*Journal of Chromatography*, 336 (1984) 125—137
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2302

# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MICROBIAL ACID METABOLITES

R.F. ADAMS*, R.L. JONES and P.L. CONWAY

*CSIRO Division of Food Research, P.O. Box 52, North Ryde, N.S.W. 2113 (Australia)*

## SUMMARY

The use of high-performance liquid chromatography with a cation-exchange column and effluent monitoring at 210 nm has been evaluated for the profiling of selected microbial metabolites including aliphatic, dicarboxylic, and phenolic acids, as an adjunct to the identification of selected bacteria, detection of bacterial metabolites in foods, and the monitoring of industrial microbial fermentations. Advantages of the technique include the simultaneous profiling of different classes of organic acids without derivatization. Most applications require only qualitative or semi-quantitative data. For others, data are given on the day-to-day reproducibility for several acids.

## INTRODUCTION

The profiling of $C_2$—$C_7$ fatty acids, and several dicarboxylic and keto acids, present as metabolites in spent cultures of bacteria, is a valuable adjunct to other procedures for the presumptive identification of anaerobic bacteria. Comprehensive procedures for the profiling of the metabolites use gas chromatography (GC) for the analytical separation [1—4]. The procedures require separate manipulation of the sample for different classes of the acids. The $C_2$—$C_7$ fatty acids may be chromatographed directly from the acidified culture media or after a one-stage solvent extraction. Most other acids require derivatization and extraction of the derivatives before GC. Metabolites other than acids have been successfully profiled by GC [4]. These include alcohols, amines, hydroxy acids and nitrosoamines. However, as other classes of compounds are included, sample manipulation becomes more demanding.

Developments in high-performance liquid chromatography (HPLC), especially column and detector technology, have shown that it is feasible to use HPLC as an alternative to GC for the determination of the acid metabolites. Earlier HPLC studies for the separation of carboxylic acids included those using detection based on pH [5], ion exchange with chemical detection [6], and detection

0378-4347/84/$03.00 

using conductivity [7]. More recently separations of carboxylic acids on cation-exchange resin columns used ion-exclusion chromatography (IEC) coupled with ultraviolet (UV) monitoring of the effluent [8–11] or conductometry [12]. Mobile phases for IEC have been water [13], dilute mineral acid [8, 10], dilute mineral acid modified with acetonitrile [11], and aqueous *n*-butyric acid [12]. The use of HPLC is attractive because, unlike GC, several classes of acids, including phenolic acids, may be determined within one analysis. Extraction, but no derivatization, of the acids is required. Procedures have been reported for the analysis of bacterial metabolites using a cation-exchange resin column with 220-nm [9] or 210-nm [10, 11] monitoring of the eluent. The bacterial metabolites in buttermilk [9], *Clostridia* cultures [10] and in cultures of a group of clinically significant bacteria [11] have been successfully analyzed.

In the two latter reports [10, 11] the object has been to aid in identifying bacteria chiefly for clinical purposes. Other important applications of the procedures are to monitor bacterial fermentations in general. Types of sample may include food products, such as cultured buttermilk [9], wines [12], starter cultures, food contaminated with bacteria, liquid from waste digesters, and body fluids. For these applications qualitative data are often sufficient.

For supplementary identification purposes, semi-quantitative results are adequate. However, HPLC can provide excellent quantitative data under controlled conditions. In this study, we present the results of evaluating HPLC procedures of Guerrant et al. [11], with minor differences, on a wide variety of sample types. These include the acid products of bacteria, including several *Salmonellae*, *Clostridia*, and *Lactobacilli*. Differentiation into hetero- and homofermentative types of *Lactobacilli* was evaluated. Fermentations of heterogeneous mixtures of bacteria present in wine-making, an artificial gut and a fruit waste digester were monitored. Generally, comparing the acid profiles of samples was most useful. Both semi-quantitative and quantitative data, including reproducibility, are presented.

## EXPERIMENTAL

### *Materials*

Acetonitrile (Acetonitrile-190, Ajax Chemicals, Sydney, Australia) was HPLC grade. Water was from a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). Analytical reagent grade sulphuric acid and diethyl ether were used. Reference carboxylic acids were obtained either from Aldrich (Milwaukee, WI, U.S.A.) or from Sigma (St. Louis, MO, U.S.A.) as free acids or alkaline salts. Standard acids were prepared first as individual stock solutions in 0.003 mol/l sulphuric acid then combined to give a diluted working reference standard. Aliquots of the working standard were dispensed into 1-ml vials (Hypo-Vials, Pierce, Rockford, IL, U.S.A.). Standards were stored at 4°C when not in use. The acids used were 99+% pure, except for lactic, pyruvic, oxaloacetic, and the aromatic acids (approx. 95% pure). The acids and concentrations of the working standard are given in Table I.

