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NOTES

Rapid Gas-Chromatographic Method for Identification of Metabolic Products of Anaerobic Bacteria

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The volatile fatty acids, alcohols, nonvolatile fatty acids, and ketones produced by anaerobes can be separated and identified by using a single type of gas-chromatographic column. A rapid and simple procedure is described.

Gas-chromatographic analysis of short-chain fatty acids and alcohols produced as metabolic end products has become a very important method of classification of anaerobic microorganisms (1–8). However, in some cases the fermentation patterns of different microorganisms are very similar, making identification difficult. In such cases it becomes important to analyze as many metabolites as possible.

The VPI *Manual* (3) gives instructions for identifying and separating volatile fatty acids (VFA), alcohols (ALOH), and nonvolatile fatty acids (NVFA), but the separation of ALOH is poor and the thermal conductivity detector is not very sensitive. The *Wadsworth Manual* (6) recommends injecting the centrifuged broth culture into the column without any purification, but that has many disadvantages: rapid contamination of the column, artifactual peaks, and ghosting (2, 7). VFA, ALOH, and NVFA can be separated and detected by the method given in the manual of Willis (8), but this requires an extra column and an additional gas-chromatographic run for the alcohols. In practice ALOH are therefore usually neglected. I describe here a gas-chromatographic method that permits the separation of VFA, ALOH, and ketones (KE) with only one injection of the sample. The NVFA can then be identified as methyl esters (MeE) by injecting the derivatized sample into the same column.

A Varian 3700 dual flame-ionization gas chromatograph was used. The instrument was interfaced with a Varian CDS 111 data system, and the recorder was a Varian G-2500 set on the 1 mV range, paper speed 1 cm/min. The silanized Pyrex glass columns (length 2 m, internal diameter 3 mm) were packed with 15% FFAP (Free Fatty Acids Phase; Applied Science) on Volaspher (Merck) of 100/120 mesh. The columns were conditioned at 100°C overnight with almost no carrier gas flow. For the next 24 h the oven temperature was 160°C and the gas flow rate was 10 ml/min. The temperature was then raised slowly (1°C/min) up to 230°C and kept isothermal for 16 h; the gas flow rate was increased to 20 ml/min. The sensitivity of the electrometer was 1.0×10^{-10} A/mV. The injection port temperature was 220°C for the VFA, ALOH, and KE and 160°C for NVFA-MeE, and the detector temperature was 250°C. The oven temperature was 60°C for 2 min and was increased then by increments of 22°C/min to the final temperature of 215°C. The carrier gas (N_2) flow rate was 38 ml/min; hydrogen and air flow rates were adjusted for optimal acetic acid response.

Standard mixtures of VFA, ALOH, and KE (see legend of Fig. 1) were prepared by diluting 1.25 g of each component (except for biacetyl and hydroxyacetone [2.5 g each] and acetoin [0.625 g]) to 25 ml with ethanol-free ether in a volumetric flask. The acetoin was first dissolved in acetic acid. The hydroxyacetone was purchased in aqueous solution; it was distilled under vacuum and dehydrated before use. A 5-ml sample of the mother solution was diluted to 50 ml with ether and kept in a freezer until used.

The standard mixture of NVFA (see legend of Fig. 2) was prepared by dissolving 100 mg of each component in a minimal amount of 2 N NaOH and diluting to 25 ml in a volumetric flask. A 10-ml sample of this standard solution was supplemented with 20 ml of methanol and 1 ml of 50% sulfuric acid. After esterification (80°C for 30 min), 10 ml of distilled water was added, and the MeE were extracted with 5 ml of chloroform. The standard mixtures were always injected before other samples, after the oven temperature and the carrier gas flow had stabi-

lized, to check the condition of the column and the detector response. All the components of the standard mixtures were from Fluka A.G. (Switzerland).

For the analysis of bacterial cultures the bacteria were grown anaerobically for 48 h in PYG broth (3). The sample preparation for VFA, ALOH, and KE took place as follows: 4 ml of the centrifuged broth culture was saturated with 1 g of sodium chloride. After addition of the internal standard (0.1 ml of a 2.5-mg/ml solution of heptanoic acid in 1 N NaOH), the sample was acidified with 0.15 ml of 2 N HCl and then extracted with 1 ml of ethanol-free (washed) ether. The test tubes were cooled with ice to help the separation of the extracts. A 3-μl portion of the ether layer was injected into the column. The retention times were calculated relative to the internal standard (see legend of Fig. 1). For the NVFA, 2 ml of the centrifuged broth, to which the internal standard (0.05 ml of a 25-mg/ml solution of benzoic acid in 1 N NaOH) had been added, was supplemented with 4 ml of methanol (Merck) and placed at −25°C, stoppered with Parafilm. This step precipitates a large amount of polypeptides derived from the medium, which would otherwise jam the column, possibly for hours. After 45 min the tubes were centrifuged at 3,500 rpm for 10 min. The liquid was then poured into glass-stoppered test tubes, 0.2 ml of 50% sulfuric acid was added, and the samples were placed in a water bath at 80°C

