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Quantitative Gas Chromatographic Analysis of Volatile Fatty Acids in Spent Culture Media and Body Fluids

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Gas chromatographic analysis of volatile fatty acids for identification of obligately anaerobic bacteria and for presumptive diagnosis of anaerobic infections is now widely practiced. However, it is difficult to compare data because only a qualitative analysis is done or only chromatograms are presented instead of quantitative data on volatile fatty acid production. We compared three stationary phases for volatile fatty acid analysis of aqueous solutions and four methods of pretreating samples for gas chromatography. Quantitative analysis could be done accurately by using Carbowax as the stationary phase after pretreatment of spent culture media with Dowex columns. If only qualitative analysis is required (e.g., for presumptive diagnosis of anaerobic infections), ether extraction and headspace analysis are equally suitable. The overall variation coefficient for volatile fatty acid production by four reference strains of obligately anaerobic bacteria after 24 h of incubation was approximately 10%.

In the 25 years since gas chromatography (GC) was developed, many workers have used this sensitive technique for taxonomy and classification of microorganisms. For species identification of obligately anaerobic bacteria GC analysis of the volatile fatty acids (VFAs), which are the principal metabolic products of interest in these organisms, is often necessary.

The recent renaissance of medical anaerobic bacteriology has increased the use of GC techniques in clinical bacteriological laboratories. Because of improvements in isolation and culture methods and a greater awareness of the clinical importance of these methods among clinicians and bacteriologists, the rate of isolation of obligately anaerobic bacteria from clinical infections has increased during the last 10 years. In addition, the development and availability of antibiotics that are active especially against obligately anaerobic bacteria have caused an increasing demand for rapid laboratory diagnosis of anaerobic bacterial infections. Unfortunately, isolation and cultivation of obligately anaerobic bacteria are laborious and time consuming. Often critically ill patients are involved, and anaerobic bacterial infections may run a fulminating course, so an early presumptive diagnosis is mandatory.

Several authors have shown that detection of VFAs by direct GC analysis of clinical specimens (4–6, 12, 16–18, 21) and blood cultures (20) is a rapid and reliable method for detection of anaerobic bacterial infections. This procedure allows a presumptive diagnosis within 60 min at a relatively low cost once the necessary capital investments have been made. Moreover, the technique is not hampered by preexisting antibiotic therapy, inadequate sampling, or delayed transport to the laboratory. For these reasons GC analysis of VFAs has become an important technique in clinical anaerobic bacteriology in medical and veterinary laboratories.

Because GC is now widely used, it would be very useful if results from different laboratories could be compared. However, this is difficult since most laboratories perform only qualitative or semiquantitative analyses of VFAs and so far only chromatograms, which are influenced by the variety of the different GC systems used, have been published. This is especially a disadvantage if data on VFA production are used for identification and taxonomy. The use of an internal standard may facilitate the comparison of chromatograms obtained in different laboratories (8), but to be really able to compare results from different laboratories it is desirable to obtain quantitative data on VFA production by obligately anaerobic bacteria. Quantitative GC analysis of VFAs has been shown to be feasible for other purposes (3).

The goal of this study was to compare different pretreatment methods and different stationary phases for quantitative analysis of VFAs in aqueous solutions. The influence of different inoculum sizes and different incubation periods on the production of VFAs by four reference strains of obligately anaerobic bacteria was also determined. The results obtained with each stationary phase were analyzed in terms of separation efficiency, analysis time, sensitivity, and variability. The pretreatment methods were compared for usefulness, level of recovery, and variability. Calibration curves were made for each VFA (C_2 through C_6) with one selected pretreatment method and stationary phase.

MATERIALS AND METHODS

Stationary phases. The following three stationary phases for packed columns were used: (i) Carbowax (Supelco Inc., Hilversum, The Netherlands), which contained 0.3% Carbopack C, 20 M Carbowax, and 0.1% H_3PO_4; (ii) Chromosorb 101 (Chrompack B.V., Middelburg, The Netherlands), a styrene divinylbenzene polymer; and (iii) SP 1200 (Supelco), which contained 10% SP 1200, 1% H_3PO_4, and acid-washed 80/100 Chromosorb W. The three stationary phases were selected because of suitability (according to the manufacturers) for analysis of VFAs in aqueous solutions.

