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Liquid Chromatographic Procedure for Fermentation Product Analysis in the Identification of Anaerobic Bacteria

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High-performance liquid chromatography with a cation-exchange resin-packed column was used to determine fermentation products of several known and unknown *Clostridium* species. The column was operated at 30°C, and isocratic elution was done with 0.013 N H_2SO_4. Sample preparation for high-performance liquid chromatographic analysis required only membrane filtration. Glucose and formate were readily determined. Quantitative results were easily obtained. Chromatograms of eight unknown strains could be matched with chromatograms of at least one of the type culture strain chromatograms. In some cases, additional testing was necessary before identification could be made. The same conclusions were reached by parallel testing with gas chromatography to determine fermentation products. High-performance liquid chromatography is simple to apply and, under some conditions, is faster than gas chromatography for fermentation product analysis.

Semiquantitative determination of fermentation products is required for definitive identification of many types of anaerobic bacteria (2). Gas chromatography (GC) is normally used for these analyses (3, 4, 7, 10), but recent developments in high-performance liquid chromatography (HPLC) have advanced it to a level such that it may be a better technique for fermentation product analysis than GC.

Turkelson and Richards (8) found that a wide variety of organic acids could be separated on a cation-exchange resin-packed HPLC column eluted with dilute mineral acid. A lower detection limit of 5 mg of acids per liter was achieved by monitoring the ultraviolet (UV) absorption of the column effluent at 210 nm. No pretreatment other than filtration was applied to the sample before chromatographic analysis. By slightly modifying the packing material it is possible to increase the range of the column to include alcohols and neutral compounds (1, 11). These results suggest that the principal products of bacterial fermentation could be determined by a single chromatographic analysis, and elaborate sample pretreatments before chromatography would be eliminated.

Analysis of bacterial fermentation products by GC normally requires sample splitting, preparation of volatile derivatives, extractions, and individual analyses of the various splits. Rizzo (5) described a GC method for fermentation product analysis that used glass capillary columns. Volatile acids, alcohols, and ketones were analyzed in an ether extract of one culture broth sample. Nonvolatile fatty acids in another sample were converted to methyl esters, which were extracted into chloroform. The chloroform extract of methyl esters was then analyzed by GC. Although the GC run times were short, considerable time was required to perform the extractions and methylation, and the results were presented in two parts.

In this study, we used a commercial cation-exchange resin-packed column to analyze the fermentation products of nine known *Clostridium* strains and eight unknown *Clostridium* strains isolated from environmental samples. Liquid chromatograms were used to aid in the identification of the unknown strains by the procedure of Holdeman et al. (3). Parallel studies with GC gave identical results.

MATERIALS AND METHODS

Microorganisms. The following type cultures were purchased from the American Type Culture Collection, Rockville, Md.: *Clostridium acetobutylicum* ATCC 824; *Clostridium bifermentans* ATCC 19299; *Clostridium butyricum* ATCC 19398; *Clostridium indolis* ATCC 25771; *Clostridium innocuum* ATCC 14501; *Clostridium pasteurianum* ATCC 6013; *Clostridium sphenodoides* ATCC 19403; *Clostridium sporogenes* ATCC 19404; and *Clostridium subterminale* ATCC 25774.

The following eight *Clostridium* strains were isolated from several natural environments: 17A, clam gut; 21A, well water, St. Petersburg, Fla.; 26A, freshwater lake sediment, Palo Alto, Calif.; 27A, well water,

Bay Park, N.Y.; 29A, well water, Visalia, Calif.; 31A, freshwater lake sediment, Palo Alto, Calif.; 16A, clam gut; and 23A, anaerobic digestor, Visalia, Calif.

Media and growth conditions. Culture techniques, media, and identification protocols have been described previously (3).

Bacteria were grown in prereduced, anaerobically sterilized peptone-yeast extract broth supplemented with 10 g of glucose per liter (PYG) and in prereduced, anaerobically sterilized chopped meat-carbohydrate medium. Citrate broth was prepared as described by Walther et al. (9). Yeast extract, peptone, and Trypticase were obtained from BBL Microbiology Systems, Cockeysville, Md.

