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WALL-COATED OPEN-TUBULAR CAPILLARY COLUMN SYSTEM FOR GAS CHROMATOGRAPHIC ANALYSIS OF NEUTRAL AND ACIDIC FERMENTATION END-PRODUCTS

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SUMMARY

The separation of 18 neutral and 17 acidic fermentation end-products by capillary column gas chromatography is described. A wall-coated open-tubular column, 30 m × 0.25 mm I.D., with SP 1000 as stationary phase, was found to combine high efficiency with rapidity of analysis. Non-volatile acids and formic acid were analysed (after methylation) either isothermally at 130°C, 160°C, 170°C and 180°C or with a temperature programme from 90°C to 160°C. Volatile acids were chromatographed isothermally at 130°C, 160°C, 170°C or 180°C; while alcohols, aldehydes, ketones, diketones, diethyl ether and acetoin could be resolved on the same column isothermally at temperatures ranging from 90°C to 170°C. Volatile neutral compounds and acids (C_4) and higher were resolved when the column was programmed from 110°C to 170°C. The column proved satisfactory for the analysis of fermentation products of a wide range of bacteria.

INTRODUCTION

Detection and identification of anaerobic bacteria can be accomplished by gas chromatographic analysis of acidic and neutral end-products of fermentation[1–3]. Identification keys incorporating fermentation data have been constructed[4] on the basis of the behaviour of named strains. Without gas chromatographic data, it would be difficult to identify some genera, and many species.

Although virtually all neutral products and fermentation acids can be resolved in the same analysis on porous polymer columns[5], non-volatile acids generally have been converted into suitable volatile derivatives before analysis. The most usual derivative has been the methyl ester[6], although butyl esters have also been employed[7]. Various routes for synthesis of esters are available and have been discussed elsewhere[8]. Esterification of formic acid has permitted its detection by flame ionization detectors. Originally, short packed columns were used[9]; latterly, longer, high-resolution packed columns have been introduced[10]. An alternative to long packed columns is the use of open-tubular columns. The increased efficiency of such columns

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has already assisted microbiologists and biochemists engaged in analysis of pyrolysates[11], carboxylic methyl esters[12,13] and diastereoisomers of amino acids as their pentafluoropropionyl aminoacyl-(+)-3-methyl-2-butyl derivatives[14]. Fermentation liquor *per se* has not yet been analysed with use of open-tubular columns; however, enantiomers of lactic and glyceric acids have now been successfully analysed as their O-acetylated menthyl esters on a 25-m column of SP 1000[15]. Similarly, acetic, propionic, butyric, valeric and capronic acids have been analysed successfully on a 45-m capillary column of free fatty acid phase (FFAP)[16].

The present study investigates the feasibility of resolving more complex mixtures of neutral and acidic end-products of fermentation, and the applicability of the technique to the analysis of bacterial culture supernatants.

EXPERIMENTAL

Bacterial cultures

In order to confirm the applicability of the chemical analytical method, the following representative organisms were included in the study: *Actinomyces bovis*, *Bacteroides fragilis*, *Bifidobacterium bifidum*, *Clostridium butyricum*, *C. noyvii*, *C. perfringens*, *C. tetani*, *Eubacterium aerofaciens*, *Streptococcus faecalis* and *Veillonella parvula*.

The identities of cultures of anaerobes were confirmed by using the VPI scheme[4]. *S. faecalis* was presumptively identified as being a Gram-positive, catalase-negative, chaining coccus, bile-tolerant and of Lancefield group D; this organism was cultured for 48 h in brain–heart infusion (Lab-M, Salford, Great Britain) at 37°C. The other organisms were grown in Robertson's cooked meat medium (Oxoid, Basingstoke, Great Britain) at 37°C for 48 h. Extraction of "fermentation" products from culture supernatants followed an existing, proven, scheme[4] with only slight modification.

Reagents

All chemical reagents were obtained from BDH (Liverpool, Great Britain), except for heptanoic acid (Sigma, Poole, Great Britain), isobutanol, *tert.*-butanol, neopentyl alcohol and active amyl alcohol (Aldrich, Wembley, Great Britain).