Standard solutions. To test the stationary phases, we used an aqueous standard solution gravimetrically blended containing 10 mmol of each of the following VFAs per liter: formic acid (C_1), acetic acid (C_2), propionic acid (C_3), isobutyric and *n*-butyric acids (C_4), isovaleric and *n*-valeric acids (C_5), isocaproic and *n*-caproic acids (C_6), and *n*-heptanoic acid (C_7). Three standard solutions, which contained 10 mmol of each gravimetrically blended VFA (C_2 through C_6) per liter in water, in GLC-broth (Biotrading,

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TABLE 1. GC conditions for the stationary phases examined

Stationary phase	Column length (m)	Oven temp (°C)		Injector temp (°C)	Detector temp (°C)	N_2 carrier gas flow rate (ml/min)	Analysis time (min)	Sample size (μl)
		Initial	Final					
Chromosorb 101	1.83	200	200	200	240	10	22	1
SP 1200	1.83	125	125	200	200	40	12	1
Carbowax	0.91	120	120	200	200	20	20	1
Carbowax	0.91	110	190	150	200	20	10	1

Wilnis, The Netherlands), and in concentrated human buffy coat cells in plasma, were used in the second series of experiments to test pretreatment methods. The buffy coat cells suspended in plasma were used to mimic body fluids and are referred to as pus below.

GC. The GC system consisted of a Packard-Becker model 433 gas chromatograph equipped with a dual-column system and flame ionization detectors in conjunction with a digital processor. The sensitivity of the detectors was 10^{-14} A, and the detectors were connected to a GC control station equipped with a printer plotter (model 433 control station; Packard-Becker, Delft, The Netherlands). Glass columns were used (1.83 m by 2 mm for Chromosorb 101 and SP 1200 and 0.91 m by 2 mm for Carbowax). All columns were conditioned overnight with the effluent end detached from the detector and at a carrier gas (N_2) flow rate of 20 ml/min. The SP 1200 and Carbowax columns were conditioned at 175°C, and the Chromosorb 101 column was conditioned at 250°C.

After conditioning, the columns were treated with water to saturate the stationary phases. This was done by injecting 5 μl of water into the Chromosorb column 20 times. The SP 1200 column was pretreated by injecting 1 μl of water 10 times. With the Carbowax column 5 μl of formic acid (0.1% [vol/vol] in water) was injected at 175°C seven times; the time interval between injections was approximately 10 min. Analyses were carried out by using the GC conditions recommended by the manufacturers of the stationary phases (Table 1). The Carbowax column was used both isothermally at 120°C and with the following temperature program: an initial oven temperature of 110°C; after 1.5 min the oven temperature was raised 20°C/min to 190°C; and the final temperature was held for 4.5 min. In addition to good peak separation, peak symmetry, and lack of tailing on the chromatograms, the columns were tested for ghosting by repeated injections with twice-distilled water and 0.1% formic acid. Sensitivity was determined by measuring the minimum levels at which each of the VFAs (C_2 through C_6) could be measured. This was done by injecting 1-μl portions of twofold dilutions of the aqueous standard solution until we identified the lowest dilution at which all VFAs (C_2 through C_6) could be measured quantitatively at the highest possible detector sensitivity. Formic acid, which is logically grouped with the VFAs, was not measured because flame ionization detectors do not respond to it. Variation was calculated after 1 μl of the aqueous standard solution was injected into each stationary phase 10 times consecutively. The variation coefficient for each VFA was estimated from the results of 10 injections and was calculated as follows: (standard deviation/mean) × 100. The variation coefficient of a column was considered to be the sum of the variation coefficients of the VFAs (C_2 through C_7) divided by the number of variation coefficients ($n = 9$). The variation in the retention times for each VFA (C_2 through C_7) was calculated in the same way. After each injection the syringe was washed with glass-distilled water, ethanol, and ethyl ether and dried by suction.

Sample pretreatment. In a second series of experiments we compared the following four previously described methods for pretreatment of spent culture media and body fluids prior to GC analysis of VFAs: vacuum distillation (22), molecular sieving (1, 15), headspace analysis (7), and ether extraction (12, 13). All concentrations of formic acid given below are volume percentages in water, and all GC analyses were done by using Carbowax as the stationary phase and the temperature program described above.

(i) Vacuum distillation. A 2-ml sample was acidified with 200 μl of 0.1% formic acid, and 1 drop of antifoaming agent 33151 (BDH Chemicals Ltd., Poole, United Kingdom) was added. The specimen flask (22) was connected with a receiver tube, which was cooled in liquid nitrogen. This receiver tube was then evacuated with a waterjet pump and kept evacuated by continuous suction, while the contents of the specimen flask were mixed with a magnetic stirrer and slowly heated over a period of 20 min from room temperature to 120°C in an oil bath. After this the specimen was reduced to complete dryness, and after the distillate was thawed at room temperature, it was directly injected into the GC apparatus.

(ii) Molecular sieving. Pasteur pipettes were filled with 1 ml of packed cation-exchange resin in the hydrogen form (AG 50 W-X_4; 200-400 mesh; Bio-Rad Laboratories, Richmond, Calif.) on phosphoric acid-treated glass wool (catalog no. 2-0383; Supelco). After the fluid was drained, these Dowex columns were ready for use. Before the columns were filled, the resin was converted to the hydrogen form by washing it with distilled water until the supernatant was clear; this was followed by washing with 1 N NaOH for 24 h. After neutralization to pH 7 by repeated washing with distilled water, the resin was washed with 4 N HCl for 2 h. Before use the resin was adjusted to pH 7. A 1-ml portion of a sample was acidified with 100 μl of 0.1% formic acid, and this preparation was passed through the resin; in addition, the resin column was rinsed twice with 0.5 ml of 0.1% formic acid. All of the effluents from the column were collected in a test tube and analyzed by GC.

