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## Note

# Gas chromatographic analysis of $C_2$–$C_5$ fatty acids in aqueous media using Carbopack B–Carbowax 20M–phosphoric acid

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The accurate determination of the lower saturated monocarboxylic acids is of interest because they are produced by many micro-organisms as metabolic products[1–3]; they also occur in various other sources of biological origin[3–5]. The use of the packing material Porapak N for the gas chromatographic determination of $C_2$–$C_5$ free fatty acids (FFA) in aqueous media has been described[2,6,7], but complete separation of the isomeric pairs of butyric and valeric acids with Porapak N was not possible within a reasonably short analysis time[7]. A relatively new column packing, Carbopack B–Carbowax 20M–phosphoric acid, has been developed specifically for the separation of $C_2$–$C_5$ FFA in dilute aqueous solution at low concentrations[3,8]. We have investigated the suitability of this packing material for the determination of FFA, with special reference to "ghosting" effects.

"Ghosting" is a major source of error during quantitative or qualitative determinations of FFA[9–11], but many workers tend to ignore or disregard this phenomenon, probably because they are unaware of recent observations on adsorption caused by the support material, metal tubing and inlet systems, glass wool and carbonaceous deposits[12]. Formic acid vapour in the carrier gas, or formic acid added to the FFA sample, can minimize "ghosting" and reduce tailing[6,7,9,10,12,13].

## EXPERIMENTAL

A Hewlett-Packard 5830A keyboard controlled gas chromatograph with a multi-function digital processor, equipped with a hydrogen-flame ionization detector, was used. The operating temperatures were: inlet, 220°; detector, 250°; oven, as specified in text. The gas flow-rates were: nitrogen, 60 ml/min; hydrogen, 65 ml/min; air, 255 ml/min. Stainless-steel columns (1.0 m × 1.6 mm I.D.), and glass columns (1.0 m × 4 mm I.D.), were packed with 3% Carbowax 20M–0.5% phosphoric acid on Carbopack B (60–80 mesh) (Supelco Inc., Bellefonte, Pa., U.S.A.) in the normal manner, with gentle tapping. It was necessary to plug both ends of the column with a minimal amount of glass wool to prevent disturbing the packing during injection, and for this purpose phosphoric acid-treated glass wool (Supelco Inc.) was used (it is more suitable for FFA analysis than is untreated glass wool[14]).

Standard aqueous solutions of FFA at concentrations ranging from 5–1000

ppm of acetic, propionic, isobutyric, *n*-butyric, isovaleric and *n*-valeric acids were injected on-column, either directly or after acidification by adding 1 ml of conc. formic acid to 4 ml of FFA sample. All chemicals used were of reagent grade. To test for "ghosting", three samples of a given FFA standard solution were injected at 15-min intervals, followed by two injections each of 2 $\mu$l of water and 2 $\mu$l of 20% formic acid to elute adsorbed acids. The extent of "ghosting" was estimated by expressing the area of a "ghost" peak as a percentage of the area of the corresponding peak in the preceding FFA sample.

To examine the suitability of the above column for the determination of microbial metabolites, a strain of *Enterobacter aerogenes* was cultured for 48 h at 37° in a medium containing 1% of Bacto-peptone (Difco) and 0.5% of D-glucose (Merck), 10 ml of medium being used per 15-ml screw-capped bottle. After incubation, the culture medium was filtered through a membrane filter (pore size 0.22 $\mu$m) under pressure[2], and the filtrate was acidified with formic acid as described above before gas chromatographic analysis.

## RESULTS AND DISCUSSION

Although small injection volumes ($\leqslant$ 1 $\mu$l) are recommended[3,8], good separation could be attained with 2 $\mu$l injection volumes, and the isomers of butyric and valeric acids were well separated (see Fig. 1). Temperature-programming could

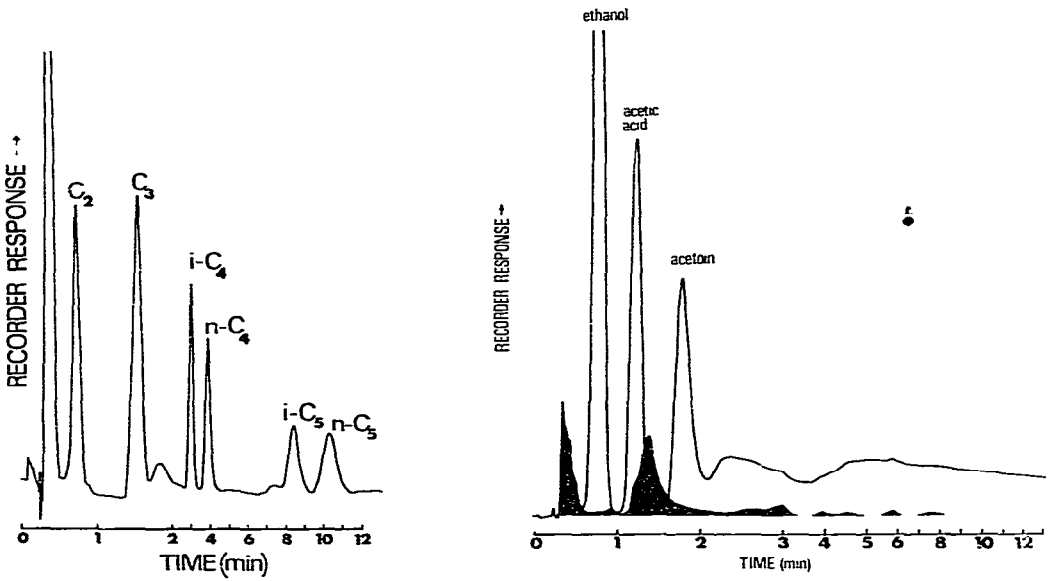


Fig. 1. Separation of acetic, propionic, isobutyric, *n*-butyric, isovaleric and *n*-valeric acids in a glass column packed with Carbopack B–Carbowax 20M–phosphoric acid. Oven temperature 185°; attenuation 8. The FFA sample contained *ca.* 8 ppm of each acid and 20% formic acid.

