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GAS CHROMATOGRAPHIC ANALYSIS OF LOW BOILING FATTY ACIDS IN BIOLOGICAL MEDIA*

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SUMMARY

Different types of Porapak packing material have been tested in order to find a suitable combination for the separation and determination of micro-quantities of formic, acetic, propionic, isobutyric, butyric, 3-methylbutyric and valeric acids present in biological media as intermediates and end-products in microbial fermentation. The best results were obtained with Porapak N (100–120 mesh). With a helium flow rate of 50 ml/min through an 85-cm stainless steel column with 2 mm I.D. and temperature programming between 165 and 215° at a rate of 4°/min, the seven acids can be completely separated in 15 min. 2-Methyl- and 3-methylbutyric acids cannot be separated.

Quantitative determination of formic acid is possible down to 200 ng by this method and the other six acids can be determined down to 20 ng. These sensitivities allow direct injection of the biological (aqueous) medium into the chromatograph, the only pretreatment being centrifuging and membrane filtration.

INTRODUCTION

Saturated fatty acids with 1–5 C-atoms occur as intermediates and end-products in different types of microbial fermentation and their separation and quantitative determination in fermentation broths is of great interest. However, in most cases only small amounts of these compounds are present (< 50 p.p.m.). Different types of sample preparation have been studied in order to increase the concentration of the acids in the final sample and to isolate them from water-soluble but non-volatile organic compounds in the fermentation broth.

Steam distillation has been found to give 4–5 fold dilution of the acids in the condensate, even when the sample is saturated with NaCl. Solvent extraction is rendered difficult by the unfavourable partition coefficients of these acids between water and organic solvents. In addition, the most favourable solvents, like ethyl acetate, methyl ethyl ketone and diisopropyl ether,[1] create a difficulty, when gas chromatographic (GC) analysis is to be used, by interfering with the retention volumes of the acids. However, gas chromatography must be considered as the most advan-

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tageous method for this purpose as it allows the separation of the acids and the quantitative determination of each of them. The difficulty of performing the separations and especially the quantitative determination of these volatile free fatty acids arises mainly from their relatively high boiling points and their adsorption on the solid support. Numerous techniques have been used to reduce or eliminate these problems. LEE AND BETHEA[2] reviewed the many combinations of liquid phases, liquid phase blends and supports reported up to 1968. Summarizing this, together with their own experience, they stated that satisfactory results could only be obtained on using a non-volatile acidic substance, such as H_3PO_4, trimer acid or isophthalic acid, as an additive to the liquid phase, and Teflon as solid support. Their best results were obtained with columns of 10% FFAP + 0.2% H_3PO_4 or 10% trimer acid + 0.8% dinonylnaphthalene disulfonic acid or 10% ethofat 60/25 + 0.5% isophthalic acid, all with 40–60 mesh Teflon as solid support. Unfortunately the concentrations of the solutions used for this work are not mentioned, but it seems that they were quite high.

In 1969, DOELLE[3] reported a method for the determination of some of the straight- and branched-chain saturated fatty acids with 1–7 C-atoms, using a column with 3.125% PEGA on Chromosorb W-AW. He tested twelve other stationary phases including Carbowax 20M, polyethylene glycol 600, Apiezon L, DEGS, LAC-269 + H_3PO_4 and E310 which he did not find suitable for this problem. With his proposed column he could quantitatively determine formic acid down to 10 μg and the other acids down to 0.1 μg. This is already a good approach to the requirements of the biologists. However, the separation of formic and acetic acids was too poor for the simultaneous determination of these two acids.

It is surprising that very little has been reported on the use of porous polymers like Porapak for this problem. Waters Associates recommend Porapak Type Q or QS for the separation of C_1–C_4 fatty acids. MAHADEVAN AND STENROOS[4] found that the addition of H_3PO_4 was necessary because these acids could not be eluted from untreated Porapak Q. In the present paper a method for the determination of straight- and branched-chain C_1–C_5 fatty acids in biological media is reported, using a Porapak N column.