Organisms were maintained in chopped meat-carbohydrate medium at 5°C with monthly transfer. Samples for chromatographic studies were obtained as follows. Bacteria were grown in PYG for 24 to 48 h until maximum growth was obtained. Cultures were centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant was filtered through a polycarbonate filter (0.45 μm; Nuclepore Corp., Pleasanton, Calif.). Filtered samples were used immediately for chromatographic analysis.

HPLC. A Waters Associates (Milford, Mass.) high-performance liquid chromatograph (model ALC/GPC 204) equipped with a model 6000A solvent delivery system, a model U6K injection system, a model R401 refractive index (RI) detector, a model 450 variable wavelength detector, and a model 730 printing integrator was used to determine fermentation products and unreacted glucose.

The column is constructed from 9.5-mm stainless steel tubing (7.8 [inside diameter] by 300 mm) and packed with 9-μ-diameter HP X-87 cation-exchange resin. The prepacked column for organic acid analysis was obtained from Bio-Rad Laboratories, Richmond, Calif. The column was operated at 30°C with 0.013 N H_2SO_4 at 0.7 ml/min as the eluent except as noted.

During continued operation with the HP X-87 resin it was found that operating pressures gradually increased, with corresponding peak broadening and resolution loss. When the operating pressure exceeded 1,500 lb/in^2, column flow was reversed, and 0.013 N H_2SO_4 was pumped through the column at 60°C for 12 to 18 h until the pressure was reduced to 900 to 1,000 lb/in^2.

GC. Volatile fatty acids were determined by direct aqueous injection of 0.1 ml of filtered culture medium into a glass column (4 mm [inside diameter] by 0.9 m) packed with Carbopack C–0.3% Carbowax 20 M–0.1% H_3PO_4 (Supelco, Inc., Bellefonte, Pa.). Operating conditions were as follows: column temperature, 110°C; injection block temperature, 150°C; detector temperature, 150°C; air flow rate, 300 ml/min; H_2 flow rate, 30 ml/min; and N_2 flow rate, 30 ml/min. Alcohols were determined by direct aqueous injection of 0.1 ml of filtered culture medium into a stainless-steel column (1.8 mm [inside diameter] by 1.8 m) packed with Carbopak C–0.2% Carbowax 1500 (Supelco Inc.). Operating conditions were as follows: column temperature, 110°C; injection block temperature, 150°C; detector temperature, 150°C; air flow rate, 300 ml/min; H_2 flow rate, 30 ml/min; and N_2 flow rate, 30 ml/min. A Perkin-Elmer model 3920 gas chromatograph (The Perkin-Elmer Corp., Norwalk, Conn.) equipped with a flame ionization detector was used.

Nonvolatile fatty acids were determined in ethyl ether extracts of methyl derivatives (3) by injection into a stainless-steel column (1.8 mm [inside diameter] by 1.8 m) packed with 10% SP1000–1% H_3PO_4 on 10/120 Chromosorb W AW (Supelco, Inc.). Operating conditions were as follows: column temperature, 145°C; injection block temperature, 170°C; detector temperature, 145°C; air flow rate, 250 ml/min; H_2 flow rate, 35 ml/min; and N_2 flow rate, 35 ml/min. A Baseline model 1010A gas chromatograph (Baseline Industries, Inc., Boulder, Colo.) equipped with a flame ionization detector was used.

Reagents. ACS reagent-grade H_2SO_4 was used. Organic acids, alcohols, and neutral compounds were purchased in the highest quality available. Volatile acids and alcohols contained only one compound, as determined by GC and HPLC. Nonvolatile acids contained only one compound, as determined by HPLC, with the exception of lactic acid, which always contained one impurity.

RESULTS

Method development. Examples of HPLC traces of solutions containing various compounds produced by *Clostridium* species during fermentation of glucose are shown in Fig. 1. The upper trace was obtained with RI detection, and the lower trace was obtained with UV detection at 210 nm. Both traces were obtained simultaneously from the same sample, which was prepared from authentic standards. The peak preceding the lactate peak is from an impurity in the lactic acid standard.