TABLE I

CONCENTRATIONS OF ACIDS IN THE WORKING STANDARD, THEIR MEAN ($n$=6) RETENTION TIMES ($t_R$) AND RESPONSE FACTORS ($RF$) RELATIVE TO $n$-VALERIC = 1.000

| Elution order | Organic acid | Mol/l | $t_R$ (min) (S.E.M.)* | $RF$ |
|---|---|---|---|---|
| 1 | Oxalic | 0.002 | 8.1 (0.04) | 2.352 |
| 2 | Oxaloacetic | 0.0001 | 9.2 (0.06) | 0.074 |
| 3 | Pyruvic | 0.004 | 11.0 (0.07) | 2.532 |
| 4 | Succinic | 0.001 | 13.4 (0.04) | 0.074 |
| 5 | Lactic | 0.01 | 15.0 (0.05) | 10.210 |
| 6 | Formic | 0.02 | 17.0 (0.08) | 1.164 |
| 7 | Acetic | 0.02 | 17.3 (0.06) | 0.751 |
| 8 | Propionic | 0.02 | 19.1 (0.10) | 0.806 |
| 9 | Isobutyric | 0.02 | 21.0 (0.09) | 1.154 |
| 10 | $n$-Butyric | 0.02 | 23.9 (0.25) | 0.841 |
| 11 | Isovaleric | 0.02 | 25.7 (0.20) | 0.999 |
| 12 | $n$-Valeric | 0.02 | 28.9 (0.18) | 1.000 |
| 13 | Caproic | 0.02 | 30.9 (0.08) | 0.481 |
| 14 | 2-Methylvaleric | 0.005 | 34.0 (0.24) | 0.051 |
| 15 | $p$-Hydroxyphenylacetic | 0.0001 | 38.6 (0.32) | 9.140 |
| 16 | Phenylacetic | 0.002 | 43.2 (0.22) | 10.647 |
| 17 | Heptanoic | 0.02 | 50.9 (0.25) | 0.376 |
| 18 | 3-Phenylpropionic | 0.0001 | 60.2 (0.49) | 0.224 |

*S.E.M. = standard error of the mean, given between parentheses.

*Apparatus*

The liquid chromatographic equipment (Perkin-Elmer, Norwalk, CT, U.S.A.) consisted of a Sigma 3B pump module, an LC 100 column oven, an LC 75 detector, and a Sigma 15 Data Station. The column, 300 × 7.8 mm (9 μm particle size), Aminex HPX-87H (Bio-Rad Labs., Richmond, CA, U.S.A.) was obtained prepacked. The injection system was a Rheodyne Model 7105 injector valve (Rheodyne, Berkeley, CA, U.S.A.).

*HPLC conditions*

The detector was operated at 210 nm at full scale sensitivity of 0.1 absorbance unit. The mobile phase was 0.003 mol/l sulphuric acid—acetonitrile (90:10, v/v). The column was operated at 50°C and a mobile phase flow-rate of 0.6 ml/min. Before use the new column was conditioned under temperature and flow conditions with the mobile phase for one day to remove high UV-absorbing residues from the column to ensure a stable baseline. Flow-rate through the column during use increased from a starting pressure of approx. 5.0 MPa to 7.0 MPa after about fifty separations. At 8.0 MPa, flow-rate through the column was reversed for 16 h or until the pressure was reduced close to 5.0 MPa. The column was then returned to its original flow direction and analyses continued. A volume of 3.5 l of the mobile phase was prepared. The column effluent was collected into a clean container, protected from dust

contamination and excessive evaporation, and recycled as a batch when about 3 l were collected. It was possible to repeat the recycling about five times before fresh mobile phase was prepared.

*Bacteria*

Clinical isolates of *Clostridium perfringens* (FRR B179) and *Clostridium difficile* (FRR B180) were each grown in 20 ml cooked-meat medium (CMM) [1] and in peptone-yeast extract glucose (PYG) broth [1] for four days at 37° C. *Salmonella infantis* (FRR B278), *Salmonella sofia* (FRR B279), *Salmonella typhimurium* (FRR B277), *Streptococcus faecalis* (FRR B343), *Staphylococcus aureus* (FRR B343) and *Staphylococcus epidermidis* (FRR B342), *Lactobacillus casei* (FRR B738) and *Lactobacillus fermentum* (FRR B737) obtained from food industry sources, were individually grown in PYG anaerobically for four days at 37° C. Quantities of sterile media were incubated and tested for low-residual acids. Acetic acid is often present to excess as a result of pH adjustment. Such media should not be used.