EXPERIMENTAL

Apparatus

Centrifuge, IEC International Centrifuge, model HRI.

Membrane filtration, Millipore A.H. microanalysis filter holder, XX50 025 00; Millipore filter, 0.22 μ.

Gas chromatograph, Hewlett Packard Model 7624 A; double column. Flame ionisation and thermal conductivity detector. 1 mV-Moseley Recorder.

Column, 85 cm long with an I.D. of 2 mm. The stainless steel column was packed with Porapak type Q (batch No. 689) or type QS (batch No. 787) or type N (batch No. 799); all 100–120 mesh, obtained from Waters Associates.

Chemicals

All acids used as standards were of the highest purity obtainable from Th. Schuchardt, Munich, Merck AG, Darmstadt (G.F.R.) and Carlo Erba, Milan (Italy).

Method

A sample of *ca.* 5 ml taken from the fermentation broth is immediately centrifuged for 20–30 min at 10000–15000 r.p.m. depending on the size of the cells present.

1 ml of the supernatant is then passed through a 0.22 μ millipore filter (pressure filtration to avoid losses), but this filtrate is rejected. Another 1 ml of the sample is then filtered, using the same filter, and this filtrate is used for the GLC analysis. During all these operations the sample is kept below 5°. About 3–5 μl are injected into the gas chromatograph, equipped with a Porapak N column. The instrument conditions were as follows.

Temperatures: injection port, 210°; flame ionisation detector, 230°; hot wire detector, 210°.

Gases: helium, 50 ml/min; hydrogen, 40 ml/min; air, 400 ml/min.

Bridge current: 195 mA.

Column temperature programme. Start: 165°; 2 min isothermal at 165°, followed by programming at 4°/min to 195° then 6–10 min isothermal at 195°. This temperature programme was used for all the chromatograms reported in this paper.

RESULTS AND DISCUSSION

Attention was paid to two problems in particular at the start of this study: first, the cleaning up of the broth sample, and secondly, the GC column used for the separation and determination of the acids. Since GC was to be used for the analyses of the acids in the broth it was thought that it would be absolutely necessary to separate the water-soluble non-volatile compounds (salts, amino acids etc.) before the injection in order to avoid drastic base line troubles. These could be caused by a kind of continuous pyrolysis in the injection port and at the top of the column. Two techniques envisaged for the cleaning-up, *viz.* steam distillation and solvent extraction, have, however, been found to be useless, as already mentioned above, when microquantities of these acids are to be determined. Our experience with directly injected samples from the fermentation of different yeasts and bacteria has shown that the phenomena of ghost peaks and base line instability only arise when there are cells or cell fragments remaining in the sample. This is difficult to avoid by centrifuging but very easy by filtration through a 0.22-μ membrane. After this two-step pretreatment, *viz.* centrifuging and membrane filtration, it was possible to use a Porapak N column for *ca.* 700 injections before the peak shape started to deteriorate. The problem of finding a suitable column packing material for these low boiling fatty acids was defined by the amount of each acid to be detected: between 300 and 500 ng of formic and at least 50 ng of the other C_2–C_5 acids per injection. No column described in the literature was found to be satisfactory for this purpose. The free acids produced visible tailing, whether or not the stationary phases had been blended with non-volatile acidic substances. This tailing may be negligible when macro amounts are injected but becomes a dominant factor with microquantities. Porous polymer beads have been used for several years as GLC column packing material and there is one type, Porapak Q, which is recommended by the manufacturer for low boiling fatty acids. Unlike MAHADEVAN AND STENROOS[4], we did not find that the addition of H_3PO_4 was necessary for the elution of the acids from this material. Very strong tailing still occurs but it is

not influenced by treatment with H_3PO_4. A chromatogram of acetic and propionic acids, 40 ng of each on Porapak Q, is shown in Fig. 1a. Fig. 1b shows the same chromatogram on the silylated material Porapak QS, and shows a remarkable