(iii) Headspace analysis. Samples (3 ml) were acidified with 300 μl of 0.1% formic acid, and these preparations were transferred to 10-ml ampoules containing 2 g of anhydrous Na_2SO_4. The flasks were sealed gas tight with rubber stoppers and protected with aluminum crimp caps before being heated in a water bath at 75°C for 10 min with occasional shaking. Then 2 ml of the gas atmosphere in the bottles was withdrawn into a Hamilton gas-tight syringe and injected into the GC apparatus. The syringe was preheated to 80°C to avoid condensation of the sample components during injection.

(iv) Ether extraction. An acidified 1-ml sample was ex-

TABLE 2. Reproducibility of GC systems, including the injection procedures[a]

Stationary phase	Variation coefficients of peak areas (%)[b] for VFA										Variation coefficients of retention times (%) for VFA									
	C_2	C_3	iso-C_4	n-C_4	iso-C_5	n-C_5	iso-C_6	n-C_6	C_7	C_2 through C_7	C_2	C_3	iso-C_4	n-C_4	iso-C_5	n-C_5	iso-C_6	n-C_6	C_7	C_2 through C_7
Chromosorb 101	3.3	4.3	1.9	3.3	2.9	4.9	2.6	3.7	5.6	3.7	0.7	0.4	0.2	0.3	0.2	0.3	0.3	0.3	0.4	0.3
SP 1200	3.0	2.9	2.5	2.5	2.2	2.4	9.1	5.3	3.7	3.7	6.0	4.0	3.6	3.1	2.1	1.8	1.2	0.9	0.5	2.6
Carbowax	1.8	2.7	2.1	3.1	2.3	2.6	1.4	1.1	1.4	2.0	1.1	0.9	0.5	0.2	0.1	0.1	0.1	0.1	0.1	0.4

[a] The results are the means of 10 injections.
[b] Calculated as follows: (standard deviation/mean) × 100.

tracted with 1 ml of ether thoroughly mixed using a Vortex mixer and then centrifuged to break the emulsion. A 1-μl portion of the ether layer was injected directly into the GC column. The test tubes containing ether were kept on ice to prevent evaporation of the ether.

Because swabs are often used to collect clinical material such as pus for bacteriological investigations, the following four types of swabs were tested: cotton swabs, charcoal-coated swabs, fetal calf serum-coated swabs, and polyester swabs (Albic B.V., Maassluis, The Netherlands). The charcoal-coated and fetal calf serum-coated swabs were prepared by soaking cotton swabs in an aqueous 1% charcoal suspension and in fetal calf serum, respectively. After drying and autoclaving the swabs were immersed in the standard pus solution, drained of excess fluid, replaced in a holder, and stored at room temperature for 2 h to simulate the delay between collection and processing of clinical specimens. The amount of the absorbed standard was measured by weighing the swab before and immediately after immersion into the standard solution and draining of the excess fluid. For VFA analysis the swab head was snapped from its stick into a microtest tube. Ether (1 ml) and 100 μl of 0.1% formic acid were added. The microtest tube was capped and vortexed for approximately 20 s. After centrifugation at 250 × *g* for 2 min, 1 μl of the ether layer was injected into the GC apparatus.

After pretreatment each sample was spiked with 2 μl of heptanoic acid (analytical grade; catalog no. A-9378; Sigma Chemical Co., St. Louis, Mo.) per ml as an internal standard and analyzed for VFAs by GC. The variation coefficient and relative error (i.e., 100 × [error/true value of each pretreatment method in combination with the Carbowax column]) for each VFA (C_2 through C_6) were calculated.

Calibration curves. Calibration curves for quantitative analyses of C_2 through C_6 VFAs in aqueous solutions were obtained after pretreatment by molecular sieving and GC analysis. Six samples of four standard solutions containing 0.1, 1, 10, or 20 mmol of each VFA (C_2 through C_6) per liter in GLC broth supplemented with cooked meat were pretreated by molecular sieving over Dowex columns. A calibration line was fit, and the correlation coefficient was calculated.

Inoculum size and incubation period. The influence of inoculum size and incubation period on the production of VFAs by obligately anaerobic bacteria in a standard medium was measured by inoculating one, three, and five colonies and a swab of continuous growth from a culture after 48 h of incubation on Wensinck agar (19) into tubes containing 5-ml portions of GLC broth supplemented with cooked meat. All inoculations were done in triplicate. After 24 and 48 h of incubation at 37°C in an anaerobic glove box, the amounts of C_2 through C_6 VFAs produced were measured by temperature-programmed gas-liquid chromatography with Carbowax after pretreatment by molecular sieving. *Bacteroides fragilis* NCTC 8560, *Fusobacterium varium* ATCC 8501, *Clostridium difficile* ATCC 9689 and *Peptostreptococcus anaerobius* ATCC 27337 were used as reference strains.

