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Comparison of high-performance liquid and gas chromatography in the determination of organic acids in culture media of alkaliphilic bacteria

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ABSTRACT

Volatile and non-volatile organic acids were analysed by high-performance liquid chromatography (HPLC) on an Ammex HPX-87H, gas-solid chromatography (GSC) on a Porapak Q and gas-liquid chromatography (GLC) on a fused-silica capillary column. The results were compared by using standard acid solutions and with culture media of two strains of alkaliphilic *Bacillus*. Whereas the resolution of acids was excellent with GLC, the quantitative reproducibility was better with HPLC. Identification of complex culture mixtures was accomplished by GC-MS. Applicability of the methods for different purposes is discussed.

INTRODUCTION

The identification and determination of acidic fermentation products formed in culture media of microbes are useful for many purposes; for example, monitoring of microbial media during the industrial preparation of various food products is important for their quality control. In addition, acids formed in microbial culture media are analysed in clinical chemistry. The third main field is in studying the metabolic pathways in bacteria.

The methods used to determine acidic metabolites in the cultivation media of microbes include thin-layer (TLC) [1], gas-solid (GSC) [2], gas–liquid (GLC) [3] and high-performance liquid chromatography (HPLC) [4]. At present, GLC and HPLC are the most popular as identification and determination are more straightforward and precise than the other available methods.

The GC procedures require separate treatments of the samples for volatile and non-volatile acids. The volatile acids can be assayed in the acidified cultural media directly [2] or after extraction with an organic solvent, whereas non-volatile acids require derivatization [5–7]. With HPLC all acids can be analysed in one sample whenever the column resolution allows [8–10]. In recent years HPLC has become more widespread owing to the improved column selectivity and detector sensitivity. Analyses of short-chain acids using HPLC and packed GC columns have been compared previously [8,11]. In this work, volatile and non-volatile acids were analysed by HPLC, GSC with a packed column and GLC with a capillary column.

EXPERIMENTAL

Bacteria and culture conditions

Bacillus circulans var. *alkalophilus* (ATCC 21783) and alkaliphilic *Bacillus* sp. 17-1 (ATCC 31007) were cultivated in a basal carbonate medium containing 1% starch, 0.5% bacto-pan-

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tone (Difco, Detroit, MI, USA), 0.5% yeast extract (Difco) and mineral salts [12]. Sodium carbonate (10%) was separately sterilized and added to obtain a 1% concentration in the final medium. The medium (200 ml) was inoculated with 10 ml of preculture in a 500-ml erlenmeyer flask on a shaker at 37°C. Samples (5 ml) were taken automatically with a sampling device at intervals of 4 h [13]. Bacterial cells were removed by centrifugation and the supematants were stored at -20°C until used.

High-performance liquid chromatography

Organic acids were separated on an Aminex HPX-87H organic acid analysis column (300 mm x 7.8 mm I.D.; 9 μm particle size) (Bio-Rad Labs., Richmond, CA, USA) equipped with an Aminex HPX-87H Micro-Guard column (30 mm x 4.6 mm I.D.) and holder (Bio-Rad Labs.). The HPLC column was connected to a Waters (Milford, MA, USA) Model 510 pump equipped with a Rheodyne (Cotati, CA, USA) Model 7125 injector (20-μl sample loop) and to a Model 2142 refractive index detector (LKB, Bromma, Sweden) with a Model C-R3A integrator (Shimadzu, Kyoto, Japan). The mobile phase was 0.004 *M* sulphuric acid. The column was operated at the room temperature and the flow-rate of the mobile phase was 0.6 ml/min.

Bacterial cultures were centrifuged at 5000 g for 30 min to remove cells. A l-ml volume of supematant was transferred into a 5-ml capped tube. A 0.25-ml volume of 9.0 *M* sulphuric acid, 0.6 g of sodium chloride and 5 ml of diethyl ether were added to the tube. The samples were treated with a vortex mixer for 1 min and centrifuged at 1000 g for 5 min. The ether phases were transferred into clean glass tubes and 1.0 ml of 0.1 *M* NaOH solution was added. The ether phase was extracted with a vortex mixer and the turbid mixture was centrifuged at 1000 g for 5 min. The diethyl ether phases were then removed. The residual diethyl ether in the aqueous phase was allowed to evaporate from the open tubes overnight at room temperature [10]. The standards for HPLC and GLC were the same and they were treated in the same way as culture samples. No internal standard was necessary with HPLC.