*Samples from mixed microbial fermentations*

Samples included waste digester and artificial gut liquids, food products including soy sauce, and wines.

*Sample pretreatment*

Bacterial cultures were centrifuged at 10,000 *g* to remove particulate matter. Aliquots of 1 ml were transferred to 5-ml tapered, capped centrifuge tubes and 0.25-ml of 9.0 mol/l sulphuric acid added with 0.6 g sodium chloride, 5 ml diethyl ether and 25 $\mu$l acetonitrile. The mixture was mixed with a vortex for 1 min and centrifuged at 1500 *g* for 5 min. Using a 5-ml variable-pipettor set to 4.5 ml the diethyl ether phase was transferred to a clean tube. To the diethyl ether was added 0.25 ml of 0.1 mol/l sodium hydroxide, the contents mixed cautiously using a vortex mixer and centrifuged at 1500 *g* for 5 min. The diethyl ether was discarded and 25 $\mu$l acetonitrile were added. Residual diethyl ether was allowed to evaporate from the open tube. The residue was mixed and 20-$\mu$l aliquots used for chromatography. Less liquid samples were first homogenized on a weight/volume basis with water, then extracted. Samples may be stored in a capped tube at 10°C for several months if taken to the step before adding the final 25 $\mu$l acetonitrile. Minor losses only may occur in the ketoacids.

Food products that have resulted from microbial fermentations, including some dairy products, sauces and wines, were processed similarly. Where quality control requirements only need be met, extraction may not be necessary providing that a clarified sample is available. Wines were profiled successfully without extraction. There is less control over peak identification, but a reproducible fingerprint is obtainable. Where extraction was not used, or where the product such as soy sauce was a concentrate, dilution with water was used. Wines were diluted × 10 and soy sauce × 50.

*External standard.* The reference standard was extracted by the same sample pretreatment as above for the quantifying of acids in test samples.

*Calculations*

Chromatograms were evaluated by use of an external standard. Identification of peaks was assumed by coincidence of retention times with the standard. To assess peak profiles of the extracts of bacterial isolates as an adjunct to identification, semi-quantitative assessment was made by comparison of peak areas of test samples with those of the standard and recording the areas as larger, equal to, or smaller than, those of the standard. Quantitative assessment was required for assignment of *Lactobacillus* spp. to hetero- or homofermentative groups based on the relative amounts of acetic and lactic acids produced [14]. For this and similar requirements, sterile media containing a range of concentrations of the acids of interest were extracted and chromatographed. Peak areas were plotted for the extracted standards and concentrations of acids in test samples were calculated from the standard plot.

*Extraction recoveries*

The ratios of the peak areas of the extracted standards to the peak areas of unextracted standards were expressed as percentage recoveries.

*Reproducibility studies*

For within-day reproducibility of the method, six extracts from a single culture of *C. perfringens* were chromatographed. To assess day-to-day reproducibility, cultures of *C. perfringens*, were grown on each of four days to give a total of four cultures. As each incubation was completed it was processed.

## RESULTS AND DISCUSSION

A chromatogram of the working standard is given in Fig. 1. Baseline separation is adequate for most of the acids. The chromatogram shows several peaks additional to those labelled. Several of these were related to impurities in the

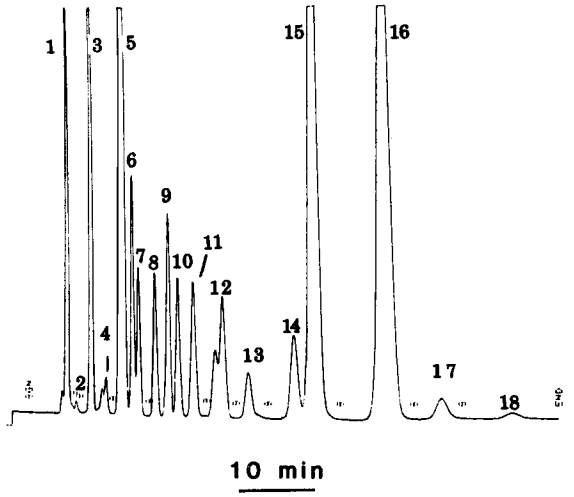


Fig. 1. Chromatogram of a standard solution of eighteen acids. The key to the numbered peaks and the concentrations used are given in Table I. The peak eluting on the leading edge of peak 12 was attributed to an impurity in the 3-phenylpropionic acid.

acids used for the standard. The peak eluting on the leading edge of peak 12 (Fig. 1) was a component of the 3-phenylpropionic acid standard.