Alcohols, and especially the sugars, have large differences in RIs relative to the 0.013 N H_2SO_4 eluent. By contrast, these hydroxylated compounds absorb very little UV light above 200 nm. The RI detector is generally the most useful detector for monitoring the fermentation processes of both glucose utilization and the products formed, namely the acids, alcohols, and ketones. Pyruvic and fumaric acids absorb strongly at 210 nm, and UV absorption is convenient for detecting these compounds. The other organic acids yield moderate UV and RI responses, UV absorption being the more sensitive test overall. Both detectors can be used to advantage, but the RI detector was used for this work.

The effect of varying eluent concentrations is shown in Fig. 2. The resolution of compounds in the H_2SO_4 normality range of 0.013 to 0.5 remained fairly constant. The retention times of the alcohols, sugars, and most of the acids remained constant; the exceptions were fumaric and pyruvic acids. Based on this finding, we chose the mildest solution, 0.013 N, for our study.

Figure 3 shows the effect of temperature on

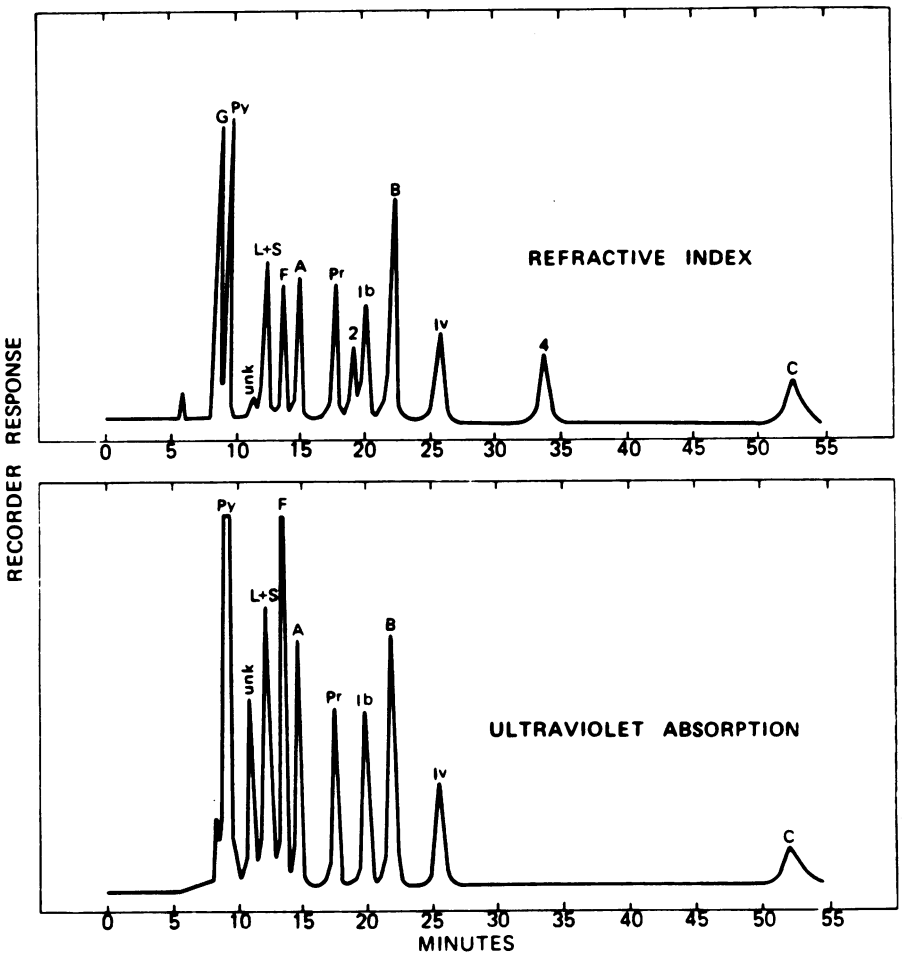

FIG. 1. *HPLC traces of solutions of various organic compounds dissolved in distilled water. RI and UV absorption were used for detection of compounds in the column effluent. The column was operated at 30°C with 0.013 N H_2SO_4 as the eluent. Abbreviations and concentrations are as follows: (G) glucose, 5 mM; (Py) pyruvate, 10 mM; (L) lactate, 7 mM; (F) formate, 15 mM; (A) acetate, 11 mM; (Pr) propionate, 7 mM; (Ib) isobutyrate, 6 mM; (2) ethanol, 9 mM; (B) butyrate, 11 mM; (Iv) isovalerate, 5 mM; (4) butanol, 5 mM; (C) n-caproate, 4 mM; (S) succinate (not present in this sample but indicated for reference); (unk) unknown.*