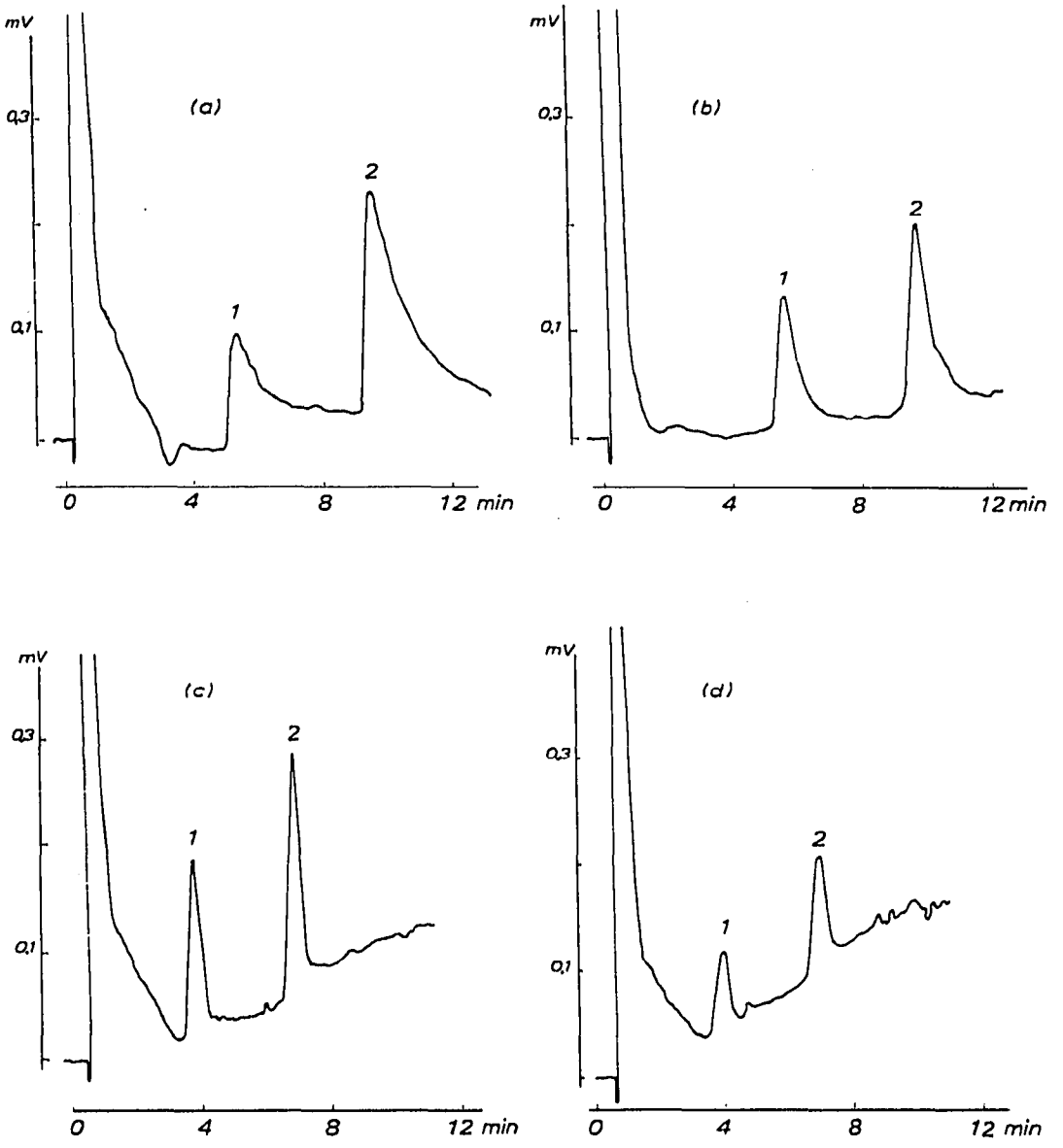


Fig. 1. Acetic (1) and propionic acid (2), on (a) Porapak Q, 40 ng each; (b) Porapak QS, 40 ng each; (c) Porapak N, 40 ng each; (d) Porapak N, 20 ng each.