Gas chromatography

Volatile and non-volatile acids were analysed on a fused-silica capillary column coated with nitroterephthalate-modified polyethylene glycol NB-351 (25 m x 0.32 mm I.D., $d_f = 0.2$ μm) (HNU-Nordion, Helsinki, Finland). Volatile acids were also analysed on a glass column (2.20 m x 2.0 mm I.D.) packed with Porapak Q (80-100 mesh) [2]. A Varian (Palo Alto, CA, USA) Model 3700 gas chromatograph equipped with a flame ionization detector connected to an HP 3388A integrator (Hewlett-Packard, Avondale, PA, USA) was used. The operating conditions for the capillary column were injector temperature 200°C, detector temperature 230°C and column temperature programmed from 60 to 200°C at 6°C/min. The flow-rates were 1.2 ml/min for the carrier gas (nitrogen), 300 ml/min for air and 30 ml/min for hydrogen. The splitting ratio was 50:1. The conditions for the packed column were injector temperature 230°C, detector temperature 250°C and column temperature programmed from 150 to 240°C at 10°C/min.

Volatile fatty acids were extracted into diethyl ether [6] with a slight modification. A 0.4-ml volume of 10 *M* sulphuric acid, 1 g of sodium chloride and 2 ml of diethyl ether were added to 2 ml of culture supematant. The amples were treated with a slowly rotating mixer for 30 min. Two phases were separated by centrifugation for 5 min at 1000 g and the ether layer was transferred into another tube with a Pasteur pipette. The standard mixture contained formic, acetic, propionic, butyric, valeric and caproic acid (2 mg/ml of each in water). Evaporation of the sample and the injection volume (0.5 μl) were corrected using hexadecane as an internal standard (20 μl/l of diethyl ether). Standard solutions were treated in the same way as the supematants from bacterial cultivations. Volatile acids were also analysed using a packed glass column directly from culture samples which were made acidic with oxalic acid [2].

For analysis of non-volatile acids, methanol (4 ml) and 10 *M* sulphuric acid (0.75 ml) were added to 2 ml of culture supematant or of the standard mixture [6]. The tubes were stoppered and stirred with a vortex mixer and kept at 60°C for 30 min. Then 2 g of sodium chloride, 2 ml of

water and 2 ml of chloroform were added and the tubes were blended and centrifuged. The chloroform layers were transferred into other tubes. The standard mixture contained pyruvic, oxalic, lactic, fumaric, malonic and benzoic acid (3 mg/ml of each in water). A small amount of sodium hydroxide was added to get all of the acids into solution.

Gas chromatography-mass spectrometry

The GC-MS system consisted of a VG Analytical (Wythenshawe, UK) Model 7070E organic mass spectrometer equipped with a VG Model 11-250 data system and a Dani (Monza, Italy) Model 3800 HR gas chromatograph. The capillary GC operating conditions were as above except that the programming rate was 4°C/min and the carrier gas was helium.

RESULTS

Analyses of standard mixtures

Results for standard mixtures of organic acids obtained by HPLC and GLC are given in Tables I and II. Relative retention times (*RRT* values) and response factors (*RF* values) of volatile acids are expressed with respect to caproic acid (Table I), whereas *RRT*s and *RF*s of non-volatile acids are compared with fumaric acid (Table II). The theoretical plate numbers (*N*) for the HPLC, GLC and GSC columns were calculated with the "Sigma 5" method for peaks of Gaussian shape and were 10000, 200 000-300000 and 6000-10 000, respectively.