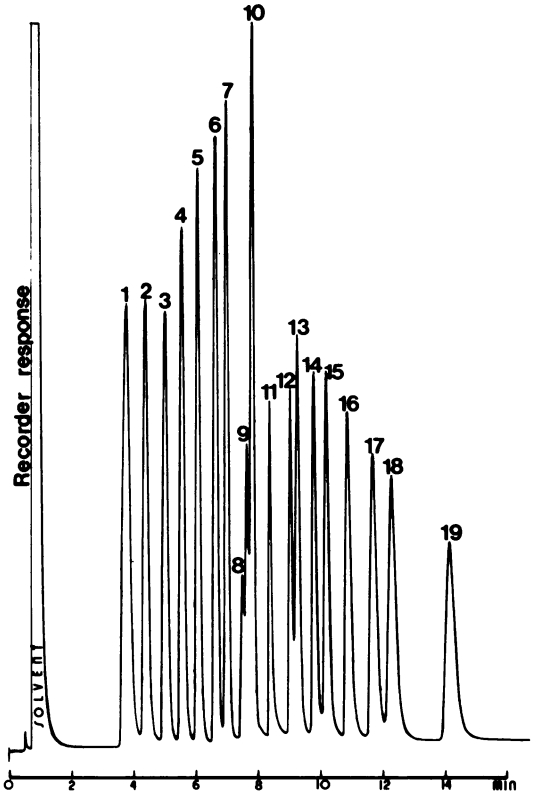

FIG. 1. *Gas-chromatographic separation of a mixture of known VFA, ALOH, and KE. Their relative retention times compared to heptanoic acid are given in parentheses: 1, Ethanol (0.27); 2, biacetyl (0.32); 3, n-propanol (0.36); 4, i-butanol (0.39); 5, n-butanol (0.43); 6, i-pentanol (0.47); 7, n-pentanol (0.49); 8, acetoin (0.53); 9, hydroxyacetone (0.54); 10, 1-hexanol (0.55); 11, acetic (0.6); 12, propionic (0.64); 13, i-butyric (0.66); 14, n-butyric (0.70); 15, i-valeric (0.73); 16, n-valeric (0.77); 17, 4-methylvaleric (0.83); 18, n-hexanoic (0.87); 19, heptanoic (1). Octanoic acid (1.18).*

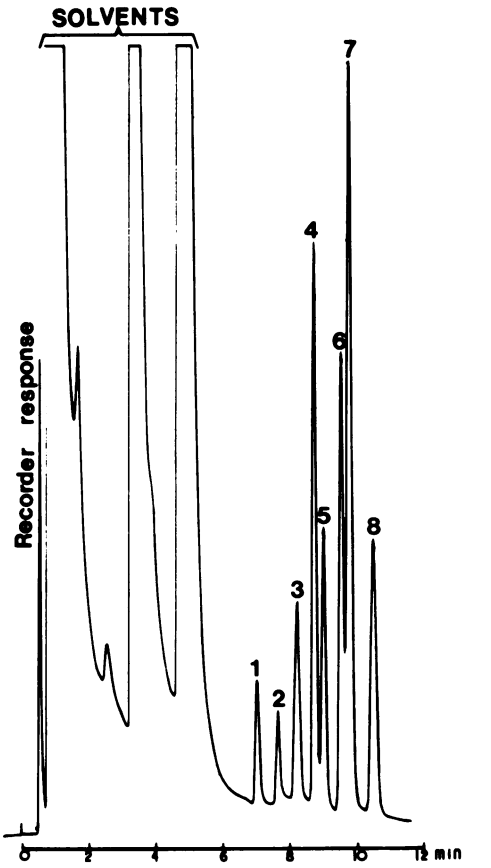

FIG. 2. *Gas-chromatographic separation of a mixture of known NVFA methyl derivatives. Their relative retention times compared to benzoic acid MeE are given in parentheses: 1, Pyruvic (0.67); 2, lactic (0.73); 3, oxalic (0.79); 4, methylmalonic (0.84); 5, malonic (0.86); 6, fumaric (0.91); 7, succinic (0.93); 8, benzoic (1). 4-Methylvaleric (0.62); n-hexanoic (0.64); octanoic (0.80).*

for 30 min. The samples were cooled down with tap water. Distilled water (2 ml) was added, and the methyl derivatives were extracted with 1 ml of chloroform. A 2-μl sample of the lower phase was injected.

The run with the standard mixture (Fig. 1) shows that all the compounds (VFA, ALOH, KE) were eluted and resolved within 14.5 min. The mixture did not contain octanoic acid. A relative retention time of 1.18 was obtained for octanoic acid by analyzing (data not shown) the metabolites of a culture of *Eubacterium alactolyticum*, which were expected to contain it (3). The standard mixture of NVFA-MeE (Fig. 2) was eluted in 10.5 min. The methyl derivatives of 4-methylvaleric, hexanoic, and octanoic acids are not present in the graph. Although these metabolites belong to the VFA group, their MeE are present in the gas chromatograms of the NVFA-MeE, e.g., 4-methylvaleric acid MeE in Fig. 3.