RESULTS AND DISCUSSION

Stationary phases. The variation coefficients for peak areas and retention times for each VFA (C_2 through C_7) in the stationary phases tested are shown in Table 2.

Chromosorb 101 was not completely satisfactory. The peaks of the chromatogram could not be completely separated because of tailing (Fig. 1A). Repeated reconditioning, overloading the columns with water, and temperature programming did not improve the chromatograms of aqueous standard solutions of VFAs, nor did saturation of the carrier gas with formic acid. Despite this, quantitative analysis was possible. The analysis time with this column was long (>20 min). No ghosting occurred, and the minimum level of detection for C_2 through C_7 VFAs was 0.04 mmol/liter.

SP 1200 was less satisfactory. The peaks of the chromatogram showed tailing, and complete separation was not possible (Fig. 1B). Reconditioning of the column was necessary after each 10 to 12 samples. At high detector sensitivity the peaks of the chromatogram became deformed, and injection of twice-glass-distilled water produced ghost peaks. This made it impossible to measure the lowest detection level. In our hands SP 1200 was not suitable for quantitative analysis of VFAs in aqueous solutions.

Carbowax performed the best and was well suited for quantitative analysis of VFAs in dilute aqueous solutions. Repeated injections of 0.1% (vol/vol) formic acid stabilized the column, and the chromatogram shown in Fig. 1D was made after more than 500 samples had been injected into the column. When isothermal analysis was used, the analysis time was rather long, and deformation of the peaks late in the chromatogram occurred (Fig. 1C). With temperature programming the analysis time was less than 11 min, and the chromatogram (Fig. 1D) improved considerably, with better separation of the early peaks. A disadvantage of Carbowax was that after ether extraction quantitative analysis of acetic acid and propionic acid was difficult, because the peaks of these acids overlapped with the ether peak. The variation coefficients of the peak areas and the retention times were smaller than those for both other stationary phases (Table 2), and the minimum level of detection for C_2 through C_7 VFAs was 0.02 mmol/liter.

Pretreatment methods. Table 3 shows the variation coefficients, relative errors, and rates of recovery for the four