Resolution is partly dependent on temperature and concentration of acetonitrile. Adjustment of these may be necessary to optimize conditions for a new column or to modify the resolution required for specific groups of the acids. The mean retention time ($n$=6) and the detector response relative to $n$-valeric acid (1.00) for each of the acids under the recommended conditions is given in Table I. The response of the detector was much greater for several acids such as the phenolic acids. Where large quantities of any acids are present in a sample, it may for some extracts be necessary to attenuate detector response in order to evaluate the peaks correctly.

TABLE II

RECOVERIES OF A MIXTURE OF SELECTED ACIDS IN 1 ml WATER EXTRACTED INTO 5 ml DIETHYL ETHER

Results were obtained from five extractions.

| Acid | Concentration (mmol/ml) | Recovery (%) (S.E.M.)* |
|---|---|---|
| Pyruvic | 0.004 | 22.4 (1.10) |
| Lactic | 0.010 | 24.0 (0.91) |
| Acetic | 0.020 | 41.2 (0.98) |
| Propionic | 0.020 | 55.2 (0.76) |
| Isobutyric | 0.020 | 69.0 (0.08) |
| $n$-Butyric | 0.020 | 61.4 (0.72) |
| Isovaleric | 0.020 | 72.1 (0.90) |
| $n$-Valeric | 0.020 | 63.3 (0.71) |
| Caproic | 0.020 | 54.0 (0.84) |
| Heptanoic | 0.020 | 49.6 (0.69) |
| Phenylacetic | 0.002 | 50.2 (0.89) |

*S.E.M. = standard error of the mean, given between parentheses.

TABLE III

REPRODUCIBILITY DATA FOR SELECTED ACIDS PRESENT IN CULTURES OF *CLOSTRIDIUM PERFRINGENS*, TYPE *C*

Within-day data were from six aliquots of a single culture. Day-to-day data were from four individual cultures started on consecutive days and processed as each incubation was completed.

| Acid | Concentration ($\mu$mol/ml of culture) | | | | | |
|---|---|---|---|---|---|---|
| | Within-day ($n$=6) | | | Day-to-day ($n$=4) | | |
| | Mean | S.D. | C.V. (%) | Mean | S.D. | C.V. (%) |
| Acetic | 8.5 | 0.42 | 4.9 | 8.3 | 0.51 | 6.2 |
| Propionic | 0.8 | 0.05 | 5.8 | 0.7 | 0.05 | 7.3 |
| $n$-Butyric | 3.3 | 0.11 | 3.4 | 3.5 | 0.18 | 5.2 |
| Isovaleric | 0.6 | 0.03 | 5.1 | 0.7 | 0.05 | 7.1 |
| $n$-Valeric | 0.7 | 0.03 | 4.7 | 0.6 | 0.03 | 4.1 |
| Phenylacetic | 0.1 | 0.006 | 6.1 | 0.1 | 0.008 | 7.8 |

Extraction recoveries were comparable to those reported by Guerrant et al. [11] for formic, propionic, fumaric and lactic acids allowing for the smaller volume of diethyl ether taken for the back-extraction step. Recoveries of these and other acids studied by us are given in Table II.

The within-day reproducibility (coefficient of variation, C.V.%) of the individual acids tested ranged from 3.4% to 6.1%. The day-to-day reproducibility (C.V.%) of the cultures of *C. perfringens* was calculated to be 4.1—7.8%. The data are given in Table III. The data show that for most quantitative applications, reproducibility is adequate. Caution would be needed where grossly different ratios of acids with close retention times are present or where unidentified components are present.