Extraction of neutral compounds and free acids

Conjugate bases were converted into undissociated acids by the addition of 1.0 ml of 10 *M* sulphuric acid to 5.0 ml of supernatant in a 1-oz, screw-cap "universal" bottle. The addition of 2.0 g of sodium chloride "salted out" polar neutral compounds, which were extracted (together with undissociated acid) into 5.0 ml of diethyl ether.

The ether extract was dried by the addition of 0.5 volume of anhydrous sodium sulphate, use of which may be preferable to anhydrous calcium chloride, which combines with alcohols. If necessary, the dried ether extract was concentrated to 25% of its initial volume by a stream of dried compressed air before analysis. Non-volatile acids extracted in this manner yielded peaks on a column of the porous polymer Chromosorb 101 (ref. 5), but not on other columns. Derivatization was therefore necessary.

Derivatization of non-volatile acids

The method selected was that of Holdeman *et al.*[4], in which non-volatile acids are esterified with methanol–sulphuric acid. For microbial laboratory use, this method appeared less demanding than methylation of sodium salts of non-volatile acids by other procedures (see below). Methylation of 4.0-ml aliquots of supernatant was performed with 8.0 ml methanol and 1.5 ml 10 *M* sulphuric acid at 60°C for 30 min in a 1-oz. screwcap bottle. Esters were salted out with 4 g of sodium chloride after addition of 4.0 ml water and 2.0 ml chloroform. The lower, chloroform, layer was dried with 0.5 volume of anhydrous sodium sulphate before concentration of the chloroform extract to 25% of its initial volume. Concentration was not vital for detection of major peaks, but did reveal trace substances.

Gas chromatographic analysis

A wall-coated open-tubular (WCOT) capillary column (30 m × 0.25 mm I.D.) of SP 1000 prepared by Phase Separations (Queensferry, Great Britain) was used for the initial analyses. The column was operated in a Pye 104 gas chromatograph equipped with a capillary column conversion kit (Phase Separations). The kit provided capillary connections, gas-control valve and injection splitter. The following analytical conditions were used: nitrogen carrier gas pressure, 8 p.s.i.; column carrier flow-rate, *ca.* 2 ml/min; nitrogen purge flow-rate, 2.5 ml/min; nitrogen split flow-rate, 30 ml/min; air flow-rate, 600 ml/min; hydrogen flow-rate, 40 ml/min. Detection of peaks was by flame ionisation detector. Computation of retention times and peak areas was by computing integrator (Spectra-Physics, Luton, Great Britain). The signal from the integrator was supplied both to an on-line teletype terminal (which produced punch-tape for subsequent data reduction) and to a Telsec chart recorder (Telsec, Luton, Great Britain). A 10-mV full-scale-deflection setting was employed; the chart speed was 0.5 cm/min. To assist in tentative peak identification, standards were chromatographed. An injection volume of 0.2 μl was employed. Analysis temperatures are shown in the tables and figures.

RESULTS

Retention data (relative to ethanol) for neutral fermentation products and chemically related compounds are listed in Table I. Higher temperatures resulted in faster analysis, though at the cost of peak resolution. Reduction in the thickness of the stationary phase film had a similar effect to increase in column temperature. Change in temperature was seen to affect members of different homologous series to differing extents, *e.g.*, diacetyl and 2-propanol. This fact proved useful in tentatively assigning peak identities after re-analysis at different temperatures. Acidic fermentation acids analysed as free acids resulted in the data shown in Table II; for convenience, data for the diethyl ether solvent are included. Table III presents data for those acids analysed as their methyl esters. The list of acids is by no means comprehensive, but is indicative of the wide range of homologous series that may be analysed on the SP 1000 WCOT column. Data for products of test organisms are listed in Table IV. Some representative gas chromatograms are depicted in Figs. 1 and 2. An analysis of an ether extract of *C. tetani* supernatant is shown in Fig. 1; a methyl ester separation is shown in Fig. 2 for *B. fragilis*.