With HPLC all volatile standard acids were separated at room temperature (Table I) with 0.004 *M* sulphuric acid as the mobile phase. However, the retention times (t_R values) were fairly long. For example, that of caproic acid was about 60 min which is three times longer than with GLC. The precision of the *RRT* values of acids with HPLC was usually very high (R.S.D. $<1\%$, $n = 5$; results not shown). Among the non-volatile acids pyruvic, malonic, lactic and fumaric acid separated as individual peaks. Oxalic acid eluted immediately after the solvent peak. and was broad compared with the other

TABLE I

RELATIVE RETENTION TIMES *(RRT)* AND RESPONSE FACTORS (RF) OF VOLATILE ACIDS WITH RESPECT TO CAPROIC ACID

The extraction recoveries are shown for HPLC. The reproducibilities of the *RF* values of HPLC and GLC analyses are expressed as the standard deviation (SD.; $n = 5$) and the relative standard deviation (R.S.D.). The concentration of each of the acids in aqueous solution was 0.2-1.0 mg/ml for HPLC and 2 mg/ml for GLC.

Acid	HPLC				GLC		
	RRT[a]	*RF*[b,c]	R.S.D. (%)	Extraction efficiency (%)	*RRT*[a]	*RF*[b,c]	R.S.D. (%)
Formic	0.24	0.33 ± 0.01	3.0	64			–
Acetic	0.27	0.48 ± 0.01	2.1	70	0.60	0.33 ± 0.01	3.0
Propionic	0.32	0.80 ± 0.23	28.8	84	0.69	0.88 ± 0.03	3.4
Butyric	0.40	0.88 ± 0.04	4.5	100	0.78	0.99 ± 0.06	6.1
Valeric	0.61	0.89 ± 0.01	1.1	100	0.89	0.91 ± 0.07	7.7
Caproic	1.00	1.00		100	1.00	1.00	

[a] *RRT* = relative retention time with respect to caproic acid.

[b] $RF = \text{response factor} = \dfrac{(\text{area})_{\text{acid}}(\text{mass})_{\text{caproic}}}{(\text{area})_{\text{caproic}}(\text{mass})_{\text{acid}}}$.

[c] ± Values are standard deviations (SD.) of five independent measurements including extractions of standard mixtures compared with caproic acid, which was assigned a value of *RF* = 1.00 in each run.

TABLE II

RELATIVE RETENTION TIMES *(RRT)* AND RESPONSE FACTORS (RF) OF NON-VOLATILE ACIDS WITH RESPECT TO FUMARIC ACID

The extraction recoveries are shown for HPLC. The reproducibilities of the RF values are expressed as in Table I. The concentration of each of the acids in aqueous solution was 3 mg/ml.

Acid	HPLC				GLC		
	RRT[a]	*RF*[b,c]	R.S.D. (%)	Extraction efficiency (%)	*RRT*[a]	*RF*[b,c]	R.S.D. (%)
Oxalic	0.42	0.69 ± 0.09	13.0	35	0.79	0.69 ± 0.05	7.2
Pyruvic	0.54	0.41 ± 0.04	9.8	47	0.55[d] 0.75[d]	0.57 ± 0.08[d]	14.0
Malonic	0.61	0.38 ± 0.01	2.6	42	0.94	0.97 ± 0.02	2.1
Lactic	0.76	0.27 ± 0.01	3.7	28	0.65	0.69 ± 0.10	14.5
Fumaric	1.00	1.00		loo	1.00	1.00	
Benzoic	–			–	1.12	0.84 ± 0.01	1.2

[a] *RRT with* respect to fumaric acid.

[b] $RF = \frac{(\text{area})_{\text{acid}}(\text{mass})_{\text{fumaric}}}{(\text{area})_{\text{fumaric}}(\text{mass})_{\text{acid}}}$

[c] Standard deviation (SD.) of five independent measurements including extractions of standard mixtures compared with fumaric acid, which was assigned a value of *RF* = 1.00 in each run.

[d] Pyruvic acid decomposed under the conditions used and yielded two peaks. The areas of the two peaks were summed and compared with fumaric acid.

standard acid peaks. Benzoic acid was not eluted from the column with 0.004 ***M*** sulphuric acid (Table II).