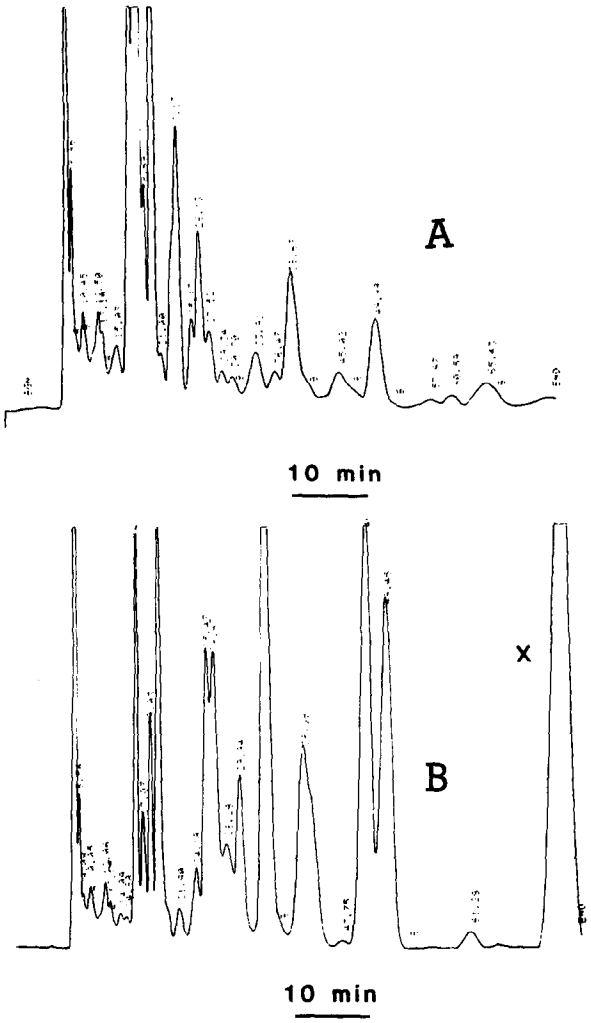


Fig. 2. The acid profile of *Clostridium perfringens* (A); and *C. difficile* (B) grown in cooked-meat medium. An important distinction is the large final peak (X) present in the *C. difficile* chromatogram. The peak retention time fits that of *p*-cresol, produced by this microorganism.

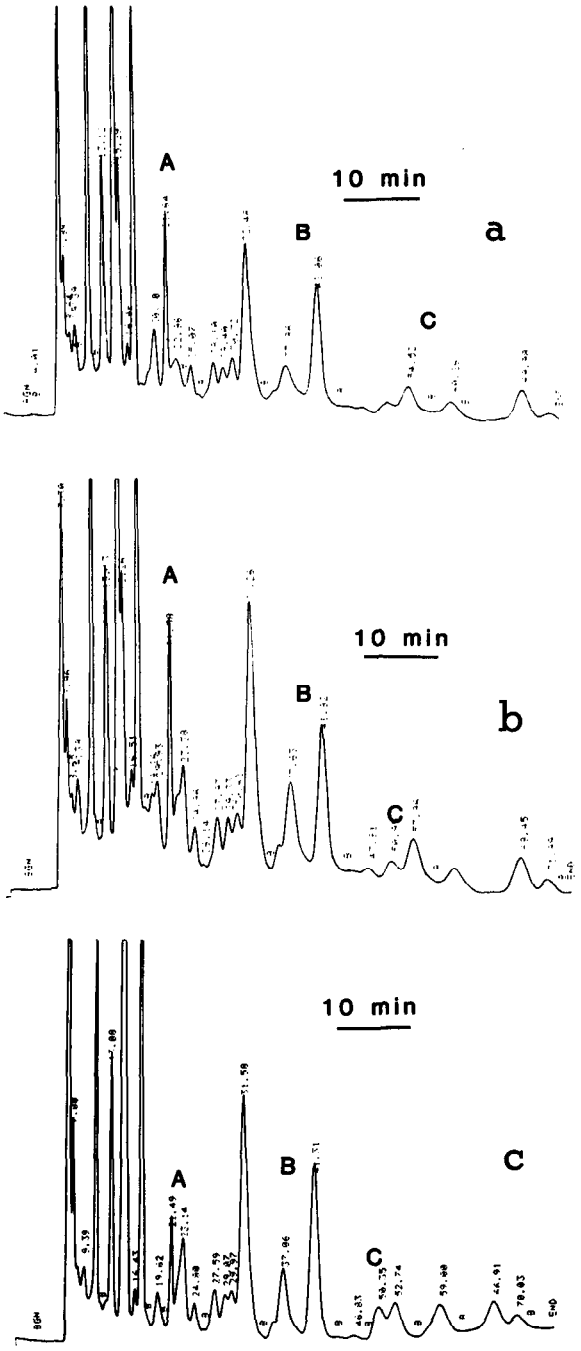


Fig. 3. Chromatograms of extracts of cultures of *Salmonella sofia* (a); *S. infantis* (b); and *S. typhimurium* (c) grown anaerobically in PYG medium. Close similarities exist between the chromatograms but differences may be noted between the peak groupings about A, B and C.

The acid profiles of bacterial cultures showed that considerable differences existed between genera and between the species tested. Fig. 2 illustrates chromatograms obtained from the cultures in CMM of *C. perfringens* (Fig. 2A) and *C. difficile* (Fig. 2B) showing major differences in quantities of several of the acids. The chromatogram of the *C. difficile* extract shows a large peak (X) which corresponds, as noted by Guerrant et al. [11] to the retention time of *p*-cresol, a metabolite expected in *C. difficile* cultures. No significant advantage to interpretation of the chromatograms was given by using CMM rather than PYG. A greater quantity of *p*-cresol was obtained with CMM for *C. difficile* cultures. Fig. 3 shows the chromatograms of extracts from the *Salmonellae*, showing close similarity within the group but useful differences were noted for the peak groups A, B, and C. Fig. 4 shows the different profiles obtained for *L. casei* and *L. fermentum*. The differences were considered to be sufficiently marked to be useful as an adjunct to other identification procedures.