TABLE I
RETENTIONS OF NEUTRAL FERMENTATION PRODUCTS AND RELATED COMPOUNDS

Compound	*Systematic name*	*Retentions relative to ethanol**				
		Column temperature (°C)				
		90	110	130	160	170
Ether	Ethoxyethane	0 783	0 867	0.913	0.958	0.951
Acetaldehyde	Ethanol	0.802	0.872	0.914	–	–
Acetone	Propanone	0 891	0.933	0.967	1.004	–
Methyl alcohol	Methanol	0.948	0 968	–	–	0.969
tert.-Butyl alcohol	2-Methyl-2-propanol	0 936	0 954	0.940	0.958	0.975
Ethyl alcohol	Ethanol	1 000	1 000	1 000	1.000	1 000
Isopropyl alcohol	2-Propanol	1.017	1.026	–	–	1.015
Diacetyl	2,3-Butandione	1 112	1.070	1.005	1 026	1.097
tert -Amyl alcohol	2-Methyl-2-butanol	1 167	1.089	1.052	1 029	1 012
sec.-Butyl alcohol	2-Butanol	1 187	1.115	1.061	–	1.031
n-Propyl alcohol	1-Propanol	1.254	1.146	1.076	1.041	1.045
Isobutyl alcohol	2-Methyl-1-propanol	1.417	1 235	1.129	1.064	1 018
Neopentyl alcohol	2,2-Dimethyl-1-propanol	1.510	1.286	1.154	1.075	1.042
–	2-Pentanol	1.572	1.328	1.165	1 080	1.045
n-Butyl alcohol	1-Butanol	1.685	1 415	1 212	1 097	1 071
Isoamyl alcohol	3-Methyl-1-butanol	2 199	1.667	1.330	1 147	1.097
"Active" amyl alcohol	2-Methyl-1-butanol	2 178	1 672	1.325	1.147	1.093
n-Amyl alcohol	1-Pentanol	2 622	1.881	1.429	1.189	1.124
Acetoin	3-Hydroxy-2-butanol	–	–	1.639	1.299	1 212

* Data obtained on SP 1000 WCOT column, 30 m × 0.25 mm I.D.

TABLE II
RETENTIONS OF ACIDIC FERMENTATION END-PRODUCTS

Acid	*Systematic name*	*Retentions relative to acetic acid**			
		Column temperature (°C)			
		130	160	170	180
Acetic	Ethanoic	1.000	1.000	1.000	1.000
Propionic	Propanoic	1.340	1.165	1.156	1.104
Isobutyric	2-Methylpropanoic	1.489	1 240	1.209	1.142
n-Butyric	Butanoic	1 876	1.414	1.380	1.246
Isovaleric	3-Methylbutanoic	2.127	1.570	1.503	1 333
n-Valeric	Pentanoic	2 940	1.865	1.785	1.496
Isocaproic	4-Methylpentanoic	3.897	2.246	2.094	1.701
n-Caproic	Hexanoic	4.693	2.537	2.390	1.857
n-Heptylic	Heptanoic	7.598	3.555	3 272	2.371
(Ether)	(Ethoxyethane)	0.411	0.627	0.654	0.753

* Data obtained on an SP 1000 WCOT column, 30 m × 0.25 mm I.D.

Examination of these chromatograms revealed minor peaks, which were not readily detectable following re-analysis on a 5-ft. packed column of Chromosorb 101. The separation of methyl esters appears to be particularly well suited to columns of SP 1000. When bacterial supernatants were analysed as methyl esters, trace peaks