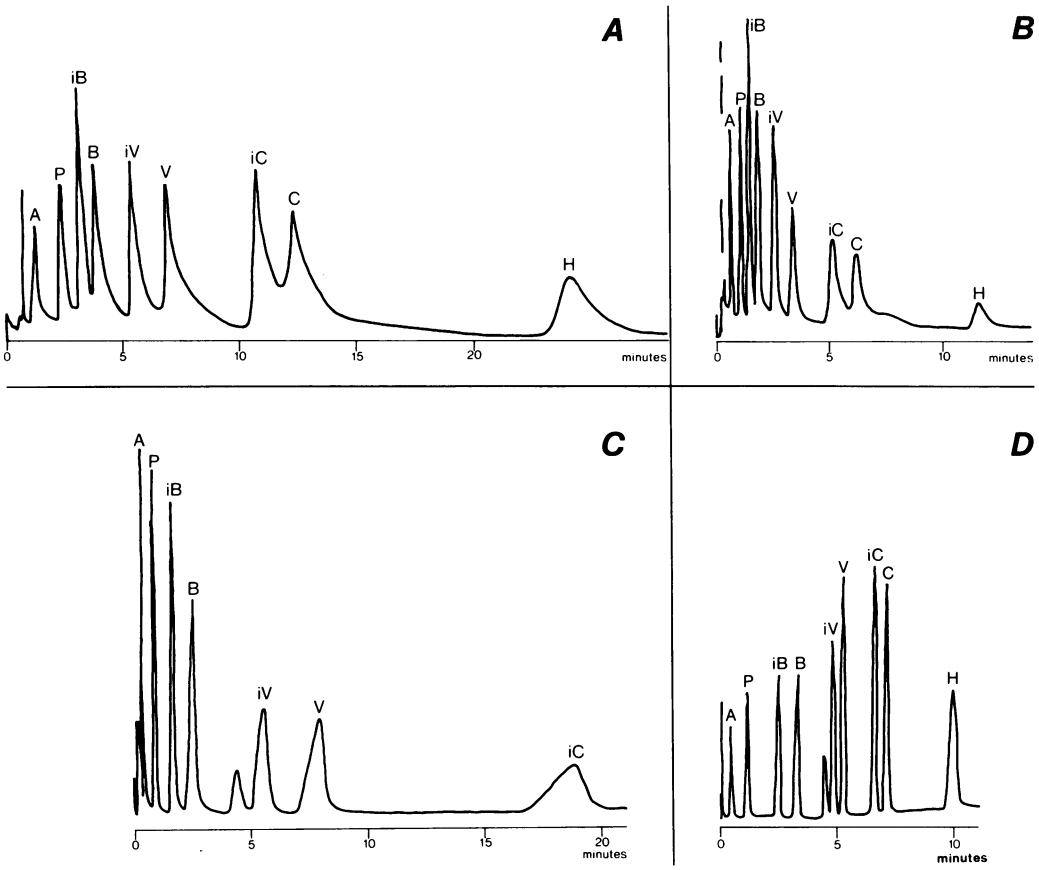

FIG. 1. Chromatograms of a standard solution containing 10 mmol of each VFA (C_2 through C_6) per liter in water, analyzed with Chromosorb 101 (A), SP 1200 (B), Carbowax (isothermally) (C), and Carbowax (temperature programmed) (D). A, Acetic acid; P, propionic acid; iB, isobutyric acid; B, *n*-butyric acid; iV, isovaleric acid; V, *n*-valeric acid; iC, isocaproic acid; C, *n*-caproic acid; H, *n*-heptanoic acid.

different pretreatment methods with samples of the three standard solutions, and Fig. 2 shows the chromatograms obtained with a standard solution containing 10 mmol of each VFA (C_2 through C_6) per liter in pus after pretreatment.

(i) Vacuum distillation. Vacuum distillation was a laborious method; approximately 30 min of treatment per sample was required. The rates of recovery with this method were between approximately 80 and 110% in spent culture media and pus. Thick and sticky pus and even tissue could be pretreated by this method.

(ii) Molecular sieving. The Dowex columns were prepared in advance and stored at 4°C until use. Large numbers of samples could be pretreated in a short time by this method. The rates of recovery were between approximately 90 and 110%. The variation coefficients for this method were of the same order of magnitude as the variation coefficients for the unpretreated aqueous standard with the Carbowax column. A drawback of this method was that only less viscous samples could be assayed. The relative errors were <10%.

(iii) Headspace analysis. Because isoacids are more volatile than the corresponding straight-chain acids, they produced much larger peaks in the chromatograms (Fig. 2D). The level of recovery of the larger VFAs was considerably lower than the level of recovery of the other acids because of their higher boiling points. This decreased level of recovery of the larger VFAs might have been due to condensation in the

TABLE 3. Comparison of four different pretreatment methods. Range of the variation coefficients, levels of recovery, and relative errors[a] after pretreatment and GC analysis with Carbowax of an aqueous standard solution, a GLC-broth standard solution, and a pus standard solution containing 10 mmol of each VFA (C_2 through C_7) per liter

Pretreatment method	Variation coefficients (%)			Levels of recovery (%)			Relative errors (%)		
	Aqueous standard solution	Pus standard solution	GLC broth standard solution	Aqueous standard solution	Pus standard solution	GLC broth standard solution	Aqueous standard solution	Pus standard solution	GLC broth standard solution
Molecular sieving	0.5–2.6	1.6–4.3	0.4–2.3	92.6–106.6	88.7–109.5	96–109	1.2–7.4	0.8–11.3	2–10.3
Headspace analysis	45.4–60.4	22.4–80.9	11.8–142	6.5–108	5.3–91	16.5–170.5	5–93.5	9–94.7	26.1–83.5
Vacuum distillation	1.5–4.9	2.2–18.2	1.7–11.4	90.6–95.8	80.6–109.4	79.4–109.8	4.2–9.4	0–19.4	1.8–20.6
Ether extraction	3.5–5.5	2.1–6.2	3.1–8.7	100.8–113.5	114–124	108–147	0.8–13.5	14–24	8–47

[a] Each standard solution was pretreated and analyzed three times.

