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Note

Gas chromatography of C_1 to C_5 fatty acids in rumen fluid and fermentation media

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Volatile fatty acids (VFA) are the main products of bacterial fermentation in the rumen and their determination is of considerable interest in ruminant nutrition and physiology. Many different substances have been used as stationary phases since the original work of James and Martin[1], but none of them seems to have proved entirely satisfactory. Doelle[2] tested 13 stationary phases and achieved the best separation with 3.125% PEGA on Chromosorb W-AW. However, the simultaneous quantitative determination of formic and acetic acids was not possible because of poor separation and there was also some overlapping of the peaks for propionic and isobutyric acids. Recently, Henkel[3] reported a very good separation of C_1–C_5 straight- and branched-chain fatty acids with the porous polymer Porapak N. Using a column of Porapak N, he was able to determine down to 200 ng of formic acid and down to 20 ng of the other acids. Temperature programming over the range 165–215° had to be used and injection of pure water before analysis was recommended so as to ensure that no acids had been retained on the column, as the porous polymers tend to give rise to ghosting effects. In testing several substances for their potential use in the analysis of VFA, we have obtained promising results with polypropylene glycol sebacate (PPGS), a compound not yet used, to our knowledge, for this purpose. The separation obtained with PPGS was better than any reported so far in the literature. This paper gives a brief description of the method.

MATERIALS AND METHODS

Apparatus

A Pye Model 64 gas chromatograph with dual flame ionization detectors was used, together with a 1 mV Honeywell recorder. For the determination of formic acid, a Pye Argon chromatograph with a 10 mV Honeywell recorder was used.

Columns. Glass columns 150 cm long and I. D. 4 mm were used with the Model 64 FID chromatograph, and 120 cm long and I. D. 4 mm with the Argon chromatograph. The columns were packed with PPGS supported on Chromosorb W, 80–100 mesh, prepared as described by Youssef and Allen[4]. The support material was impregnated by dissolving PPGS in methylene chloride, adding the support and remov-

ing the solvent in a rotary evaporator at 40° under moderate vacuum. The columns were then packed and pre-heated in the normal way.

Temperatures. For the FID chromatograph the injection port was maintained at 180°, the column at 135° and the detector at 200°; for the Argon chromatograph, the column and detector were maintained at 113°.

Gases. Argon was used as the carrier gas. The flow-rates of the gases were: FID chromatograph, argon 33 ml/min, hydrogen 33 ml/min and air 500 ml/min; Argon chromatograph, argon 75 ml/min, unless otherwise indicated.

Reagents

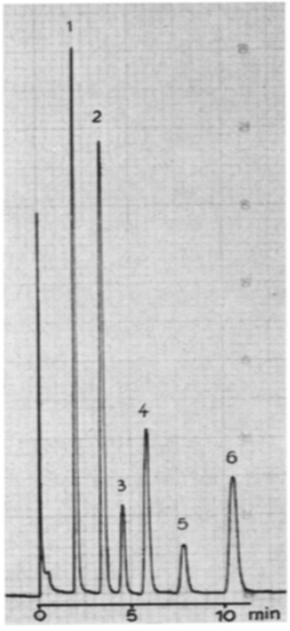
PPGS was obtained from Th. Schuchardt, Munich (G.F.R.). Formic and acetic acids were obtained from POCH, Gliwice (Poland), propionic acid from Reanal (Budapest, Hungary), butyric acid from Koch Light (Colnbrook, Great Britain), isobutyric acid from BDH Chemicals Ltd. (Poole, Dorset, Great Britain), and valeric, isovaleric and 2-methylbutyric acids from Fluka AG (Buchs, Switzerland).

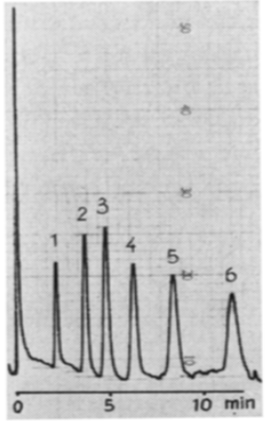
Sample preparation

Samples for the FID chromatograph were prepared as described by Erwin *et al.*[5]. For the determination of formic acid, deproteinized samples of fermentation broth were evaporated in a rotary evaporator and the dry residue was extracted with 5 *N* H_3PO_4 in acetone. A standard solution of formic acid treated in the same way served as a control of the recovery. An alternative procedure was steam distillation of deproteinized samples, titration, evaporation and dissolution in H_3PO_4–acetone.