Volatile acids were analysed by GLC directly from diethyl ether phases of acidified samples and non-volatile acids from chloroform after derivatization with methanol–sulphuric acid. All the volatile and methyl esters of non-volatile standard acids were separated well. The acids showed sharp, symmetrical peaks with baseline resolution, except pyruvic acid, which appeared as two peaks. The ***RF*** of formic acid was near to zero when using flame ionization detection (FID). The R.S.D. (n = 5) of the ***RRT*** values was < 1% with GLC.

With GLC the ***RF*** values of volatile acids were higher than with HPLC, except for formic and acetic acid. The R.S.D.s (n = 5) of the ***RF*** values with HPLC ranged from 1.1% (valeric acid) to 4.5% (butyric acid), but for propionic acid it was 28.8% (Table I). This high R.S.D. was due to a "negative" peak near the signal from the acid. Generally, the variation of ***RF*** values of compounds other than propionic acid with GLC was higher (from 3.0 to 7.7%) than with HPLC (Table I). The ***RF*** values of methyl esters of non-volatile compounds were also higher with GLC than the ***RF*** values of the free acids with HPLC (Table II). The ***RF*** values of non-volatile compounds had more variation than volatile acids when analysed with both methods.

The amounts of extracted acids were compared with unextracted standards using HPLC (Table I). The recoveries were *ca.* 60, 70, and 80% for formic, acetic and propionic acid, respectively, and 100% for butyric, valeric and caproic acid when the concentration of each acid was 1 mg/ml or less. The extraction recovery of butyric and longer chain volatile acids decreased with increasing chain length and acid concentration. The extraction recoveries were smaller for non-volatile acids, ranging from *ca.* 30% (lactic acid) to 50% (pyruvic acid), with the exception of fumaric acid, for which the recovery

was 100%. The increased concentration did not interfere with the extraction of non-volatile compounds.

Analyses of fermentation media

GLC and HPLC results for the culture media of alkaliphilic ***Bacillus*** sp. 17-l are illustrated in Fig. 1. Acetic acid was the most abundant (Fig. 1A and B). The culture sample also contained other volatile acids such as propionic, isobutyric, butyric and isovaleric acid, detected by both GLC and HPLC (Fig. 1A and B).

The standard solution of volatile acids (Fig. 2A) and culture samples of ***Bacillus circulans*** var. ***alkalophilus*** were subjected to GSC with a column of Porapak Q (Fig. 2B). Whereas little