improvement of the peak shape. Encouraged by these results we tested all the Porapak types which are available at present and found that Porapak N is the best material for the separation of C_1–C_5 fatty acids. In Figs. 1c and 1d the chromatograms of acetic and propionic acids, each at 40 and 20 ng, are shown respectively. The peaks are sufficiently symmetrical and narrow to allow peak height measurement to be used for quantitative analyses. The method is applicable down to 20 ng, except for formic acid,

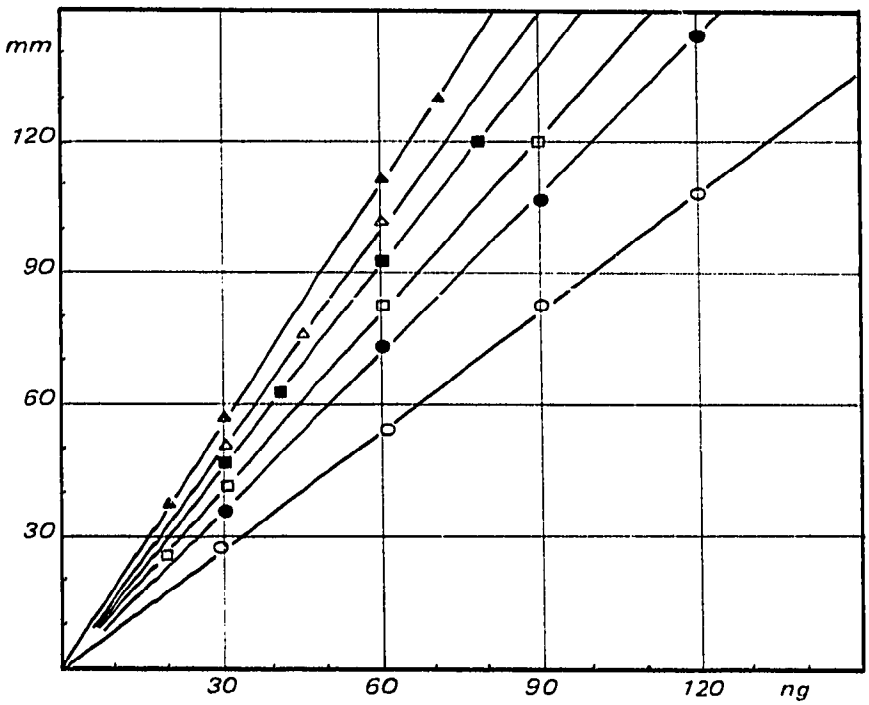


Fig. 2. Calibration curves for acetic (○—○), propionic (●—●), 2-methylpropionic (■—■), butyric (□—□), 3-methylbutyric (▲—▲) and valeric acid (△—△). Flame ionisation detector; range 1, attenuation 16. Size of injection sample 5 μl. Peak height in mm plotted against ng injected.