retention times of the various compounds. Increasing the temperature shortened the retention time of nearly all of the compounds somewhat; glucose was affected very little, and the retention time of ethanol actually increased. Notably, fumaric, pyruvic, and succinic acids changed positions relative to the other compounds as the temperature changed. Succinic and lactic acids were not resolved at 30°C but could be separated at 50°C. At 50°C, however, pyruvic acid and glucose were marginally separated, ethanol shifted to overlap isobuyric and butyric acids, and acetic, formic, and fumaric acids were poorly resolved. Changing the temperature may be very useful for obtaining a desired separation under some other circumstances, however.

Component peak areas were determined to be linear functions of concentration for both detectors when tested within the ranges found in the culture broths. Minimum detectable concentrations were determined from the area of a peak that was twice as high as the noise exhibited by the recorder base line. Minimum detectable amounts for the RI detector were as follows (in millimoles per liter): glucose, 0.01; pyruvate, 0.02; lactate, 0.02; succinate, 0.03: formate, 0.10; acetate, 0.06: propionate, 0.04; ethanol, 0.08; isobutyrate, 0.03; butyrate, 0.04; isovalerate, 0.03; butanol, 0.04; and *n*-caproate, 0.08. These con-

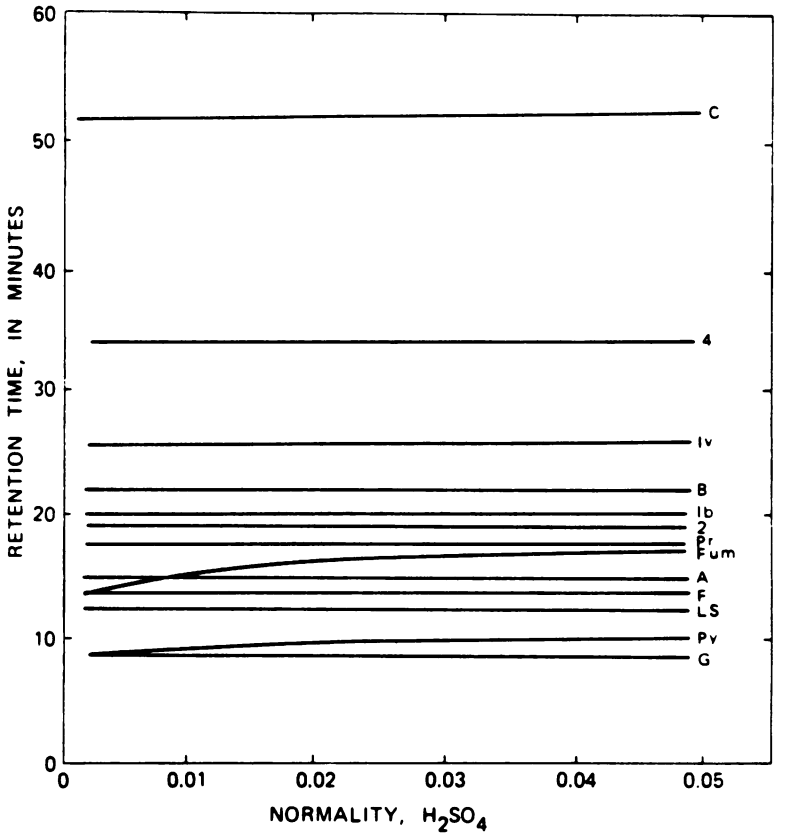

FIG. 2. *Effect of eluent acid concentration on the retention time of various compounds in an HP X-87 column at 30°C. See legend to Fig. 1 for definitions of abbreviations; Fum, fumerate; LS, Lactate-succinate.*