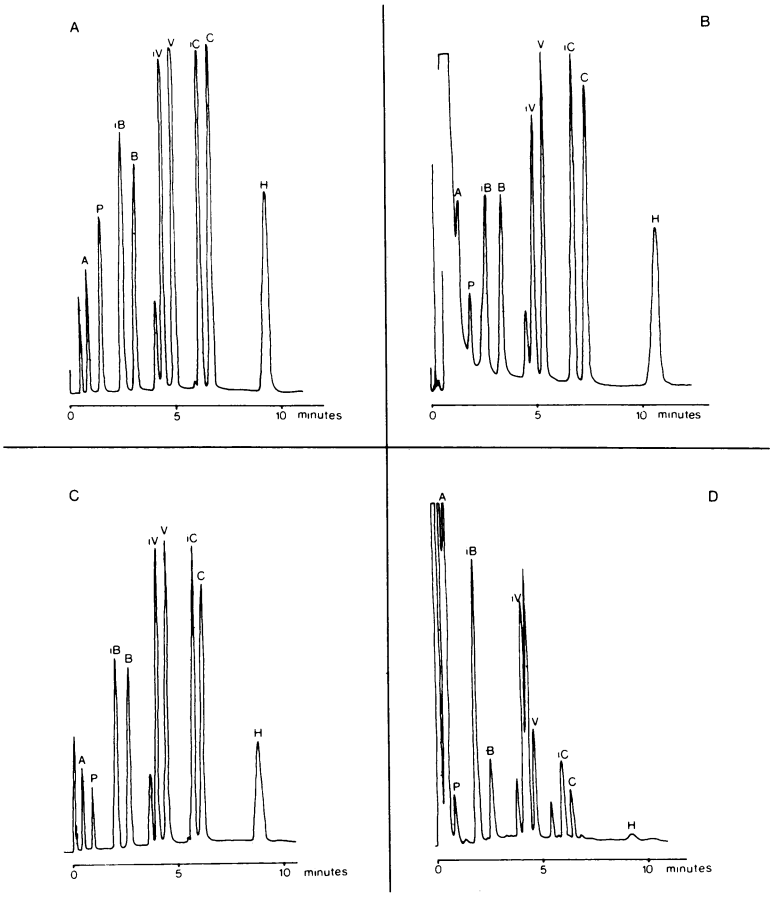

FIG. 2. Chromatograms of a standard solution containing 10 mmol of each VFA (C_2 through C_6) per liter in pus after pretreatment by vacuum distillation (A), ether extraction (B), molecular seiving (C), and headspace analysis (D). GC analysis was performed by using Carbowax as the stationary phase and temperature programming. For an explanation of abbreviations, see the legend to Fig. 1.

syringe and absorption to glass of the larger VFA molecules. For these reasons headspace analysis is unsuitable when quantitative analysis is required. The minimum levels of detection of C_3 through C_6 VFAs could not be determined for this method.

(iv) Ether extraction. With Carbowax as the stationary phase, acetic acid and propionic acid could not be properly measured quantitatively, as the peaks of C_2 and C_3 acids on the chromatograms were not separated from the ether peaks. The levels of recovery for the other VFAs (C_4 through C_6) were between 100 and 147%. Qualitative analyses of pus samples taken with charcoal-coated swabs proved to be more sensitive than analyses of samples taken with other swabs. This was due to the fact that the charcoal-coated swabs absorbed more standard pus solution (0.34 ± 0.05 g) than nylon swabs (0.12 ± 0.05 g). Serum-coated swabs and plain cotton swabs absorbed almost nothing.

Calibration curves. The results of an analysis of GLC broth standards containing different concentrations of C_2 through C_6 VFAs are shown in Table 4. A typical example of

TABLE 4. Levels of recovery of C_2 through C_6 VFAs by GC analysis following molecular sieving of a GLC broth standard solution containing different concentrations of VFAs

Concn (mmol/liter)	No. of samples	Levels of recovery (mmol/liter)							
		C_2	C_3	iso-C_4	n-C_4	iso-C_5	n-C_5	iso-C_6	n-C_7
0.1	3	0.08 ± 0.01 (80)[a]	0.04 ± 0.02 (40)	0.06 ± 0.01 (60)	0.06 ± 0.01 (60)	0.07 ± 0.01 (70)	0.08 ± 0.01 (80)	0.09 ± 0.01 (90)	0.16 ± 0.02 (160)
1	3	0.87 ± 0.21 (87)	0.74 ± 0.14 (74)	0.74 ± 0.09 (74)	0.80 ± 0.08 (80)	1.07 ± 0.08 (107)	1.04 ± 0.10 (104)	1.12 ± 0.06 (112)	1.20 ± 0.08 (120)
10	3	10.55 ± 0.56 (106)	10.11 ± 0.46 (101)	9.90 ± 0.57 (99)	10.03 ± 0.51 (100)	9.98 ± 0.55 (100)	10.19 ± 0.48 (102)	9.91 ± 0.46 (99)	9.92 ± 0.40 (99)
20	3	20.36 ± 0.66 (102)	21.79 ± 0.32 (109)	21.82 ± 0.22 (109)	22.57 ± 0.56 (113)	22.33 ± 0.23 (112)	23.36 ± 0.23 (117)	19.60 ± 0.35 (98)	20.48 ± 0.17 (102)

[a] Mean ± standard deviation. The numbers in parentheses are percentages.