RESULTS AND DISCUSSION

The analysis was performed under isothermal conditions as this procedure provided adequate separation, a relatively short analysis time of about 12 min and satisfactory peak shapes for all of the components. The most suitable concentration of PPGS was 10–15%. With 5% PPGS, the separation of individual acids was not complete and the peaks appeared bunched; a concentration of 20% PPGS increased retention times without improving resolution. The Chromosorb W used as support was routinely treated with H_3PO_4, but the omission of this treatment had no effect on the quality of resolution. In Fig. 1, a chromatogram of a standard mixture of C_2–C_5 fatty acids in proportions similar to those found in the rumen is shown. Peak symmetry is good and tailing is negligible. Separation is complete, with no overlapping of peaks. Amounts of individual acids down to 25 ng could be determined. A chromatogram of 50 ng each of C_2–C_5 fatty acids is shown in Fig. 2. No memory effect was observed after the injection of water. The linearity of the graph of peak heights against concentration in the range 50–200 ng is shown in Fig. 4. A chromatogram of rumen fluid from a cow fed predominantly on hay is presented in Fig. 3. The quality of separation is similar to that of pure acids. About 2500 analyses were carried out on the column with no signs of a decrease in the quality of separation. A portion of the stationary phase at the injection point, about 10–15 mm long, had to be replaced about once a week as the non-volatile residue of samples that accumulated there formed a charred plug which caused increased tailing of the peaks. Filtration of





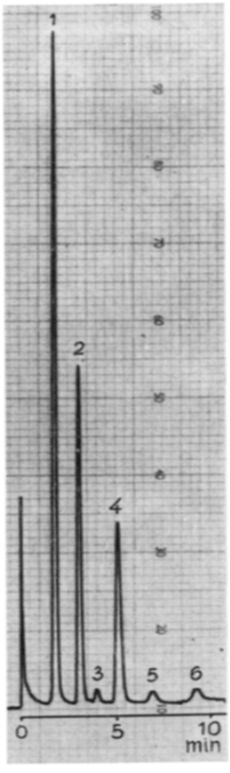


Fig. 1. Separation of VFA mixture containing 1.5 μg of acetic (1), 1.0 μg of propionic (2), 0.1 μg of isobutyric (3), 0.4 μg of *n*-butyric (4), 0.1 μg of isovaleric (5) and 0.25 μg of *n*-valeric acids (6). 15% PPGS on Chromosorb W, FID, attenuation 5×10^2, injection size 5 μl.

Fig. 2. Separation of 50 ng each of acetic (1), propionic (2), isobutyric (3), *n*-butyric (4), isovaleric (5) and *n*-valeric acids (6). 15% PPGS on Chromosorb W, FID, attenuation 1×10^2, injection size 1 μl.

Fig. 3. Separation of acetic (1), propionic (2), isobutyric (3), *n*-butyric (4), isovaleric (5) and *n*-valeric acids (6) in rumen fluid from a cow fed on a hay and concentrate diet. 15% PPGS on Chromosorb W, FID, attenuation 5×10^2, injection size 1 μl.

samples through a millipore filter, as recommended by Henkel[3], might perhaps prevent this to a certain extent.

Formic acid cannot be determined by FID and usually thermal conductivity (TC) detectors are used for this purpose. We had no TC detector at our disposal and used an argon ionization detector, which has the disadvantage that water, because of its high ionization potential, drastically reduces the sensitivity and only samples in organic solvents can be injected on to the column. Standard solutions of C_1–C_5 fatty acids were prepared in acetone, and Figs. 5 and 6 show chromatograms of a mixture of these acids in microgram and nanogram amounts. The separation of formic and acetic acids is very good. At higher detector potentials (higher sensitivity) considerable tailing of the solvent peak occurred and an impurity in the acetone impaired the symmetry of the propionic acid peak. It is therefore essential to keep the