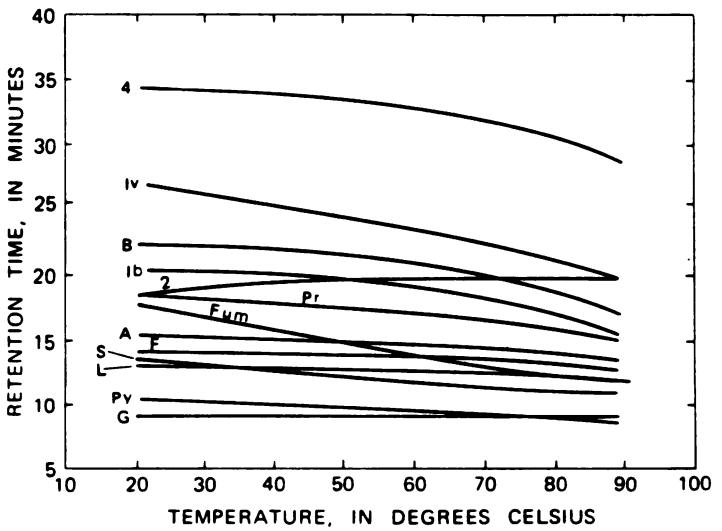

FIG. 3. *Effect of column temperature on the retention time of various compounds, with 0.013 N H_2SO_4 as the eluent. See lengd to Fig. 1 for definitions of abbreviations; Fum, fumerate.*

centrations are comparable to the limits specified by Holdeman et al. (3) as criteria for determining the presence or absence of a given compound. At 10 times the minimum detectable concentration, the relative standard deviation ranged from 0.5 to 4% for the acids and 3 to 5% for the alcohols.

Application to identification of *Clostridium* isolates. HPLC traces of uninoculated peptone-yeast extract broth and PYG cultures of eight type strains and eight unknown strains are shown in Fig. 4.

Substantial peaks representing lactate-succinate and acetate were seen on traces of uninoculated peptone-yeast extract broth. The presence of lactate and acetate in uninoculated peptone-yeast extract broth was confirmed by GC. Another substantial peak with the retention time of ethanol was also present on the HPLC traces, but ethanol was not found in uninoculated peptone-yeast extract broth by GC. Furthermore, a strong UV absorbance at 210 nm was associated with this retention time. As ethanol gives only a weak UV response at 210 nm, this suggests that an interfering compound was present in the medium. The identity of this material is unknown.

Corrections had to be made for the amounts of acetate and lactate present in the uninoculated medium and for the contribution of the spurious ethanol peak. We have found that the amounts of these compounds vary somewhat from batch to batch; therefore, medium blanks corresponding to the culture broths being tested should be used for correction purposes.

After the appropriate corrections were made, the products indicated in the HPLC traces of the type strains generally conformed to the official strain descriptions of the International Committee on Systematic Bacteriology (6) given by Holdeman et al. (3).

HPLC traces of the eight unknown strains could be matched with one or more of the type strain traces. Some ambiguities arose and, of course, these could be resolved only by determining additional characteristics. Unknowns 16A, 21A, and 26A formed one cluster of remarkably similar HPLC traces, despite the diversity of environments from which these strains had originally been isolated. HPLC traces of these three unknown strains could be superficially matched with the trace of either *C. acetobutylicum* ATCC 824 or *C. pasteurianum* ATCC 6013. According to the official descriptions of these two species (3), *C. acetobutylicum* can hydrolyze esculin, whereas *C. pasteurianum* cannot. It was found that unknowns 16A, 21A, and 26A and *C. acetobutylicum* ATCC 824, the type strain of the species, gave a strong positive response for esculin hydrolysis; therefore, the unknowns were classified as *C. acetobutylicum*, even though the lactate-succinate peaks on the unknown traces more closely matched the lactate-succinate peak on the *C. pasteurianum* trace than on the *C. acetobutylicum* trace.

Similarly, unknowns 17A, 23A, and 31A formed another cluster of similar HPLC traces. These HPLC traces matched the traces of *C. bifermentans* ATCC 19299 well, except that pyruvate was indicated on the trace of the type strain but not on the traces of the unknown strains. Unknowns 17A, 23A, and 31A were shown to be *C. bifermentans* strains by additional testing.

The HPLC trace of unknown 27A was matched with the trace of *C. butyricum* ATCC 19398, and the trace of unknown 29A was matched with the trace of *C. sporogenes* ATCC 19404. These tentative identifications were confirmed by additional testing.