TABLE III
RETENTIONS OF METHYL ESTERS OF NON-VOLATILE AND OTHER ACIDS

Acid	*Systematic name*	*Retentions relative to methyl lactate**					
		Column temperature (°C)					
		90	*100*	*110*	*120*	*140*	*160*
Formic	Methanoic	0.199	0.332	0.405	0 482	0 604	–
Acetic	Ethanoic	0 212	0 348	0 420	–	0.623	–
(Methyl alcohol)	(Methanol)	0 226	0.363	0 434	0 503	0.640	0 758
Propionic	Propanoic	0.233	0.372	0 444	–	–	–
(Chloroform)	(Trichloromethane)	0 284	0.431	0 499	0 561	0 688	0 795
Pyruvic	2-Oxopropanoic	0.826	0 748	0 782	0.811	0 868	0 916
Lactic	2-Hydroxypropanoic	1.000	1.000	1.000	1.000	1 000	1.000
Malonic	Propanedioic	2 220	2.303	2.086	1.885	1.550	1.312
Fumaric	*trans*-Butenedioic	2 628	2.733	2 473	2 209	1.787	1 463
Succinic	Butanedioic	3 447	3 433	3.016	2 629	2.014	1 574
Malic	2-Hydroxybutanedioic	–	–	–	–	8.694	4 748
α-Ketoglutaric	2-Oxopentanedioic	–	–	–	–	–	5 046

* Data obtained on an SP 1000 WCOT column, 30 m × 0.25 mm I D

TABLE IV
MAJOR NEUTRAL AND ACIDIC FERMENTATION END-PRODUCTS OF SOME BACTERIA
For analytical conditions, see legends to Figs 1 and 2 Peaks· 2 = ethanol; 3 = 1-propanol; 4 = 1-butanol; F = formic acid; A = acetic acid; P = propionic acid; B = butyric acid; L = lactic acid; S = succinic acid.

Microorganism	*Products**	
	Neutral, and free acid	*Methyl esters*
Actinomyces bovis	A	S, L
Bacteroides fragilis	A, P	S
Bifidobacterium bifidum	A, 2	L
Clostridium butyricum	B, 2, A	L
Clostridium noyvii	B, P, A	S**
Clostridium perfringens	B, A	L
Clostridium tetani	4, 2, B, 3, A	
Eubacterium aerofaciens	2, A, F	L
Streptococcus faecalis	A, 2	L, F
Veillonella parvula	P, A	S**

* Arranged in order of descending peak area for each organism.
** Trace amount.

(previously undetected) were noted. Subsequently, shorter columns, 5 m in length were tested. These afforded a similar number of theoretical plates to a 5 ft. × 0.25 in. O.D. packed column of Chromosorb 101. However, separations could be effected in <3 min at 15 p.s.i. nitrogen and at 140°C for methyl esters (or at 130°C for free acids and neutral products). In no case would the system reported in this paper have resulted in mis-identification of any of the strains tested. Although the extraction procedure must have removed traces of high-boiling lipophilic compounds from