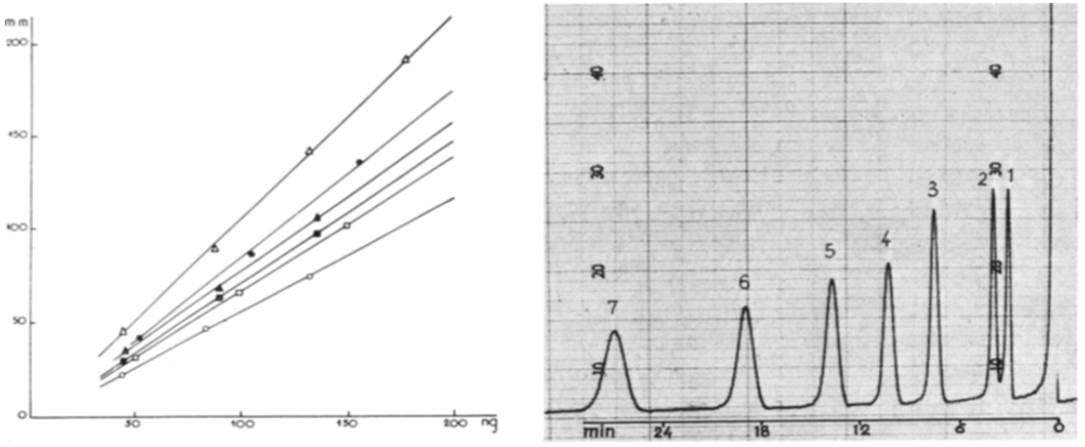


Fig. 4. Standard curves (peak heights *versus* nanograms injected) for acetic (□—□), propionic (●—●), isobutyric (△—△), *n*-butyric (▲—▲), isovaleric (■—■), and *n*-valeric acids (○—○). 15% PPGS on Chromosorb W, FID, attenuation 1×10^2, injection size 5 μl.

Fig. 5. Separation of 0.5 μg each of formic (1) and acetic acids (2) and 1 μg each of propionic (3), isobutyric (4), *n*-butyric (5), isovaleric (6) and *n*-valeric acids (7). 15% PPGS on Chromosorb W, argon ionization detector, detector potential 1250 V, attenuation $\times 1$, injection size 5 μl.

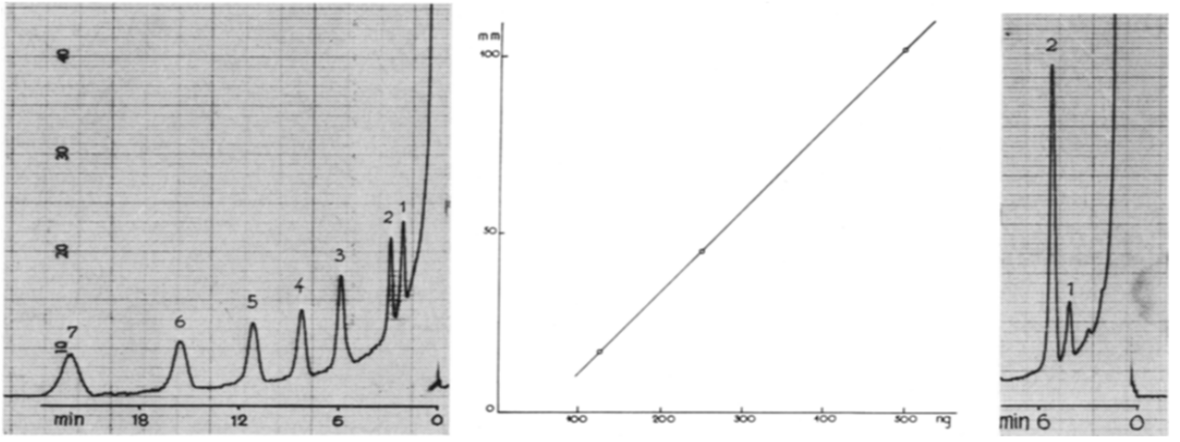


Fig. 6. Separation of 50 ng each of formic (1) and acetic acids (2) and 100 ng each of propionic (3), isobutyric (4), *n*-butyric (5), isovaleric (6) and *n*-valeric acids (7). 15% PPGS on Chromosorb W, argon ionization detector, detector potential 1750 V, attenuation $\times 1$, argon flow-rate 100 ml/min, injection size 1 μl.

Fig. 7. Standard curve (peak height *versus* nanograms injected) for formic acid. 15% PPGS on Chromosorb W, argon ionization detector, detector potential 1250 V, attenuation $\times 1$, injection size 5 μl.

Fig. 8. Separation of formic (1) and acetic acids (2) in fermentation fluid of a pure culture of *Borrelia* sp. grown in glucose medium. 15% PPGS on Chromosorb W, argon ionization detector, detector potential 1500 V, attenuation $\times 1$, injection size 1 μl.

sample size as small as possible. Amounts of formic acid down to 50 ng could be determined. The standard calibration curve for formic acid is given in Fig. 7. The separation of formic and acetic acids was best at 90°, with no overlapping of peaks, but retention times were excessively long, particularly if the samples contained acids up to *n*-valeric. The temperature of 113° was chosen for isothermal operation as it gave satisfactory separation of formic and acetic acids and the duration of analysis was reasonable. Fig. 8 shows the separation of formic and acetic acids, fermentation products of a rumen spirochaete, *Borrelia* sp., grown in a medium with glucose as energy source. Neither of the methods of sample preparation was found to be satisfactory as none of them gave 100% recovery of formic acid, although both of them yielded satisfactory chromatograms. With the use of a TC detector, these cumbersome procedures could be eliminated and temperature programming would reduce the retention times and improve the peak shapes of the heavier components, although the latter are normally determined with an FID.

Isovaleric and 2-methylbutyric acids could not be separated, irrespective of the column length and separation conditions used.

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