Results obtained from fermentation product analysis by GC led to identical classifications of unknown strains.

HPLC traces of PYG cultures of *C. indolis* ATCC 25771 and *C. sphenoides* ATCC 19403 were virtually identical. The properties of *C. indolis* and *C. sphenoides* are very similar, and we were unable to differentiate these two species on the basis of morphology or physiological properties. Walther et al. (9) had reported that *C. sphenoides* can ferment citrate, with the production of acetate and ethanol, whereas 16 other *Clostridium* species were not able to utilize citrate. Walther et al. (9) did not include *C. indolis* in the strains tested. We decided to test the possibility that citrate fermentation might be used to differentiate between *C. indolis* and *C. sphenoides*.

The citrate broth medium described by Walther et al. (9) was inoculated with *C. indolis* and *C. sphenoides*. After 72 h of incubation at 30°C, the cultures were centrifuged, filtered, and injected into the high-performance liquid chromatograph under the same conditions as those described for the analysis of PYG cultures. HPLC traces of uninoculated citrate broth and citrate broth cultures of *C. indolis* (indistinguishable from uninoculated medium) and *C. sphenoides* are shown in Fig. 5.

There was only sparse growth in the *C. indolis* culture, and the absence of products in the HPLC trace of the citrate broth culture indicated that *C. indolis* cannot utilize citrate. Rough calculations with integrated peak areas, with the uninoculated culture broth serving as a citrate standard, indicated that approximately 1.7 mol of acetate and 0.3 mol of ethanol were produced from each mole of citrate utilized, in