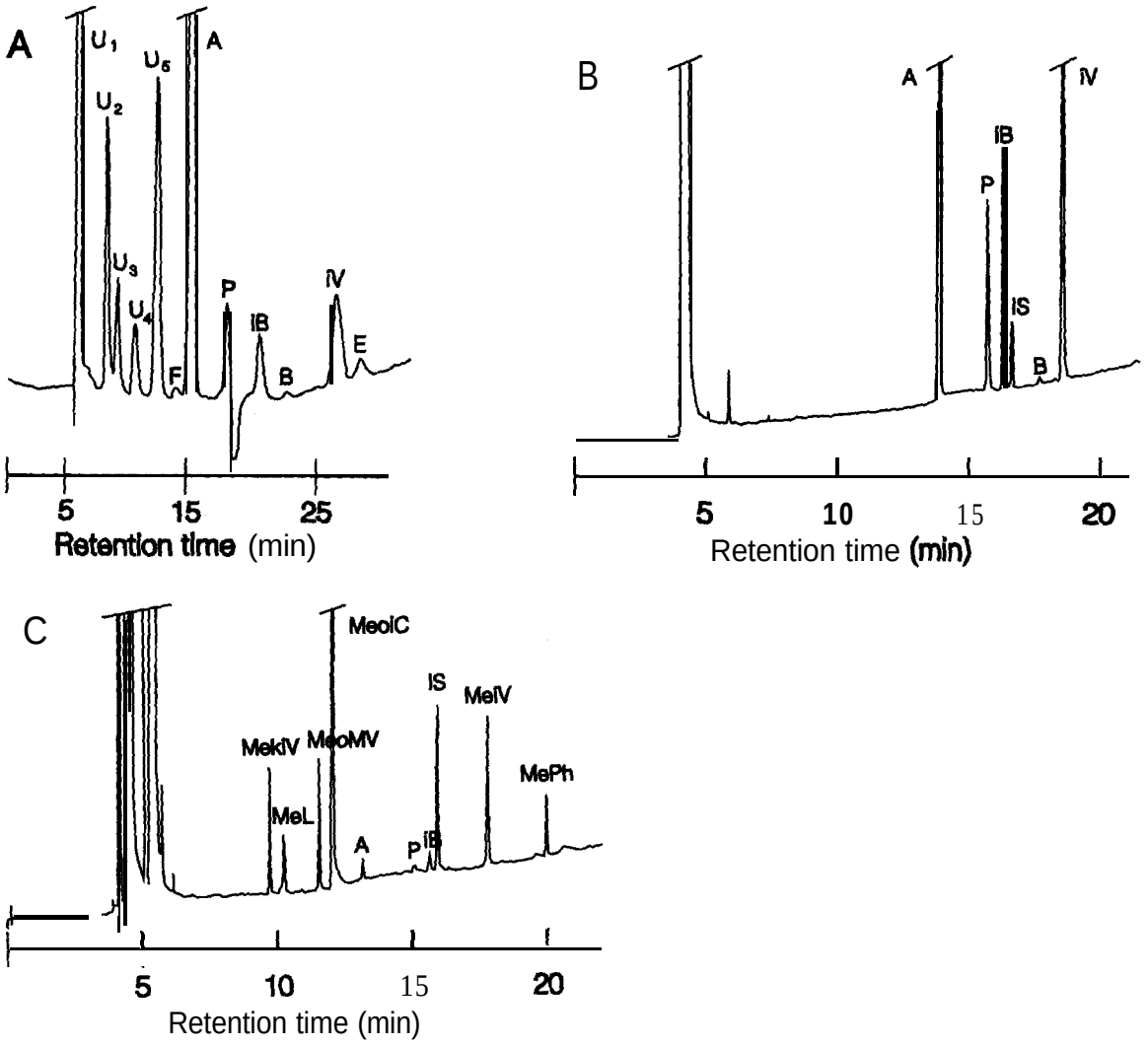

Fig. 1. Separation of short-chain acids from culture medium of alkaliphilic ***Bacillus*** sp. 1'7-1 (A) by HPLC as free acids, (B) by GLC as free acids and (C) by GLC as methyl esters. The compounds are abbreviated as follows: A = acetic acid; B = butyric acid; F = formic acid; iB = isobutyric acid; iV= isovaleric acid; MeiV= methyl isovalerate; MekiV= methyl a-ketoisovalerate; MeL = methyl lactate; MeoiC = methyl a-oxoisocaproate; MeoMV = methyl D-α-oxo-β-methylvalerate; MePh = methyl phenylacetate; IS = internal standard = hexadecane (20 μl/l); U_1–U_5 = unidentified components; E = diethyl ether. Concentrations of identified compounds (mg/ml) in selected peaks: (A) F = 0.02, A = 3.40, P = 0.30, iB = 0.20, B = 0.01, iV = 0.35; (B) A = 3.45, P = 0.27, iB = 0.24, B = 0.01, iV = 0.40; and (C) MekiV = 0.30, MeL = 0.17, MeoMV = 0.32, MeoiC = 1.50 and MePh = 0.13. Injection volumes: (A) 20; (B) 0.5; and (C) 2 μl.