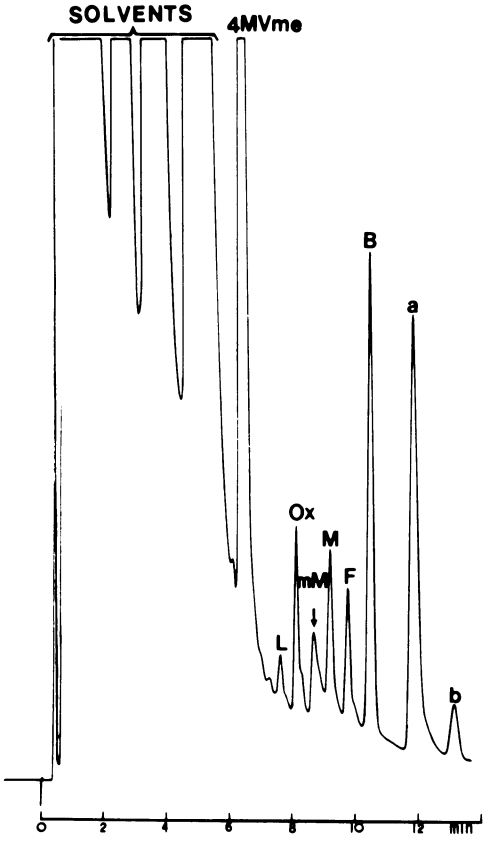

FIG. 3. *NVFA from Clostridium bifermentans, as methyl derivatives: 4MVme, 4-methylvaleric; L, lactic; Ox, oxalic; mM, methylmalonic; M, malonic; F, fumaric; B, benzoic; a, (1.13); b, (1.26).*

This method has been successfully used for anaerobes in this laboratory. As an example, the gas chromatograms of *Clostridium bifermentans*, isolated from cock cecum contents and identified by the API 20 Anaerobe System (Analytab Products Inc., La Balme-Les Grottes, France) in this laboratory, are shown in Fig. 3 and 4. The patterns are clearly resolved and allow the exclusion of *Clostridium perfringens*, *C. subterminale*, *C. botulinum*, *C. capovitale*, *C. sordellii*, and *C. sporogenes* (3).

The method thus gives a large amount of information without being too complicated or time-consuming. The suggested gas-chromatographic program gives adequate resolution of all fatty acids, ALOH, and KE commonly expected among metabolic products of anaerobic bacteria. The same program is used for the NVFA-MeE. Satisfactory results are obtained with short elution times. The elution times could be made even shorter, but a shorter program could easily give rise to wrong interpretations of the fermentation patterns, especially by misinterpretations

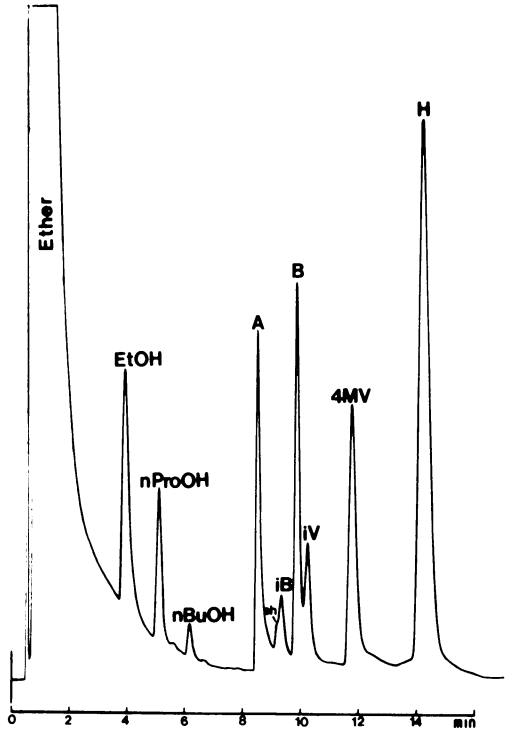

FIG. 4. *Gas chromatogram of an extract of Clostridium bifermentans. EtOH, Ethanol; nProOH, n-propanol; nBuOH, n-butanol; A, acetic acid; sh, shoulder from traces of propionic acid; iB, i-butyric acid; B, n-butyric acid; iV, i-valeric acid; 4MV, 4-methylvaleric acid; H, heptanoic acid.*

of possible unknown peaks. The separation of the peaks looks good, and even for the compounds (acetoin, hydroxyacetone, 1-hexanol) present in the range between *n*-pentanol and acetic acid there is no hesitation in identification if the retention times are calculated relative to an internal standard. Although these three compounds are not present in commonly used standard mixtures, they have been found in cultures of clostridial species.

The method is especially suitable for routine laboratory use, since the same column and the same operating conditions can be used for all work. The preparations of the two samples required is also easy. The treatment with methanol at freezer temperature seems a useful precaution in the preparation of NVFA samples, since peptides, which otherwise interfere with the gas-chromatographic separation, are present in all media suitable for routine work.

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