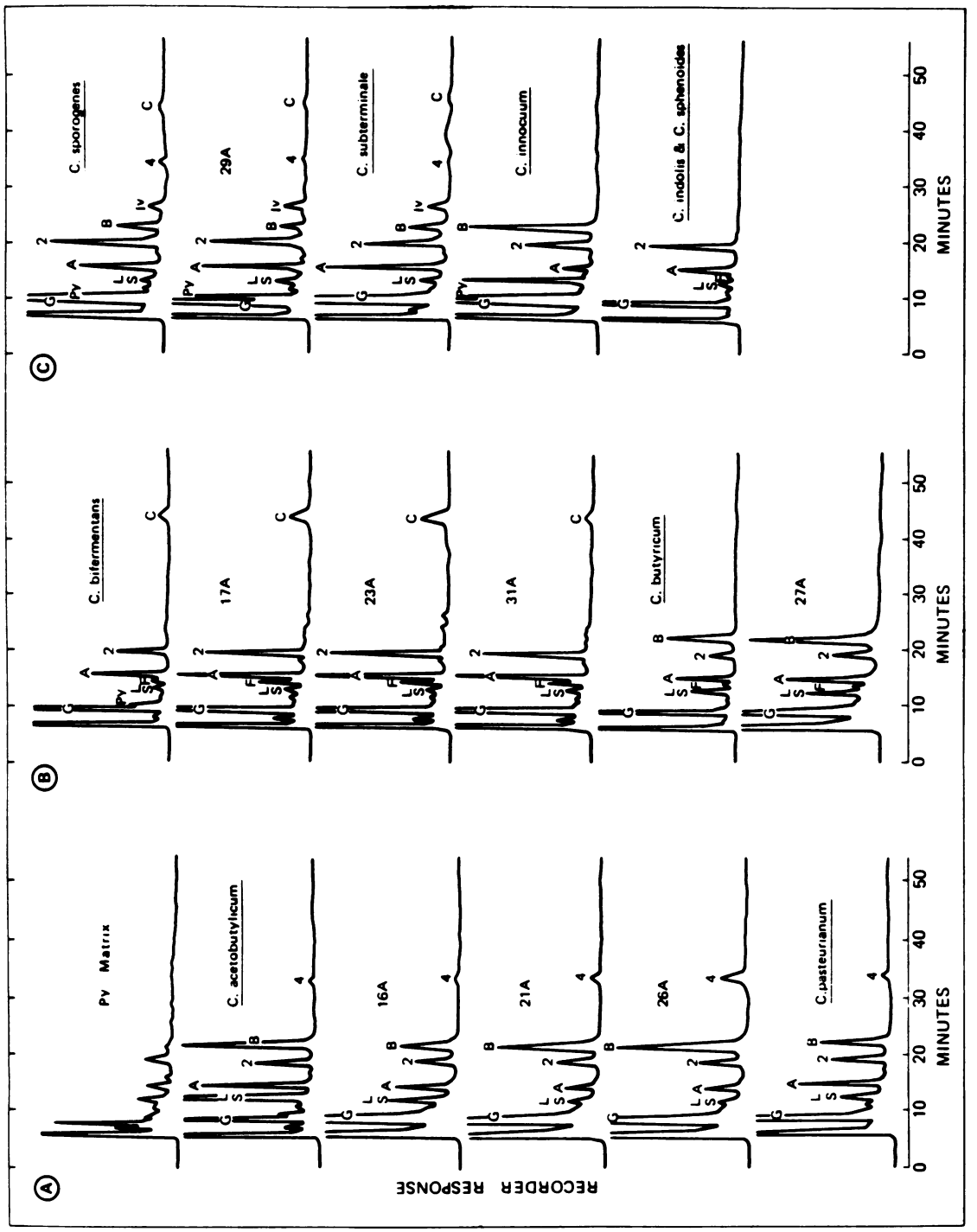

FIG. 4. *HPLC traces of eight known strains and eight unknown strains of Clostridium grown in PYG. The column was operated at 30°C with 0.013 N H_2SO_4 as the eluent. See legend to Fig. 1 for definitions of abbreviations.*

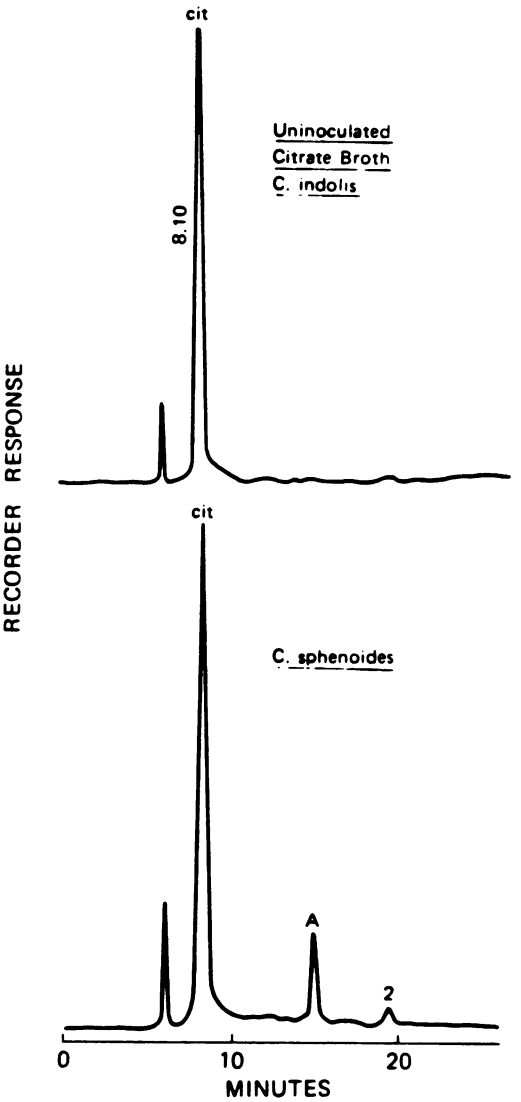

FIG. 5. *HPLC traces of uninoculated citrate broth and 24-h citrate cultures broth of C. indolis (upper trace) and C. sphenoides (lower trace). The column was operated at 30°C with 0.013 N H_2SO_4 as the eluent. See legend to Fig. 1 for definitions of abbreviations; Cit, citrate.*

agreement with the findings of Walther et al. (9).

Deterioration of column performance caused by peak broadening gradually occurred during use of the column. This happened more rapidly with PYG and other complex media than with defined media consisting only of carbohydrates and growth factors in mineral salt solutions. Column performance could be restored by reversing column flow and pumping at a low flow rate at an elevated temperature for a few hours. One of our columns that has been used for over 500 injections still gives satisfactory results after six regeneration cycles.

