V. B. D. SKERMAN, *A Guide to the Identification of the Genera of Bacteria*, 2nd Ed., Williams and Wilkins, Baltimore, 1967, p. 247.
4 N. R. BACHUR, *Anal. Biochem.*, 13 (1965) 463.
5 H. W. DOELLE, *Bacterial Metabolism*, Chap. 6, Academic Press, New York, 1969, in press.
6 C. W. MOSS AND V. J. LEWIS, *Appl. Microbiol.*, 15 (1967) 390.
7 C. W. MOSS, V. R. DOWELL, JR., V. J. LEWIS AND M. A. SCHEKTER, *J. Bacteriol.*, 94 (1967) 1301.
8 V. J. LEWIS, C. W. MOSS AND W. L. JONES, *Can. J. Microbiol.*, 13 (1967) 1033.
9 R. T. O'BRIEN, *Lab. Pract.*, 8 (1967) 78.
10 R. K. THAUER, K. JUNGERMANN, H. HENNINGER, J. WENNI[illegible] AND K. DECKER, *European J. Biochem.*, 4 (1968) 173.
11 H. W. DOELLE, *J. Gas Chromatog.*, 5 (1967) 582.
12 H. W. DOELLE, *Antonie van Leeuwenhoek J. Microbiol. Serol.*, 32 (1966) 373.
13 A. T. JAMES, *Methods Biochem. Anal.*, 8 (1960) 10.
14 M. L. VORBECK, L. R. MATTICK, F. A. LEE AND C. S. PEDERSON, *Nature*, 187 (1960) 689.
15 M. H. YUEH AND F. M. STRONG, *J. Agr. Food Chem.*, 8 (1960) 491.
16 L. D. METCALFE AND A. A. SEMITY, *Anal. Chem.*, 33 (1961) 363.
17 H. BRANDENBERGER AND S. MÜLLER, *J. Chromatog.*, 7 (1962) 137.
18 A. S. APPLEBY AND J. E. O. MAYNE, *J. Gas Chromatog.*, 5 (1967) 266.
19 I. R. HUNTER, *J. Chromatog.*, 7 (1962) 288.
20 H. W. OCKERMAN, T. N. BLUMER AND H. B. GRAIG, *Food Res.*, 29 (1964) 23.
21 C. W. GEHRKE AND W. M. LAMKIN, *J. Agr. Food Chem.*, 9 (1961) 85.
22 E. M. EMERY AND W. E. KOERNER, *Analyt. Chem.*, 33 (1961) 146.
23 A. MURAI AND Y. TACHIKAWA, *J. Chromatog.*, 14 (1964) 100.
24 H. GERSHON AND J. A. A. RENWICK, *J. Chromatog.*, 20 (1965) 134.
25 R. B. JACKSON, *J. Chromatog.*, 22 (1966) 251.
26 R. B. HUGHES, *J. Sci. Food Agr.*, 11 (1960) 47.
27 R. B. JACKSON, *J. Chromatog.*, 16 (1964) 306.
28 G. W. LANIGAN AND R. B. JACKSON, *J. Chromatog.*, 17 (1965) 238.
29 I. R. HUNTER, V. H. ORTEGREN AND J. W. PENCE, *Anal. Chem.*, 32 (1960) 682.
30 W. R. MAYBERRY AND G. J. PROHAZKA, *J. Gas Chromatog.*, 3 (1965) 232.

31 R. G. ACKMAN AND J. C. SIPOS, *J. Chromatog.*, 13 (1964) 337.
32 R. C. CRIPPEN AND C. E. SMITH, *J. Gas Chromatog.*, 3 (1965) 37.
33 L. W. PACKETT AND R. W. MCCUNE, *Appl. Microbiol.*, 13 (1965) 22.
34 G. J. FAICHNEY, *J. Chromatog.*, 27 (1967) 482.
35 I. OLSEN AND J. M. MERRICK, *J. Bacteriol.*, 95 (1968) 1774.
36 A. T. JAMES AND A. J. P. MARTIN, *Biochem. J.*, 63 (1956) 144.
37 V. M. RIDDLE, *Anal. Chem.*, 35 (1963) 853.
38 J. R. P. CLARKE AND K. M. FREDERICKS, *J. Gas Chromatog.*, 5 (1967) 99.