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Detection of Alcohols and Volatile Fatty Acids by Head-Space Gas Chromatography in Identification of Anaerobic Bacteria

LENNART LARSSON,[1]* PER-ANDERS MÅRDH,[2] AND GÖRAN ODHAM[3]

Department of Medical Microbiology,[2] Laboratory of Ecological Chemistry,[3] and Department of Technical Analytical Chemistry,[1] University of Lund, S-220 07 Lund, Sweden

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A head-space gas chromatographic technique for the analysis of volatile bacterial metabolites is described. *Bacteroides fragilis, Clostridium perfringens,* and *Propionibacterium acnes*, cultured in a glucose-containing peptone yeast extract medium, were studied. The head-space technique was compared with the injection of the complete liquid culture medium, and solvent extracts thereof, into the gas chromatograph. Volatile fatty acids could be detected by all three methods, whereas alcohols produced by *C. perfringens* and *P. acnes* were detectable only in the head-space chromatograms. Both FFAP and Porapak Q were used as gas chromatography stationary phase. Porapak Q was found more suitable than FFAP for the separation of alcohols. The head-space technique requires a minimum of preparation before the analysis and is well suited to automation.

Gas chromatography (GC) is today routinely applied in the identification of anaerobic bacteria. When cultured in a peptone yeast extract medium supplemented with glucose (PYG medium) (8), many anaerobes produce alcohols and free fatty acids which, after extraction, can be analyzed by GC either in their natural state or after being converted to more volatile derivatives. A flame ionization or thermal conductivity GC detector is generally used. From the qualitative and quantitative characteristics in the chromatograms so obtained, many anaerobes can be identified as to genus and sometimes even as to species (8).

Bacterial metabolic products in a broth medium can also be detected by GC by injection of the entire medium into the gas chromatograph (3, 12). This technique has been used as an alternative to extraction methods in the identification of anaerobes. Thermal conductivity detectors can not be satisfactorily used in such analyses.

GC of the head-space atmosphere has also been used in studies of microorganisms. In this technique, the volatile microbial metabolites above a culture medium are sampled by a gas-tight syringe and injected into the gas chromatograph (6). The head-space GC technique has been applied mainly in dairy research, e.g., for the evaluation of microbial contamination of food (1, 2, 5, 11). It has also been used in analyses of fungi (10) and in studies on bacteria of the genus *Clostridium* (4).

In the present communication, we describe a head-space GC technique as developed for the identification of microorganisms, particularly anaerobic bacteria. Head-space chromatograms from studies of *Bacteroides fragilis, Clostridium perfringens,* and *Propionibacterium acnes* are compared with those obtained from analyses of complete culture media that had been incubated with the same organisms and extracts thereof. The usefulness of different GC stationary phases is considered.

MATERIALS AND METHODS

Organisms. Two freshly isolated strains of each of *B. fragilis, C. perfringens,* and *P. acnes* were used. The strains were isolated and identified according to the recommended procedures (8).

Medium. PYG medium, containing 1% (wt/vol) of glucose, was employed (8). Before incubation, nitrogen was bubbled through the medium at 75°C for 15 min to remove volatile compounds. The medium was then made up to its original volume by adding sterile distilled water.

Standard solutions. In the comparative studies, a standard solution was used, consisting of 0.005 ml of each of ethanol and propanol, 0.001 ml of each of butanol and amyl alcohol, and 0.1 ml of each of acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, and caproic acid in 100 ml of distilled water. The head-space technique was also employed when studying a solution consisting of 0.1 ml of each of heptanoic, octanoic, and nonanoic acid in 100 ml of the standard solution described, and a solution of 0.1 ml of caproic acid in 100 ml of distilled water.

Culture technique and preparation of samples. The bacteria were inoculated into 12-ml screw-cap bottles containing 10 ml of PYG and incubated at 37°C for 40 h. For comparison purposes, uninoculated culture medium, incubated under the same conditions,

was tested. The culture medium and 10 ml of the standard solution containing alcohols and lower fatty acids were acidified to pH 2 using diluted H_2SO_4 (1:3 [vol/vol] in water) and divided into three 3-ml aliquots. Each sample was analyzed by one of the three GC methods described below. The caproic acid solution and that containing higher fatty acids were analyzed by the head-space technique only.