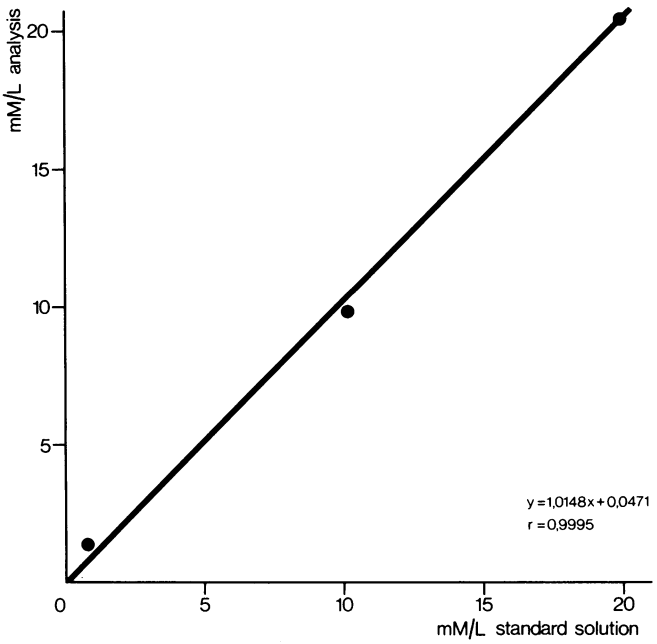

FIG. 3. Standard curve of caproic acid.

a calibration curve of one VFA (caproic acid) is shown in Fig. 3. With the exceptions of acetic and propionic acids the variation coefficients of the other VFAs were approximately 10% or less at concentrations between 1 and 20 mmol/liter.

Inoculum size and incubation period. The results of a C_2 through C_6 VFA analysis after 24 h of incubation are shown in Table 5. The inoculum size seemed not to be very critical, as the standard deviations did not significantly increase if the results obtained after inoculation of one, three, or five colonies were calculated together or separately. However, after inoculation of the broth culture with a swab of continuous growth, the standard deviations tended to increase in some organisms and for specific VFAs. After inoculation of one, three, or five colonies the variation coefficients were between 1 and 15%. After 48 h of incubation the standard deviations increased.

We chose quantitative analysis of VFAs in aqueous solutions because this method simplifies and speeds up pretreatment of samples, but mostly because organic extraction methods, such as ether extraction, do not extract all VFAs equally, which makes quantitative analysis more difficult. Quantitative analyses of VFAs in aqueous solutions with Chromosorb 101 and SP 1200 proved to be difficult, mainly because of tailing. This caused incomplete separation of the peaks and was due to the instability of these columns if aqueous solutions were injected.

Despite results obtained by other workers (2, 10, 11), saturating the carrier gas with formic acid did not improve the performance of the Chromosorb 101 column at all, but proved to be deleterious to our GC equipment. The analysis time with this column was relatively long (>20 min). With the Carbowax column we used temperature programming to improve the chromatogram and to shorten the elution time. VFA concentrations as low as 0.02 mmol/liter could then be measured in aqueous solutions. The quality of the column improved while it was used. The separation of the peaks was complete, the overall variation coefficient was 2% (range, 1 to 3%), and the elution time was short (less than 12 min). The latter is of prime importance when many samples are to be

TABLE 5. Formation of VFAs after 24 h of incubation of four reference strains of obligately anaerobic bacteria

Anaerobic bacterium	Inocula	C_2		C_3		iso-C_4		n-C_4		iso-C_5		n-C_5		iso-C_6		n-C_6	
		Concn (mmol/liter)[a]	VC (%)[b]	Concn (mmol/liter)	VC (%)	Concn (mmol/liter)	VC (%)	Concn (mmol/liter)	VC (%)	Concn (mmol/liter)	VC (%)	Concn (mmol/liter)	VC (%)	Concn (mmol/liter)	VC (%)	Concn (mmol/liter)	VC (%)
C. difficile	1, 3, 5[c]	14.37 ± 1.05[d]	7	1.10 ± 0.16	14	4.96 ± 0.38	8	5.47 ± 0.79	14	0.61 ± 0.03	4	0.65 ± 0.10	15	7.54 ± 0.32	4	0.36 ± 0.03	9
	1, 3, 5, Swab	14.26 ± 0.89	6	1.08 ± 0.14	13	4.93 ± 0.32	6	5.43 ± 0.65	12	0.64 ± 0.06	10	0.66 ± 0.08	12	7.53 ± 0.26	3	0.36 ± 0.03	9
P. anaerobius	1, 3, 5	15.11 ± 0.18	1	1.04 ± 0.03	3	5.19 ± 0.14	3	0.76 ± 0.02	3	0.83 ± 0.03	3	0.10 ± 0.01	6	7.96 ± 0.06	1	0.24 ± 0.01	2
	1, 3, 5, Swab	15.17 ± 0.19	1	1.08 ± 0.08	7	5.29 ± 0.23	4	0.83 ± 0.13	16	0.81 ± 0.06	7	0.10 ± 0.01	8	8.10 ± 0.27	3	0.26 ± 0.03	13
B. fragilis	1, 3, 5	7.31 ± 0.25	3	9.77 ± 0.61	6	0.10 ± 0.01	10			1.37 ± 0.04	3						
	1, 3, 5, Swab	7.46 ± 0.37	5	10.64 ± 1.81	17	0.12 ± 0.05	38			1.58 ± 0.41	26						
F. varium	1, 3, 5	21.43 ± 0.54	3	5.41 ± 0.05	1			16.55 ± 0.27	2								
	1, 3, 5, Swab	21.85 ± 0.94	4	5.67 ± 0.53	9			17.27 ± 1.46	8								

