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GAS CHROMATOGRAPHIC ANALYSIS OF BACTERIAL AMINES AS THEIR FREE BASES

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SUMMARY

Columns of Chromosorb 103, Tenax-GC, Amine 220 plus potassium hydroxide on Chromosorb W, and Carbowax 20M plus potassium hydroxide on Chromosorb W were compared for their ability to separate bacterial amines as their free bases in aqueous solution. A 1.52 m × 0.6 cm O.D. column of Chromosorb 103 separated eleven amines when operated isothermally at 185°C. A further four high-boiling amines could be separated at 240° C. The other packings separated only eight amines isothermally, except for Tenax-GC which separated seven of the free bases. Chromosorb 103 performed less well than Carbowax 20 M plus potassium hydroxide with respect to number of plates or peak resolution. The maximum number of amines separated, thirteen, required Chromosorb 103 programmed from 170° C to 230° C at 3° C min^{-1} after an initial holding time of 20 min. It was possible tentatively to identify amines in culture supernatant fluid of *Proteus mirabilis*, viz. ethylamine, isobutylamine and isoamylamine, after direct injection of culture supernatant fluid.

INTRODUCTION

The trace analysis of amines is important because they are commonly found in foodstuffs [1], tobacco leaf [2], human urine [3] and blood [4] and also in micro-organisms [5]. The production of amines by micro-organisms and their subsequent release into the growth medium has been known for many years and several mechanisms for amine production have now been described [5—7].

However, the gas chromatographic (GC) separation of complex mixtures of free amines at very low concentrations in aqueous solutions is hampered by technical difficulties associated with the analysis of highly polar solutes; for example, adsorption and decomposition of the compounds in the column,

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ghosting phenomena, badly tailed peaks and very low detector response. A common method of overcoming these problems is to convert such polar compounds to relatively non-polar derivatives that are characteristically more suitable for GC analysis. Several derivatives such as Schiff bases [8], carbethoxyamines [9], dinitrophenyl amines [10], trimethylsilylated [11] and acetylated [12] derivatives have been used for this purpose. Another successful approach has been to employ less reactive column packing materials to reduce the interaction with solutes. Examples of this approach include the use of porous polymers [13], and the deactivation of supports by treatment with alkali [14]. Wall-coated open tubular [15], and support-coated open tubular columns [12] which minimise column-solute interactions have also been used, though much less widely than packed columns. Where large numbers of samples are to be analysed, a simple and rapid technique is required. Therefore, the object of the present study was to compare various analytical conditions and column packings to determine the optimum GC parameters for analysis of complex mixtures of bacterial amines as their free bases, in aqueous solution.

EXPERIMENTAL

Reagents

Methylamine, ethylamine, isopropylamine and *n*-propylamine were the gift of W. Goddard. All other amines were purchased from BDH (Liverpool, Great Britain) and were analytical-grade reagents. Glass columns, liquid phases and supports were obtained from Phase Separations (Clwyd, Great Britain).

Column packings

The following packings were prepared and evaluated; 10% (w/w) Amine 220 plus 10% (w/w) potassium hydroxide, 10% (w/w) Carbowax 20M plus 2% (w/w) potassium hydroxide, both coated onto Chromosorb W acid washed DMCS treated support (85—100 mesh). The porous polymers, Tenax-GC and Chromosorb 103, were used as supplied.

All packings were tested as packed glass columns of 1.52 m × 0.6 cm O.D. For potassium hydroxide packings, the support was treated with aqueous alkali prior to treatment with a solution of organic stationary phase in methanol. All columns were conditioned by heating at 8°C min^{-1} to 20°C below the maximum recommended temperature for the stationary phase, then held at that temperature with the carrier gas flowing through the column for 24 h. An exception was Amine 220 plus potassium hydroxide which was heated at 2°C min^{-1} to 160°C.

Bacterial cultures

In order to confirm the applicability of various techniques to the analysis of bacterial amines, actual cultures were examined. *Proteus mirabilis* was grown in 50 ml broth under an atmosphere of carbon dioxide at 35°C for 24 h. After centrifugation at 3000 *g* for 15 min, the culture supernatant fluid was made basic with 1.5 g potassium carbonate and 0.1 ml 10 *N* sodium hydroxide. GC analysis was by direct injection of 5-μl samples onto the column being tested.

GC analysis

Initially all packings were tested isothermally using the following oven temperatures: Amine 220 plus potassium hydroxide, 60°C and 100°C; Carbowax 20M plus potassium hydroxide, 60°C and 150°C; Chromosorb 103, 185°C and 240°C; Tenax-GC, 120°C and 165°C.

Subsequently, the most satisfactory packing (see Results), Chromosorb 103, was evaluated in dual 2.74 m × 0.6 cm O.D. columns heated from 170°C to 230°C at 3°C min^{-1} after an initial holding time of 20 min.