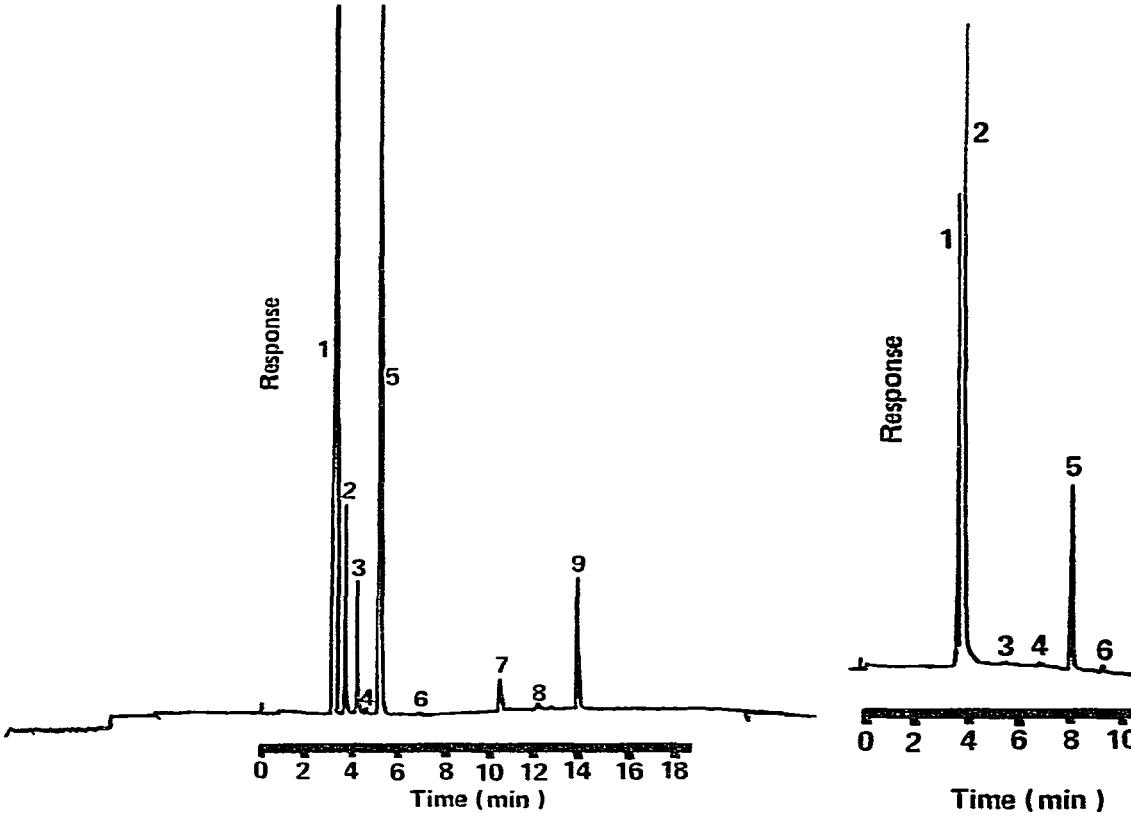

Fig 1 Ether extract of acidified supernatant of *C tetani* analysed on a 30 m × 0 25 mm I D. SP 1000 WCOT column, programmed from 110°C to 170°C at 10°/min after a 7-min hold Peaks· 1 = solvent; 2 = ethanol; 3 = 1-propanol; 4 = 2-methyl-1-propanol; 5 = 1-butanol; 6 = 1-pentanol; 7 = acetic acid; 8 = 2-methyl-propanoic acid; 9 = *n*-butyric acid

Fig 2. Methyl esters of acids of *B fragilis* analysed at 150°C isothermally on a 30 m × 0.25 mm I.D. SP 1000 WCOT column Peaks· 1 = methanol; 2 = chloroform; 3 = lactate; 4 = malonate; 5 = succinate; 6 = unknown

spent media, such compounds did not appear to interfere in the separation of more volatile substances. This may have been due to over-night reconditioning of the column. Analysis of control uninoculated media revealed the presence of low levels of acetic and lactic acids.

DISCUSSION

Perfectly adequate techniques for the gas chromatographic analysis of simple mixtures of fermentation products on packed columns, have been described elsewhere[4]. However, the time required for each analysis places a limit on the number of samples that a clinical laboratory can handle each day. On advantage of the SP 1000 WCOT column described herein is the reduction in analysis time that is possible when a shorter column is used. A second advantage is that some modern gas chromato-

graphs are designed primarily for use with capillary, rather than packed, columns. If none of the higher efficiency offered by the 30-m column is sacrified in favour of a more rapid analysis, then complete separation of isomers of fermentation products should yield additional chemotaxonomic data. The separations obtained in this study compare favourably with separations of methyl esters of acids from white Riesling wine obtained on a 20-ft. packed column[17], or a capillary column separation of acids[16]. The conditions used would not favour rapid elution of tricarboxylic acid intermediates as their methyl esters, although trimethylsilyl derivatives might well be eluted with ease[18].

Methylation with methanol and sulphuric acid[4] was preferred to other, more sophisticated, techniques. In our hands, methanol and sulphuric acid proved a simple, rapid, reliable and economic technique. All of the other esterification techniques suffer from one disadvantage or another: boron halides in methanol[19] have a very limited shelf life; boron halides in butanol are not readily available in all countries; diazomethane[20] is carcinogenic, acutely toxic and spontaneously inflammable; and thionyl chloride[21] is exceedingly corrosive and cannot form with formic acid an acyl chloride for subsequent methylation by methanol.

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