Analysis of ether extracts of the medium. Diethyl ether (1 ml) was added to one of the samples. After shaking, the screw-cap bottle was centrifuged, and the ether phase was transferred to 2-ml test tubes. Approximately 0.1 g of solid anhydrous $MgSO_4$ was added. After 10 min at room temperature, 2 μl of the ether solution was injected into the gas chromatograph.

Analysis of complete broth medium. One of the samples was centrifuged at 5,000 × *g* for 5 min to remove bacterial cells. Then, 2 μl of the supernatant was injected.

Head-space analysis. The samples were transferred to 10-ml ampoule flasks containing 2 g of anhydrous Na_2SO_4. The caproic acid solution was also analyzed, with and without varying amounts of salt. The flasks were sealed with rubber stoppers and protected with a Teflon sheet and aluminum crimp caps before being heated in a water bath at 75°C for 10 min with occasional shaking. The caproic acid solution was also analyzed after being incubated for 2, 4, 6, and 8 min in a water bath at 25, 35, 45, 55, 65 and 75°C. Then 2 ml of the gas atmosphere in the bottles was withdrawn with a Hamilton gas-tight syringe and injected into the gas chromatograph. The syringe was heated to 80°C to avoid condensation of sample components during injection.

GC equipment and test conditions. Hewlett-Packard (model 5750) and Perkin-Elmer (model 3920) gas chromatographs, equipped with dual flame ionization detectors, were used. In the case of the Hewlett-Packard instrument, a 2-m stainless steel column (ID 2 mm) packed with 5% FFAP (Varian) on Chromosorb G AW DMCS 80/100 mesh was employed. The nitrogen carrier gas flow was 30 ml/min. The injector and detector temperatures were 200°C, and the temperature of the column was 135°C. When the standard solution containing higher fatty acids was run, the column temperature was 160°C. The Perkin-Elmer instrument was equipped with a 2-m glass column (ID 2 mm) packed with Porapak Q 100/120 mesh. Here, the injector and detector temperatures were 250°C, and that of the column was 195°C. Nitrogen, at a flow rate of 30 ml/min, was used as carrier gas. The attenuation used was 640 in all analyses.

Mass spectrometry. Mass spectra were run on a Varian Mat model 112 GC-mass spectrometry combination. The GC column consisted of a 2-m stainless steel tube (ID 2 mm) packed with Porapak Q (80/100) mesh). The carrier gas flow and the temperature of the injector and column were the same as described for the Perkin-Elmer instrument. The ion source was held at 200°C, and the electron energy was 80 eV.

RESULTS

Analysis of standard solutions using the three different GC techniques. Chromatograms obtained by the three GC techniques in analyses of the standard solutions containing alcohols and lower fatty acids are shown in Fig. 1. The peaks representing alcohols (a-d) were largest in the head-space chromatograms. These peaks were partly hidden under the large solvent peak obtained when using the ether extraction technique, and also by the broad "solvent" peak obtained when injecting the aqueous standard solution directly into the gas chromatograph. This broad peak also appeared when distilled water was injected. The fatty acids studied (e-l) were readily detectable when using all three sampling techniques. Larger acetic and propionic acid peaks were obtained with the direct injection technique than with the extraction and head-space techniques. Peaks representing the other acids (h-l) were, as expected, largest when the ether extraction technique was used. In the head-space chromatograms, the peaks representing isoacids were considerably larger than