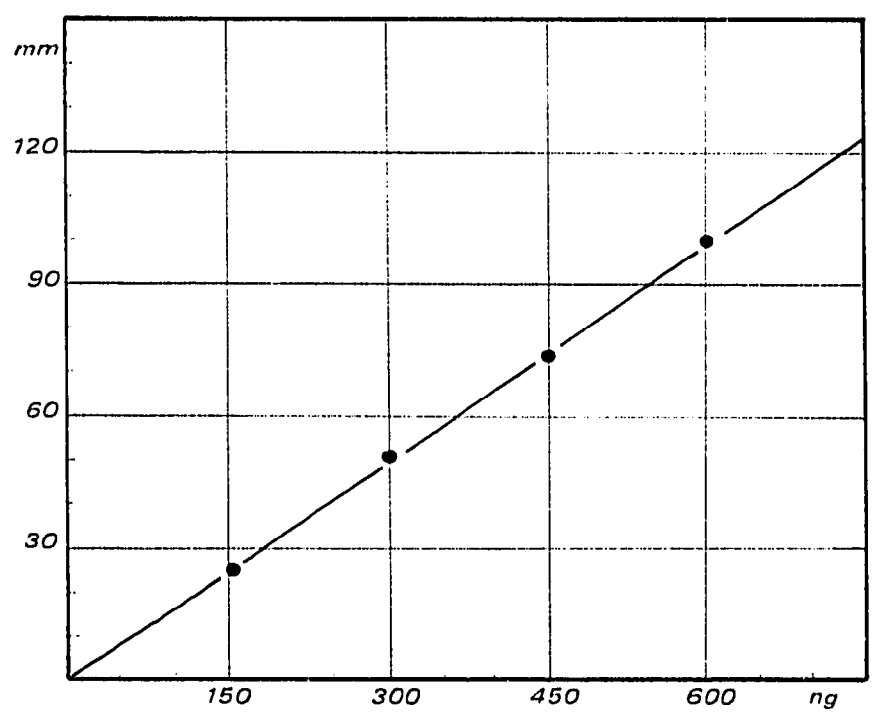
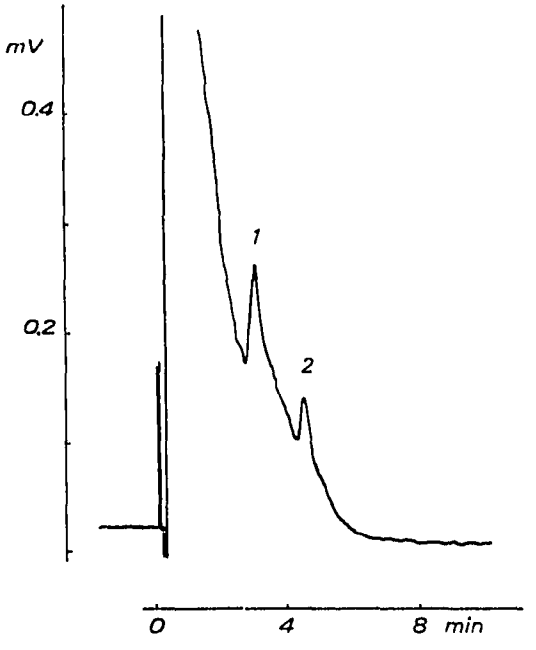


Fig. 3. Calibration curve for formic acid. Thermal conductivity detector: attenuation 1. Size of sample injected 5 μl. Peak height in mm plotted against ng injected.

where the limit is 200 ng. The standard calibration curves are given in Figs. 2 and 3. The lowest amounts detectable are *ca.* 100 ng for formic acid and 10 ng for the other six acids. This means that on injecting 5 μl, formic acid can be determined down to 40 p.p.m. and the other acids down to 4 p.p.m. (The difference in sensitivity between formic and the other acids is because formic acid gives no true signal with the FID so that a thermal conductivity detector must be used.) Fig. 4 shows the chromato-