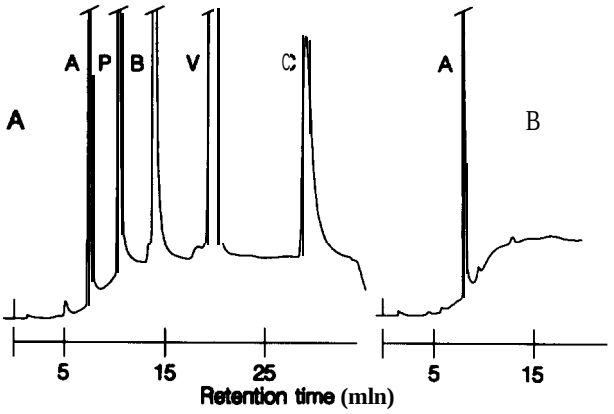

Fig. 2. GSC of (A) standard volatile short-chain acids and (B) culture medium of *Bacillus circulans var. alkalophilus* with Porapak Q. Standard solution contained (A) acetic, (P) propionic, (B) butyric, (V) valeric and (C) caproic acid. Formic acid was included in the standard mixture and the small peak (F) before acetic acid may represent it. Concentrations of the standard acids (A-C) were 2 mg/ml. The calculated concentration of A in (B) was 1.7 mg/ml. Injection volume, 1 μl.

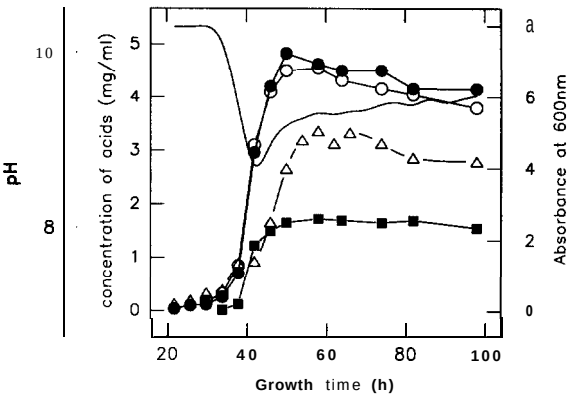

Fig. 3. Production of acetic and formic acid in cultivation medium of *Bacillus circulans* var. *alkalophilus*. Acetic acid was determined using (0) HPLC and (●) GLC and formic acid was determined using (■) HPLC. The growth (A) **and pH (line without symbols) curves are included.**

or no response was observed from formic acid, acetic acid was readily detected. Two small unknown peaks in the chromatogram after acetic acid (Fig. 2B) were already present in the uninoculated medium. Such compounds did not appear with the diethyl ether-extracted samples. Because there is no necessity for sample preparation, chromatography with Porapak Q is a valuable method when only a low resolution of volatile acids is required.

More detailed analyses of volatile and non-volatile acids of the bacterial culture media were done using GC-MS. The MS data bank system (see Experimental) was very helpful in the identification of methyl esters of unknown GLC peaks, showing the presence of α-keto-isovaleric, D-α-oxo-β-methylvaleric, α-oxoiso-caproic and phenylacetic acid (MekiV, MeoMV, MeoiC and MePh, respectively, in Fig. 1C). These acids are also produced by several anaerobic bacteria [14].

The culture media of alkaliphilic *Bacillus* sp. 17-1 contained lactic acid. Its concentration was lower when analysed by GLC (MeL in Fig. 1C) than HPLC (U, in Fig. 1A). Succinic acid eluted at the same retention time as lactic acid with HPLC. However, succinic acid did not exist in the culture medium of this bacterium as it was not detected by GLC. The large lactic acid peak in HPLC may contain other acids, e.g. oxo-acids, which were identified by GC-MS. In the HPLC trace there were four other peaks (U,, U_2, U_3 and U_4, Fig. 1A) which were not identified. Two of them (U, and U_4) eluted close to pyruvic and malonic acid, but these acids were not detected with GLC. One peak (U,) may belong to a sugar. Peak U_1 that comes with the solvent peak was much higher than the normal solvent peak and hence the media contain substances that are not separated with this column.

The kinetics of the appearance of acetic and formic acid in the culture medium with variation in pH during the growth of the alkaliphilic *Bacillus* are shown in Fig. 3. Acetic acid measured by GLC and HPLC correlated well with each other (Fig. 3). The formic acid curve was drawn according to the HPLC results because there was no FID response.