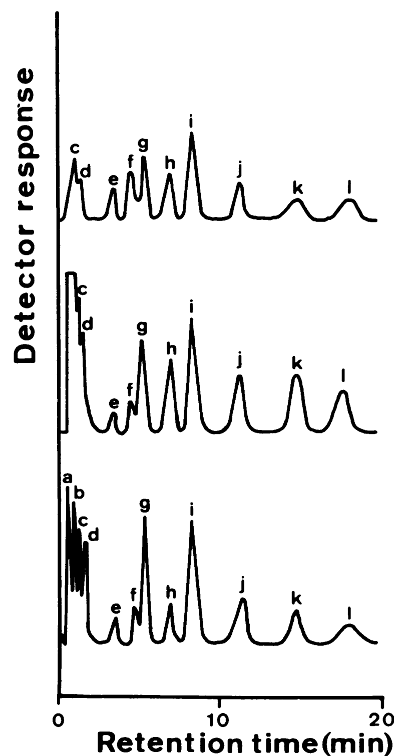

FIG. 1. *Chromatograms of a standard solution of alcohols and fatty acids. Direct injection of the sample (upper panel), injection of ether extracts (center), and head-space analysis (lower panel). An FFAP stationary phase was used. Symbols: a, ethanol; b, propanol; c, n-butanol; d, n-amyl alcohol; e, acetic acid; f, propionic acid; g, isobutyric acid; h, butyric acid; i, isovaleric acid; j, valeric acid; k, isocaproic acid; and l, caproic acid.*

those of the corresponding straight-chain acids.

Head-space analysis of the caproic acid solution. The relative peak area of caproic acid plotted against the temperature of the sample is shown in Fig. 2. The amount of caproic acid in the gaseous phase was almost 10 times as large at 75°C (used in this study) as at 45°C. Temperature equilibration required 6 min at 75°C.

Addition of Na_2SO_4 ("salting out") was found to increase the peak heights markedly. From a practical point of view, we found it advantageous to use such large amounts of Na_2SO_4 that saturation was reached. The salt used was of reagent grade and, as indicated by chromatograms of the pure salt, did not contain any volatile compounds.

Head-space analysis of the higher fatty acids. The head-space chromatograms obtained by analysis of the solution containing heptanoic, octanoic, and nonanoic acid displayed only a small peak of octanoic acid and a hardly visible nonanoic acid peak. Octanoic acid was therefore considered to be the highest straight-chain fatty acid that could be detected under the prevailing conditions.

Analysis of broth cultures using the three different GC methods. Chromatograms obtained by GC analysis of PYG medium incubated with *B. fragilis*, *C. perfringens*, and *P. acnes* are shown in Fig. 3. The three GC techniques gave similar chromatograms with respect to the fatty acids from each species. Acetic (e), propionic (f), butyric (h), and isovaleric acid (i) produced by the bacteria studied were easily detected by all three methods. In the head-space chromatograms, however, a few peaks with short retention times were observed. The largest of these peaks were identified by mass spectrometry. The mass spectra and GC retention times of the compounds produced by *C. perfringens* proved identical with those of authentic ethanol, propanol, and *n*-butanol. *P. acnes* produced only

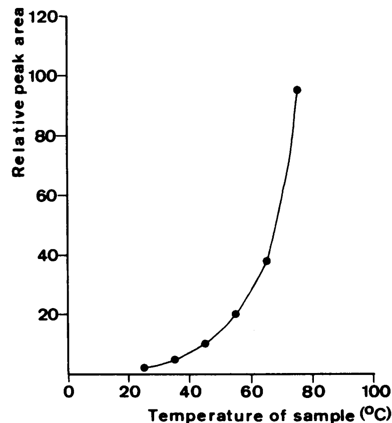

FIG. 2. *Relative peak area of caproic acid in relation to sample temperature. Head-space analysis using an FFAP stationary phase.*