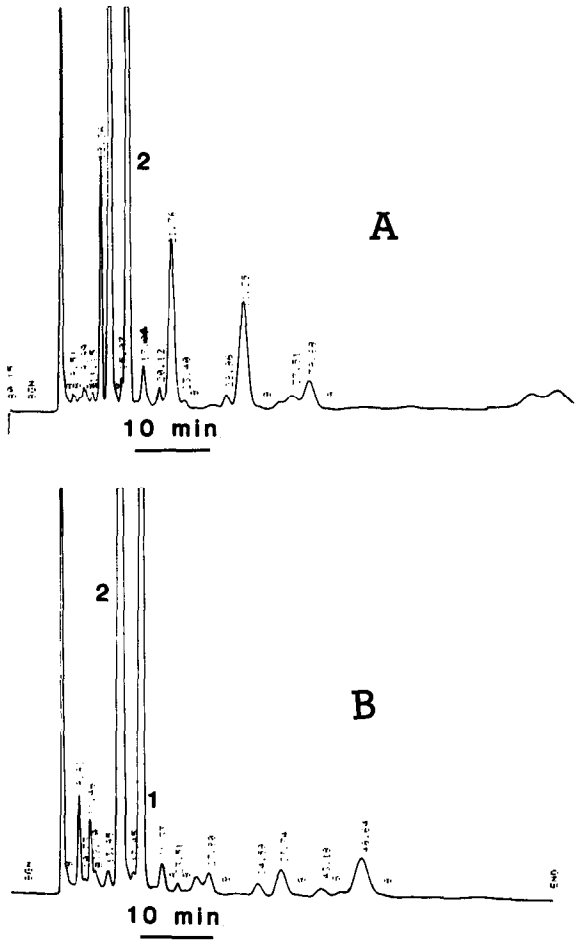


Fig. 4. Chromatograms of extracts from *Lactobacillus casei* (A); and *L. fermentum* (B). The two profiles are distinctive. Additionally, the ratios of acetic (peak 1) to lactic (peak 2) permitted assignment of *L. casei* and *L. fermentum* to homofermentative and heterofermentative, respectively.

Distinctive differences were also found for the acid profiles of the *S. faecalis*, *S. aureus* and *S. epidermidis* cultures (data not shown). Further data are needed to confirm such use for a wide range of bacterial species.

The basis for assignment of a *Lactobacillus* as hetero- or homofermentative is whether it produces acetic acid greater or less than 50% of the lactic acid produced [14]. The *L. casei* (Fig. 4A) and *L. fermentum* (Fig. 4B) were readily assignable to homo- and heterofermentative by this criterion, the values of acetic:lactic ratios being 7.2:92.8 and 68.5:31.5, respectively. The reproducibility of acetic acid was about 4.9% C.V. and that for lactic acid about 5.2% C.V. on a within-day basis. For these *Lactobacilli* the variation would not significantly effect the result. For other species where the ratio is closer interpretation may be difficult, but in such cases modification of the mobile phase may improve resolution and consequently the reproducibility.

The potential of the HPLC procedure for some food products is indicated in Fig. 5 showing chromatograms obtained for diluted wine samples, a red wine (A), a port wine (B), and one obtained for a soy sauce (C) obtained by a mixed fermentation including *Lactobacilli*, yeasts and *Fungi*. The wines were not extracted and must therefore have contained a mixture of acids, bases and neutrals. The soy sauce was extracted but contained several compounds not identifiable. However, the profile itself was considered to be a probable useful indicator of product integrity and stability. The chromatogram was generally characteristic of several samples of soy sauce of the same make. Benzoic acid is a common preservative in some food products, including some soy sauces. The peak X on the chromatogram (Fig. 5C) was provisionally assigned to benzoic acid.

The profiles obtained from the fluid of a citrus waste digester [15] were useful in indicating the healthy or otherwise status of the digester. In Fig. 6 the profiles of a 'healthy' and a 'bad' fermentation are given. A healthy digester was considered to be one producing close to theoretical quantities of methane. For a similar type of mixed fermentation sample, the procedure was used successfully with the supernatant of an artificial gut. The aim in this case was to study changes in metabolism of a mixed culture.