DISCUSSION

The foregoing results have demonstrated the utility of HPLC in the identification of several unknown *Clostridium* isolates. We have also used HPLC for analysis of products produced by other genera of bacteria grown on PYG and other media and by *Clostridium* species grown on several other media. Although out experience with other bacteria and with other media is not extensive, we are convinced that HPLC is equally as useful for those cases as for the examples presented above.

The actual machine time required for HPLC analysis can be as long as 70 min if *n*-heptanoic acid is present in the mixture. Much faster analysis is possible with GC. Either the procedure described by Rizzo (5) which uses a flame ionization detector or the procedure of Holdeman et al. (3) which uses a thermal conductivity detector requires less than 45 min of machine time (30 min for volatile acids and 15 min for nonvolatile acids). Sample preparation before injection into the gas chromatograph involves several separate operations and requires about 35 min, including a minimum reaction time of 30 min for methylation of nonvolatile acids. Fermentation product analysis by GC thus requires upwards of 50 min if the nonvolatile acids are included. If qualitative determination of only volatile acids and alcohols will suffice, the analysis can be completed in less than 20 min. In our experience, the frequency of isolation of heptanoic acid-producing organisms has been low, and we routinely stop elution of the HPLC column 60 min after injection. The slightly longer time required for HPLC analysis is probably offset by its simplicity, however.

The ease of quantitative determination of carbohydrates and formate with the RI detector is a major advantage of HPLC over GC. Carbohydrates can be determined readily by GC, but this determination requires an additional derivitization step and a different column operating under different conditions than those used for acids and alcohols. The results of single HPLC determination can be used to determine a partial fermentation balance, as was demonstrated by the dissimilation of citrate by *C. sphenoides*.

Formate poses problems in GC analysis because if a thermal conductivity detector is used, the acid must be extracted into a nonaqueous solvent, and extraction efficiency is low and variable. The sensitivity of a flame ionization detec-

tor for formate is low. Thus, quantitative determination of formate by GC is unsatisfactory.

The conditions chosen for the demonstration runs, namely, a column temperature of 30°C and 0.013 N H_2SO_4 as the eluent, were convenient for this work but may not be optimal. Indeed, the inability to separate lactate and succinate (this separation is essential when working with strains of *Bacterioides fragilis*) is one shortcoming of these conditions. Separation of lactate and succinate can be achieved at 50°C, but then certain other difficulties arise. Doubtless other sets of operating parameters might prove to be better for various purposes; nevertheless, we believe that HPLC should be considered as the first choice for fermentation product analysis.

LITERATURE CITED

1. **Bio-Rad Laboratories.** 1979. New organic analysis HPLC column. Bio-Rad Laboratories, Richmond, Calif.
2. **Buchanan, R. E., and N. E. Gibbons (ed.).** 1974. Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
3. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
4. **Powell, R. R., Jr., and T. M. Hawkins.** 1973. Laboratory methods in anaerobic bacteriology. CDC Laboratory Manual. Publication no. (HSM) 73-8322. Center for Disease Control, Atlanta, Ga.
5. **Rizzo, A. F.** 1980. Rapid gas-chromatographic method for identification of metabolic products of anaerobic bacteria. J. Clin. Microbiol. **11**:418-421.
6. **Skerman, V. B. D., V. McGowan, and P. H. A. Sneath.** 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. **30**:225-420.
7. **Sutter, U. L., V. L. Vargo, and S. M. Finegold.** 1975. Wadsworth anaerobic bacteriology manual, 2nd ed. Extension Division, University of California, Los Angeles.
8. **Turkelson, V. T., and M. Richards.** 1978. Separation of the citric acid cycle acids by liquid chromatography. Anal. Chem. **50**:1420-1423.
9. **Walther, R., H. Hippe, and G. Gottschalk.** 1977. Citrate, a specific substitute for the isolation of Clostridium sphenoides. Appl. Environ. Microbiol. **33**:955-962.
10. **Willis, A. T.** 1977. Anaerobic bacteriology: clinical and laboratory practice, 3rd ed. Butterworth & Co., Ltd., London.
11. **Wood, R. L., L. Cummins, and T. Jupille.** 1980. Recent developments in ion exchange chromatography. J. Chromatogr. Sci. **18**:551-558.