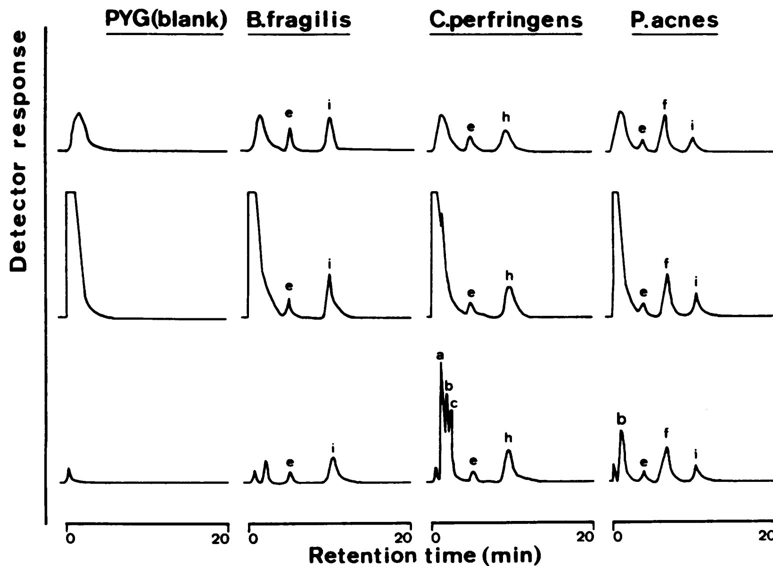

FIG. 3. *Chromatograms of noninoculated glucose-containing PYG medium and cultures of B. fragilis, C. perfringens, and P. acnes in PYG medium. Analysis of complete medium (upper panel) and ether extracts of the medium (center), and head-space analysis (lower panel). An FFAP stationary phase was used. For symbols, see legend to Fig. 1.*

propanol. The alcohol peaks observed could not be identified in the chromatograms obtained by the other two methods tested.

A chromatogram of incubated PYG medium blanks treated with nitrogen at 75°C for 15 min is also shown in Fig. 3. Analysis of untreated medium indicated the presence of considerable amounts of volatiles, which could be appreciably decreased by the treatment described. The nature of these volatiles was not further investigated. When analyzed by the head-space technique, untreated PYG medium volatiles hid the ethanol and propanol peaks. However, all the other compounds could be detected.

Comparison between different stationary phases. In Fig. 4, head-space chromatograms of the standard solution containing alcohols and fatty acids, using FFAP and Porapak Q as stationary phases, are shown together with chromatograms of *C. perfringens*. Porapak Q showed a superior separation of the alcohols. On the other hand, the FFAP phase separated the higher fatty acids more rapidly than did the Porapak Q phase.

DISCUSSION

In the characterization of anaerobic bacteria using GC analyses, volatile fatty acids have so far been the principal compounds of interest. Only in cases when no volatile acids (or merely acetic acid) are detected, samples are methylated and analyzed with respect to nonvolatile fatty acids (8). Alcohols have been less often used for species differentiation of anaerobes. However, the head-space technique described appears to be highly suitable for the detection of small amounts of alcohols and other volatile compounds. The additional information obtained from such compounds may prove valuable for species characterization of bacteria.

The addition of an inorganic salt, such as Na_2SO_4, was found to increase the yields considerably. The salt decreases the solubility of the organic compounds and hence increases the concentration of these compounds in the vapor above the sample. The water bath should be kept at a constant temperature, since variations in temperature affect the size of the peaks obtained (Fig. 2). Straight-chain fatty acids up to octanoic acid can be detected by the head-space technique described. Because of their greater volatility, isoacids give somewhat higher peaks than corresponding normal acids. One disadvantage of the head-space technique is that the size of the chromatographic peaks does not represent the true relative proportions of the volatiles present in the sample. However, this is not critical in GC identification of bacteria.

GC analysis of ether extracts of acidified broth cultures has so far been the technique most commonly used in the identification of anaerobic bacteria. This method facilitates concentration of the higher fatty acids (Fig. 1). Nevertheless, appreciable amounts of acetic and propionic acid remain in the aqueous phase after extraction, resulting in relatively small peaks for these acids. The lower alcohols too remain to a great extent in the aqueous phase. To increase the concentration in the ether phase, "salting out" has been used (8). Such a procedure was, however, not performed in the present study. The solvent peak partly hides those of the lower alcohols and possibly also of other highly volatile compounds. Such compounds need lower column temperatures (increased retention time) or temperature programming, if the ether extraction technique is used. This technique is also more laborious than the other two methods discussed.