All analyses were performed in a PYE 104 gas chromatograph equipped with dual flame ionization detectors. Peak times and areas were recorded by a minigrator computing integrator (Spectra-Physics, Luton, Great Britain). Gas chromatograms were recorded using a 10-mV f.s.d. W + W flat bed chart recorder operated at 30 cm h^{-1}. Gas flow-rates were as follows: air, 600 ml min^{-1}; hydrogen, 40 ml min^{-1}, nitrogen, 45 ml min^{-1}.

Methods for evaluation and comparison of columns

The evaluation of gas chromatograms was carried out by determining the following (1) number of theoretical plates; (2) peak resolution factors; (3) maximum number of amines resolved in an isothermal analysis; (4) total analysis time per elution temperature, with regard to the maximum and minimum operating temperatures of the liquid phases.

TABLE I

COMPARISON OF COLUMN PACKINGS FOR ANALYSIS OF BACTERIAL AMINES

Column packing	Operating temperature* (°C)	Maximum numbers of amines separated	Number of plates per meter**	Peak resolution***
10% (w/w) Amine 220 plus 10% (w/w) potassium hydroxide	60 100	8 3	450	2.0
10% (w/w) Carbowax 20M plus 2% (w/w) potassium hydroxide	60 150	8 5	850	2.0
Chromosorb 103	185 240	11 4	650	1.6
Tenax-GC	120 165	7 5	450	1.4
Chromosorb 103 (temperature programmed)	At 170°C for 20 min then to 230°C at 3°C/min, final holding time of 5 min	13	—	—

*Isothermal, except for temperature programmed Chromosorb 103. Higher temperatures were used to separate only the less volatile amines.

**Calculated as the mean number of plates for all peaks.

***Calculated as the mean resolution for all peak pairs.

TABLE II

RETENTION DATA RELATIVE TO *n*-BUTYLAMINE FOR AMINES ANALYSED AS FREE BASES IN AQUEOUS SOLUTION

Amine	Synonym	Column packings				
		Amine 220 plus potassium hydroxide (60°C isothermal)	Carbowax 20M plus potassium hydroxide hydroxide (60°C isothermal)	Tenax GC (120°C isothermal)	Chromosorb 103	
					185°C isothermal	Temperature programmed from 170 to 230°C (see Table I)
Methylamine	Aminomethane	0.18	0.29	–	0.19	0.18
Ethylamine	Aminoethane	0.26	0.36	0.14	0.29	0.25
Isopropylamine	2-Aminopropane	–	–	–	0.42	0.37
n-Propylamine	1-Aminopropane	0.45	0.55	0.36	0.54	0.51
tert.-Butylamine	–	–	–	0.35	0.54	–
sec.-Butylamine	–	–	–	0.63	0.84	0.75
Isobutylamine	–	0.69	0.74	0.64	0.84	0.82
n-Butylamine	1-Aminobutane	1.00	1.00	1.00	1.00	1.00
Triethylamine	–	–	–	–	–	1.32
2-Methylbutylamine	–	–	–	–	–	1.69
Isoamylamine	3-Methylbutylamine	1.68	1.51	1.26	1.65	–
n-Amylamine	Pentylamine	2.33	1.74	2.08	1.87	1.93
Pyrrolidine	Tetrahydropyrrole	1.90	1.98	2.51*	1.36	–
n-Hexylamine	1-Aminohexane	–	–	–	–	3.00
Ethanolamine	1-Aminoethanol	–	–	2.54*	2.19	–
1,3-Diaminopropane	1,3-Propanediamine	–	–	–	2.66	2.80
Di-*n*-butylamine	–	–	–	–	–	3.78
Putrescine	1,4-Diaminobutane	–	–	–	–	3.78
Cadaverine	1,5-Diaminopentane	–	–	–	–	4.72

*These two amines are not resolved.

RESULTS

The results of comparing different column packings, with respect to analysis of free amines in aqueous solution, are shown in Table I.

Resolution could be improved by use of lower temperatures but only at the expense of peak broadening and longer analysis time under isothermal conditions. The data in Table I, therefore, represent a compromise. On the basis of the isothermal tests, Chromosorb 103 proved the most satisfactory packing since it permitted adequate separation of eleven amines at 185°C; also it did not suffer from the ghosting observed with alkali-washed support-packings. When Chromosorb 103 was used as the packing in 2.74 × 0.6 cm O.D. columns with temperature programming, the number of amines resolved was increased to thirteen. Individual retention data relative to *n*-butylamine are presented in Table II. A separation of thirteen standard amines as their free bases by temperature programmed GC on Chromosorb 103 is depicted in Fig. 1. An additional two bases, putrescine and cadaverine, could be separated provided that di-*n*-butylamine was excluded from the standard mixture (Table II). The use of longer columns resulted in increased analysis time which could not be reduced by use of a higher final temperature owing to excessive column bleed. The application of the method to a culture of *Proteus mirabilis* yielded the chromatogram shown in Fig. 2. Peaks were tentatively identified as ethylamine, isobutylamine and isoamylamine with the largest peak having the retention time of the neutral fermentation end-product, ethanol. Fig. 3 shows a gas