Fig. 2. Chromatogram of an *Enterobacter aerogenes* culture (the black area shows the profile of the uninoculated medium). A glass column packed with Carbopack B–Carbowax 20M–phosphoric acid was used, with the oven temperature at 160° for 0.5 min, then programmed to 195° at 10°/min. Attenuation 64.

improve this separation and shorten the analysis time, and base-line drift during programming was very slight. No peak for formic acid was observed under these operating conditions.

Typical examples of the "ghosting" effect are presented in Table I. From these results it was evident that (*i*) "ghosting" occurred even with pure aqueous FFA samples, (*ii*) acetic acid exhibited a greater degree of "ghosting" than the other acids, (*iii*) the magnitude of "ghosting", relative to the acid concentration in the preceding FFA samples, was greater with samples containing low FFA concentrations (*i.e.* the problem of "ghosting" increases with decreasing FFA concentration"), (*iv*) water was less effective for eluting "ghost" peaks than a 20% formic acid solution, and (*v*) the addition of formic acid to the FFA sample greatly diminished "ghosting". These results support previous reports[7,10–13].

TABLE I

APPEARANCE OF "GHOST" PEAKS WITH SEQUENTIAL INJECTION OF WATER AND 20% FORMIC ACID

The column conditions were as in the legend to Fig. 1.

| *Injection No.* | *Liquid injected* | *Extent of "ghosting", %* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Acetic acid* | *Propionic acid* | *Isobutyric acid* | *n-Butyric acid* | *Isovaleric acid* | *Valeric acid* |
| *After 3 injections (each 2 µl) of FFA solution (100 ppm)* | | | | | | | |
| 1 | Water | 4.4 | 1.9 | 0.7 | 1.3 | 0.3 | 0.4 |
| 2 | Water | 4.5 | 1.0 | 0.2 | 0.6 | 0.0 | 0.0 |
| 3 | 20% Formic acid | 9.2 | 3.6 | 2.1 | 4.2 | 2.8 | 5.3 |
| 4 | 20% Formic acid | 3.9 | 0.2 | 0.0 | 0.0 | 0.0 | 0.2 |
| *After 3 injections (each 2 µl) of FFA solution (100 ppm) plus 20% formic acid* | | | | | | | |
| 1 | Water | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 2 | Water | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 3 | 20% Formic acid | 0.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 4 | 20% Formic acid | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *After 3 injections (each 2 µl) of FFA solution (1000 ppm)* | | | | | | | |
| 1 | Water | 1.2 | 0.8 | 0.4 | 0.7 | 0.6 | 0.9 |
| 2 | Water | 0.8 | 0.4 | 0.2 | 0.4 | 0.4 | 0.7 |
| 3 | 20% Formic acid | 5.6 | 2.8 | 1.5 | 3.2 | 2.3 | 4.8 |
| 4 | 20% Formic acid | 0.5 | 0.1 | 0.1 | 0.2 | 0.05 | 0.2 |
| *After 3 injections (each 2 µl) of FFA solution (1000 ppm) plus 20% formic acid* | | | | | | | |
| 1 | Water | 0.06 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 2 | Water | 0.02 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 3 | 20% Formic acid | 0.19 | 0.06 | 0.06 | 0.12 | 0.09 | 0.1 |
| 4 | 20% Formic acid | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

"Ghosting" was markedly more serious with the stainless-steel column, which agrees with reports that metal columns or metal inlets strongly adsorb FFA[11,14]. Although the stainless-steel column at an oven temperature of 165° gave a good separation of the FFA, the peaks had shoulders and some tailing was present, which was troublesome in the analysis of low concentrations (*ca.* 10 ppm) of FFA. In appear-

ance, these peaks resembled those obtained by Ottenstein and Bartley[14], who used a metal inlet and a column packed with 10% of SP-1200–1% phosphoric acid on Chromosorb W AW (80–100 mesh).

A chromatogram of the medium in which *Enterobacter aerogenes* was cultured is shown in Fig. 2. It was found that alcohols higher than ethanol, *e.g.*, propanol, butanol and butane-2,3-diol, were eluted as broad, low peaks similar to these shown in Fig. 2 after a retention time of 2 min; for all practical purposes, these alcohols cannot be determined with Carbopack B–Carbowax 20M–phosphoric acid. In this respect, such column packings as Porapak Q and Porapak N appear to be more suitable for the general analysis of microbial culture media, as the compounds listed above can be eluted as symmetric peaks from these packings (although the retention times are rather long)[15,16]. In conclusion, it can be stated that Carbopack B–Carbowax 20M–phosphoric acid is an excellent column packing, especially with glass columns, for the determination of volatile fatty acids in ppm quantities in samples in which alcohols other than ethanol are not of interest; it also proved to be highly suitable for determining ethanol, acetone, diacetyl and acetoin.

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