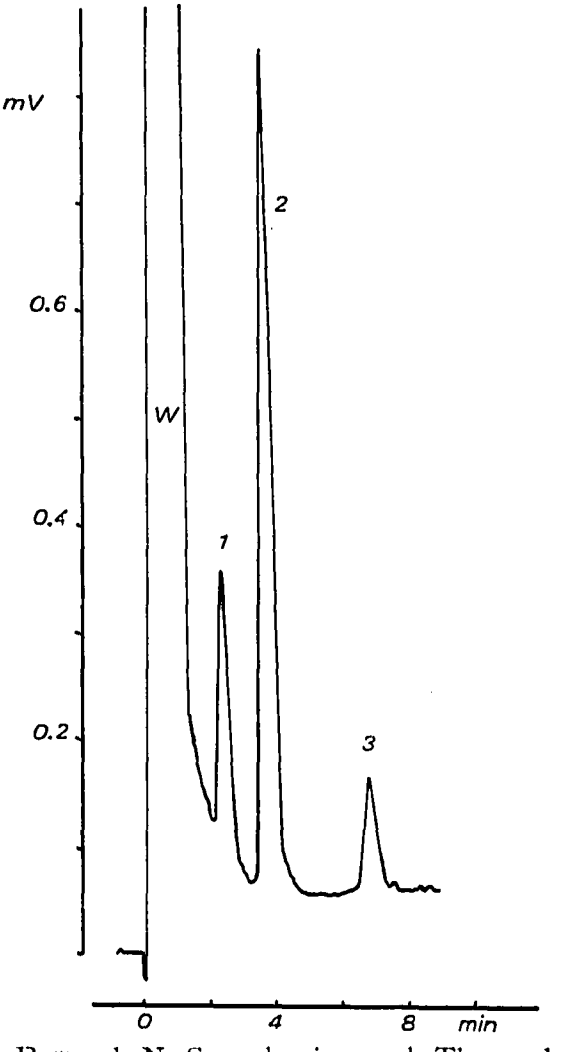


Fig. 4. 150 ng formic acid (1) + 90 ng acetic acid (2) on Porapak N. Sample size 5 μl. Thermal conductivity detector: attenuation 1.

Fig. 5. Separation of water (W) and formic (1), acetic (2) and propionic acids (3) on Porapak N. TC-detector.

gram of 150 ng of formic acid (Peak 1). The injection of pure water before starting an analysis, especially in the low concentration range, is essential to check that there are no acids retained on the column.

85 cm has been found to be the optimal column length, as with shorter columns the separation of water and formic acid becomes difficult. Fig. 5 shows the separation of water and formic, acetic and propionic acids and Fig. 6 the separation of acetic, propionic, butyric, 2-methylpropionic, 3-methylbutyric and valeric acids. 2-Methyl- and 3-methylbutyric acid cannot be separated, even with much longer columns.

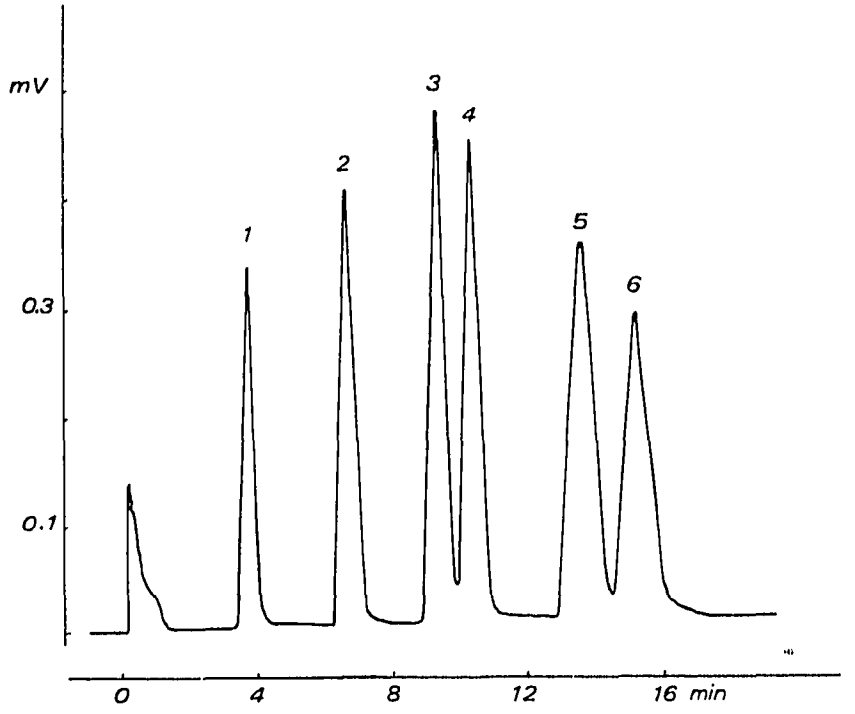


Fig. 6. Separation of acetic (1), propionic (2), 2-methylbutyric (3), butyric (4), 3-methylbutyric (5) and valeric acids (6) on Porapak N. Detector, FID.

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