Analysis of an acidified culture medium by direct injection into a gas chromatograph is an easily performed method. One advantage is that the peak areas reflect the true concentrations of the compounds in the medium. The chromatographic peaks obtained are smaller than those in chromatograms obtained by the other techniques used at the same attenuation, except for acetic and propionic acid, due to the absence of a concentration step before the GC analysis. The identity of the large, broad peak in the very early part of the chromatogram was not established. Since flame ionization detectors do not detect water, this peak is assumed to represent column material or collected impurities liberated by the water injected.

In head-space techniques, only vaporized material is introduced into the gas chromatograph. Material of high molecular weight, e.g., extracellular lipids present in ether extracts, may appear in a later analysis. In GC analysis of bacterial culture media injected directly into the gas chromatograph, nonvolatile material rapidly clogs the insert liner and the first part of the column. Consequently, these parts of the GC system must be cleansed frequently and the packing material renewed. To minimize this disadvantage, only the supernatant of liquid bacterial cultures should be injected. It is even preferable that a pre-column that adsorbs nonvolatile material of both low and high molecular weight should be installed.

FFAP is one of the stationary phases most frequently used in GC analysis of fatty acids and alcohols produced by bacteria. However, other packing materials have been employed, e.g., SP-1220 (7). Marshall and Porter (9) recently described the efficient separation of fatty acids on glass capillary column coated with 1,

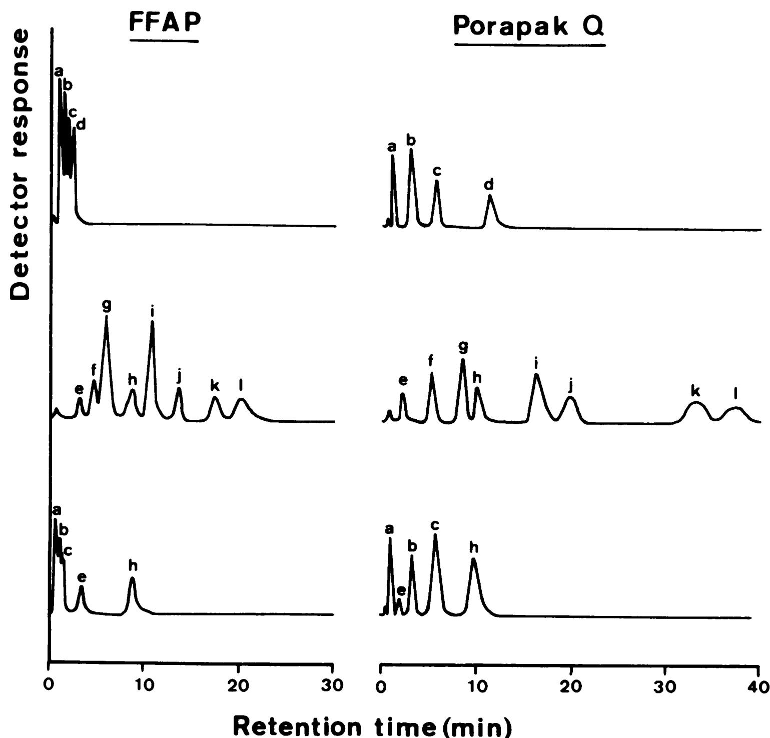

FIG. 4. *Chromatograms of standard solutions of alcohols (upper panel), fatty acids (center), and C. perfringens (lower panel), obtained by head-space analysis using FFAP and Porapak Q as stationary phases. For symbols, see legend to Fig. 1.*

15-pentadecanedicarboxylic acid. Porapak Q has also been used (12). In our experiments, this latter phase proved suitable for analysis of alcohols and fatty acids.

As exemplified in the present study using *B. fragilis*, *C. perfringens*, and *P. acnes* as test organisms, the head-space technique provided more information than was obtained by the other two techniques employed. The head-space technique is also less laborious than these methods, and is suitable for automation. For example, after incubation, the culture media can be transferred to ampoule flasks containing H_2SO_4 and Na_2SO_4 and placed in an automatic head-space analyzer (4). After a few minutes of incubation in a water bath, the vapor phase above the media can be automatically sampled and injected into the gas chromatograph.

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