From these applications, useful information involving relatively little effort was obtained both on cultures of pure bacterial isolates and of mixed microbial populations. Every effort was made to ensure that the same batch of media was used for any series of samples. It would be advisable to check media of different batches against standard bacterial isolates to ensure similarity of acid profiles. Different makes of media of the same type would be expected to give different profiles, although this was not checked. Where use of different media is unavoidable, the re-running of standard bacterial isolates is essential.

The use of retention time is not specific for the identification of analytes. Because of this, the complexity of the sample matrices presents limitations to the use of the procedures for quantitation. The extraction procedure and the type of column packing does improve selectivity for the acid metabolites but there will be other acids and compounds present some of which may interfere because of coincidence with the retention times of the analytes. The procedure has greatest value used qualitatively, such as for the development of profiles of microbial metabolites for quality control purposes and semi-quantitatively as an aid to bacterial identification.

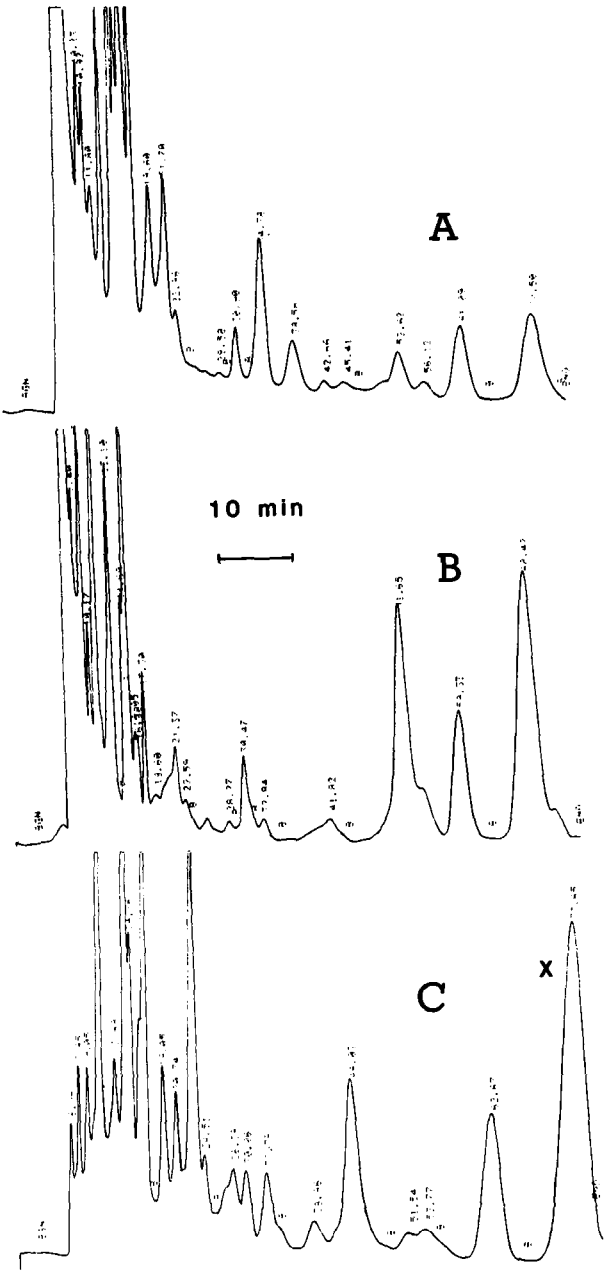


Fig. 5. Chromatograms of a red wine (A), a port wine (B) and a soy sauce (C). The wines were not extracted, being chromatographed directly as a 1:10 dilution in water. The soy sauce was extracted after dilution 1:50 with water. The wine sample would contain bases and considerable quantities of neutral compounds as well as the acids. The chromatograms show that relatively uncluttered profiles are obtainable both from the unextracted wine samples and a complex sample such as soy sauce. The final peak (X) of C was designated benzoic acid because of identical retention time and because benzoic acid is used as a preservative in some soy sauces.