DISCUSSION

HPLC with an Aminex HPX-87H column has certain definite advantages over GLC for the separation of organic acids. All of the resolved acids can be analysed in one sample without derivatization. Filtered culture media can be

used without extraction [8], but this procedure may leave potentially interfering compounds in the samples [9]. Especially for the assay of formic acid with other readily evaporating acids and tricarboxylic acids, which are difficult to derivatize, HPLC is beneficial. In addition, the determination of carbohydrates from unextracted culture samples simultaneously with acids is possible with the Aminex-87H column when a refractive index detector is used [8]. However, some procedures are needed to decrease the t_R values or to improve the resolution of some acids. The t_R values of acids shorten on increasing the temperature of the eluent [15] or on increasing the proportion of acetonitrile from 5 to 11% for fumaric acid and certain aromatic carboxylic acids [9]. A higher column temperature (50°C) and 10% acetonitrile separated succinic and lactic acid, in addition to benzoic acid, although the retention time of the latter was still long [10].

The recovery of acids was studied in sodium hydroxide extracts of the HPLC samples (see Experimental). The recovery from standard mixtures ranged from 28% to 100% (Table I), which was higher than that reported previously [10] for diethyl ether extracts of pyruvic, lactic, acetic, propionic, butyric, valeric and caproic acid. The efficiency of extraction of volatile fatty acids increased with increasing carbon chain length when the concentration of acids was ≤ 1.0 mg/ml (Table I). When the concentration of each volatile acids was >1.0 mg/ml the extraction recovery decreased for butyric and longer chain acids. Related results were also obtained after double extractions with diethyl ether-sodium hydroxide from water [16].

The GLC samples were prepared according to the method of Drucker [6]. Other methods such as derivatization with boron trifluoride-butanol [7] have many steps and boron halides in methanol have a very limited shelf-life [6]. Methylation with methanol and sulphuric acid is a simple and rapid technique, especially for large numbers of samples. The disadvantage of the methanol-sulphuric acid method is that not all of the tricarboxylic acid-cycle acids, e.g., citric acid, are detected and two peaks appeared from pyruvic acid after derivatization. For identification of TCA-cycle acids and for avoiding multiple peak formation from double bonds or from oxo-groups, trimethylsilyl derivatization has been used [17,18].

There was no FID response to formic acid and in general methyl esters of volatile acids were difficult to analyse because of the ease of evaporation and retention near the solvent peak. For example, the boiling point of methyl formate is only 31.8°C. These problems can be overcome by using another kind of detector or by using derivatization with butanol-sulphuric acid [19].

Poor resolution can bring about not only difficulties in identification of the chromatographic peaks but also errors in quantitative results. An advantage of GLC is the extremely high resolution of modern capillary columns. In this study, both volatile and methyl esters of non-volatile acids were separated well and the retention times remained reasonable (Fig. 1B and C). It has been stated that a stationary phase of very polar character allows the resolution of the largest number of methyl esters of acids [14].

With HPLC the relatively low resolution may produce problems. Parallel tests with high-resolution GLC can exclude possible errors. For example, the absence of succinic acid (peak U,) in Fig. 1A was readily excluded with the aid of the results in Fig. 1C. Despite the generally much lower resolution of HPLC, aromatic acids are well separated with high k' values on commonly used HPLC columns [9]. For aromatic acids, an advantage of HPLC is the feasibility of utilizing the high sensitivity and selectivity of UV detectors [9]. Unfortunately, the detection of non-aromatic acids does not permit many alternatives.

An advantage of GC is the ease of the simultaneous use of MS for the identification of peaks in the chromatograms. MS combined with HPLC is a less advanced and not readily available method. However, the use of HPLC in the analysis of acids from microbial culture samples has become more common in recent years, apparently because of the facility of sample preparation and because different types of short-chain acids can be determined in a single analysis. The reproducibility of HPLC at the quantitative level is also better than that of GLC. However, when

the definite identity of various acids from bacteria is required, GC–MS is the method of choice.

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