[a] $n = 3$.
[b] VC, Variation coefficient.
[c] One, three, or five colonies were used to inoculate GLC broth.
[d] Mean ± standard deviation.

analyzed. For qualitative and quantitative analysis of VFAs in aqueous solutions Carbowax appeared to be the best column material in our hands. However, if ether extracts were injected into the Carbowax column, the peaks of acetic and propionic acids could not be readily separated from the solvent peak in the chromatogram. However, lowering the initial column temperature to 60°C and changing the temperature program could have solved this problem, but at a cost of a considerably longer elution time. An extra peak was found just before the isovaleric acid peak. This peak was found in all of the standards and after injection of pure analytical grade isovaleric acid solutions. Mass spectrographic analysis revealed that this peak was caused by 2-methyl-butyric acid. This peak was also often present in spent culture media and in clinical samples. Direct GC analysis of the supernatants of acidified spent culture media has been proposed (14). This is easy to perform but has the considerable disadvantage that nonvolatile compounds tend to block the injector and the column. Pretreatment extends the life-span of GC columns and, by removing interfering substances, improves the quality of chromatograms. This is especially important for quantitative analysis.

The vacuum distillation pretreatment gave good results. The differences in the levels of recovery among the VFAs might have been due to absorption of larger VFA molecules to the glass wall of the connecting tube. Because vacuum distillation is laborious and time consuming, it does not seem very suitable as a pretreatment method for large numbers of samples. Thick, sticky pus and even tissue could be pretreated by this method.

With headspace analysis only vaporized material is introduced into the GC apparatus, but volatile compounds other than VFAs in the sample are also vaporized and may cause interfering peaks on the chromatogram; this makes identification of the VFAs difficult and sometimes even impossible. Because the peak areas do not proportionally represent the true concentrations of the VFAs present in a sample, quantitative analysis is not possible.

Ether extraction is the most often used method of extraction for VFAs. Nevertheless, appreciable amounts of acetic and propionic acids remain in the aqueous phase after extraction, resulting in relatively poor levels of recovery of these acids. The recovery rate for the other VFAs is between 100 and 145%. For quantitative analysis ether extraction is more laborious than molecular sieving, because special precautions must be taken to prevent evaporation of the ether. An advantage of this method is that more viscous samples can be readily pretreated, and even swabs moistened with pus can be used. This is important as often just a swab dipped into pus is delivered as a specimen to the laboratory, despite the fact that sending swab specimens for isolation of obligately anaerobic bacteria should be strongly discouraged. Charcoal-coated swabs can adsorb enough VFAs from broth cultures and pus for qualitative analysis of the VFAs by GC. As cotton and wood may contain VFAs, workers should test each batch of swabs before use. Swabs for GC analysis should also be transported in the dry state. Transport medium does not interfere with GC analysis, but extensive dilution into the medium of the VFAs drastically reduces the rate of detection of VFAs, which might only partly be compensated for by evaporating the ether to concentrate the VFAs present. High-molecular-weight material (e.g., extracellular lipids) present in ether extracts may cause tailing, may appear on the chromatogram in a later analysis, or may even block the GC columns.

A simple, rapid, and reliable method is pretreatment of samples with columns packed with cation-exchange resin. This not only removes large molecules and debris from the sample by (molecular) sieving, but also removes other molecules and ions by cation exchange. The level of recovery of the VFAs from pus and the GLC broth standard was between 90 and 110%, with a variation coefficient of only 3 ± 1% with the GLC broth standard. This is on the same order of magnitude as the variation coefficient of the Carbowax column used for analysis. A disadvantage of this pretreatment method is that viscous clinical specimens have to be diluted. Care should be taken to prevent emulsification during dilution. The calibration curves show that at concentrations between 1 and 20 mmol/liter quantitative analysis of C_2 through C_6 VFAs is fairly accurate and make it feasible to present quantitative data on VFA production by obligately anaerobic bacteria instead of chromatograms. This is important for identification and taxonomic purposes.

Despite the fact that the inoculum size did not seem to be very critical if VFA production was measured after 24 h of incubation, standardization of culture media, culture conditions, and inocula is necessary to make sure that different laboratories obtain the same results with the same strain or species.

Carbowax is the packed column material of choice for qualitative and quantitative analysis of VFAs in biological aqueous solutions. In our hands, molecular sieving with Dowex columns is the best pretreatment method when quantitative analysis is required (e.g., for identification and taxonomic purposes). The combination of a Carbowax column and pretreatment of spent culture media by molecular sieving with a Dowex column makes it feasible to present quantitative data on VFA production of obligately anaerobic bacterial species instead of chromatograms. For qualitative analysis (e.g., presumptive diagnosis of anaerobic bacterial infections) ether extraction and headspace GC are good alternatives for pretreatment of samples.

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