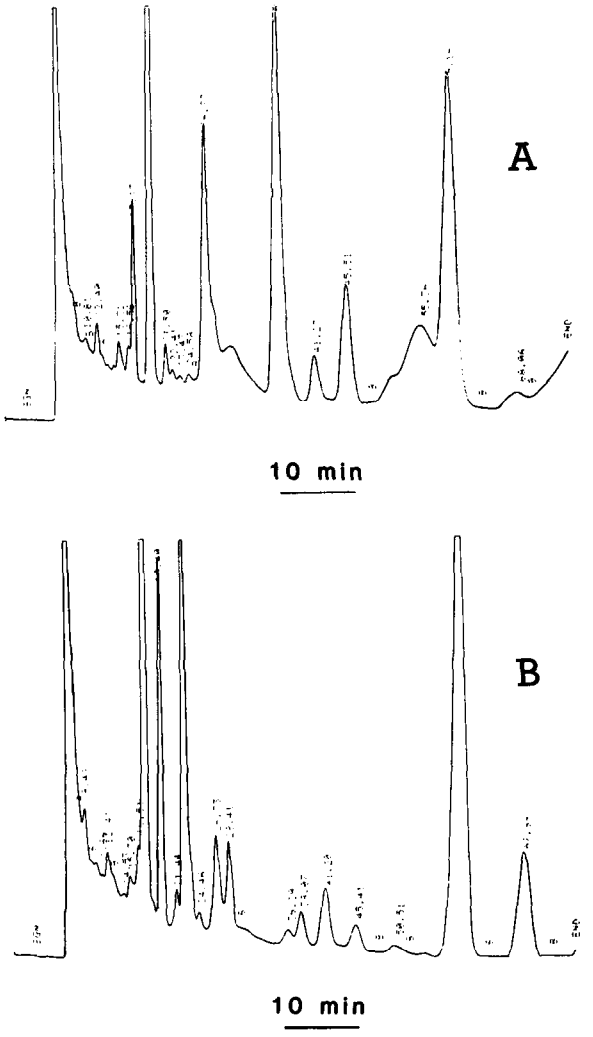


Fig. 6. Chromatograms of an extract of fluid from a fruit waste digester. (A) Profile obtained from a healthy digester. (B) Profile from a digester that was not operating efficiently. The distribution of peaks shows that metabolites are more uniformly spread throughout A, compared with B.

The column used for this report has been in use for greater than 800 analyses and about 100 separations of standards. This was without use of a guard column. The use of a guard column may be advisable, but can introduce minor losses of resolution. To maintain maximum efficiency of the analytical column every effort was made to ensure that the injected solution was particle-free. Additionally, the column was back-flushed weekly and when not in use, a flow-rate of 0.1 ml/min was maintained.

The column temperature of 50°C was chosen to give adequate resolution and to permit a higher flow-rate before back-pressure in the system became too high.

In our hands the extension of the procedures of Guerrant et al. [11] has

proved useful for a wide range of studies involving microbial fermentations. Although as presently recommended the procedure does not analyze important metabolites such as alcohols, acetone, acetaldehyde and diacetyl, a large amount of information was obtainable from a single analysis.

REFERENCES


1 L.V. Holdeman, E.P. Cato and W.E.C. Moore (Editors), Anaerobe Laboratory Manual, Virginia Polytechnic Institute and State University, Blacksburg, VA, 4th ed., 1977, p. 122.
2 V.L. Sutter, D.M. Citron and S.M. Finegold, Wadsworth Anaerobic Bacteriology Manual, C.V. Mosby, St. Louis, MO, 3rd ed., 1980, p. 53.
3 V.R. Dowell, Jr. and T.M. Hawkins, Laboratory Methods in Anaerobic Bacteriology, CDC Laboratory Manual, Centers for Disease Control No. 77-8272, U.S. Government Printing Office, Washington, DC, 1977, p. 77.
4 J.B. Brooks, in J.C. Giddings, E. Grushka and J. Cazes (Editors), Advances in Chromatography, Vol. 15, Marcel Dekker, New York, 1977, p. 1.
5 R. Farinotti, M. Caude, G. Mahuzier and R. Rosset, Analusis, 7 (1979) 449.
6 M. Nakajima, Y. Ozawa, T. Tamimura and Z. Tamura, J. Chromatogr., 123 (1976) 129.
7 H. Small, T.S. Stevens and W.C. Bauman, Anal. Chem., 47 (1975) 1801.
8 V.T. Turkelson and M. Richards, Anal. Chem., 50 (1978) 1420.
9 R.T. Marsili, J. Chromatogr. Sci., 19 (1981) 451.
10 G.G. Ehrlich, D.F. Goerlitz, J.H. Bourell, G.V. Eisen and E.M. Godsy, Appl. Environ. Microbiol., 42 (1981) 878.
11 G.O. Guerrant, M.A. Lambert and C.W. Moss, J. Clin. Microbiol., 16 (1982) 355.
12 P.R. Monk and P.G. Iland, Food Technol. Aust., 36 (1984) 16.
13 D.J. Walker and P.R. Monk, Appl. Microbiol., 22 (1971) 741.
14 R.E. Buchanan and N.E. Gibbons (Editors), Bergey's Manual of Determinative Microbiology, Williams and Wilkins, Baltimore, MD, 8th ed., 1974, p. 577.
15 A.G. Lane, Food Technol. Aust., 31 (1979) 201.