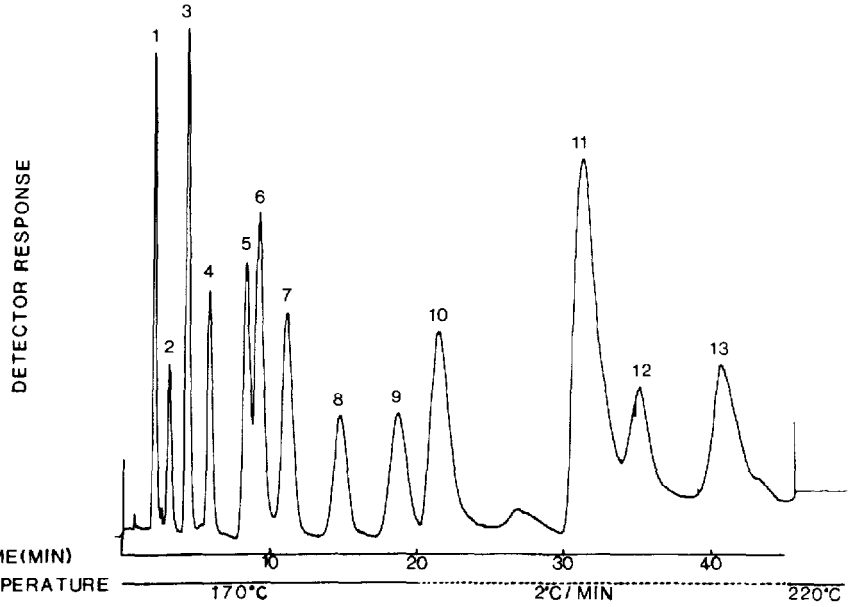

Fig. 1. Thirteen amines separated as their free bases in aqueous solution by analysis on 2.74 m × 0.6 cm O.D. columns of Chromosorb 103 using temperature programming. Peaks: 1 = methylamine; 2 = ethylamine; 3 = isopropylamine; 4 = *n*-propylamine; 5 = *sec*-butylamine; 6 = isobutylamine; 7 = *n*-butylamine; 8 = triethylamine; 9 = 2-methylbutylamine; 10 = *n*-amylamine; 11 = 1,3-diaminopropane; 12 = *n*-hexylamine. 13 = di-*n*-butylamine. Data for a separation programmed at 3° C/min are shown in Table II.

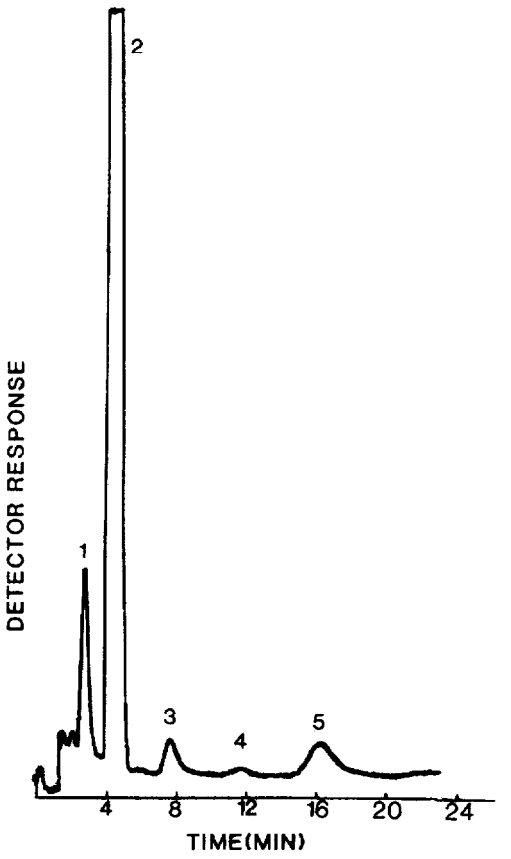

Fig. 2. Amines of *Proteus mirabilis* analysed as their free bases on a column of Chromosorb 103 (1.52 × 0.6 cm O.D.), isothermally at 165°C. Peaks: 1 = ethylamine; 2 = ethanol; 3 = isobutylamine; 4 = unknown peak; 5 = isoamylamine.

chromatogram for a mixture of standards of ethylamine, ethanol, isobutylamine, isoamylamine and *n*-amylamine added to un-inoculated culture medium. No such peaks were observed when an un-inoculated medium was analysed as a negative control.

DISCUSSION

The occurrence of amines in a number of micro-organisms has led to the suggestion that their analysis in head-space gas samples might assist microbial identification [13]. However, the lack of really satisfactory methods for analysis of amines has delayed detailed taxonomic studies on bacterial amines despite the far-sighted studies of Brooks et al. [16] who separated thirteen amines after extraction and formation of their less-polar HFBA derivatives. In a previous study, Dunn et al. [17] assessed the performance of ten column packings for the separation of a small number of primary, secondary and tertiary amines. In their hands, Chromosorb 103 proved "inconsistent" and "difficult to pack". This porous polymer does tend to expand on heating,

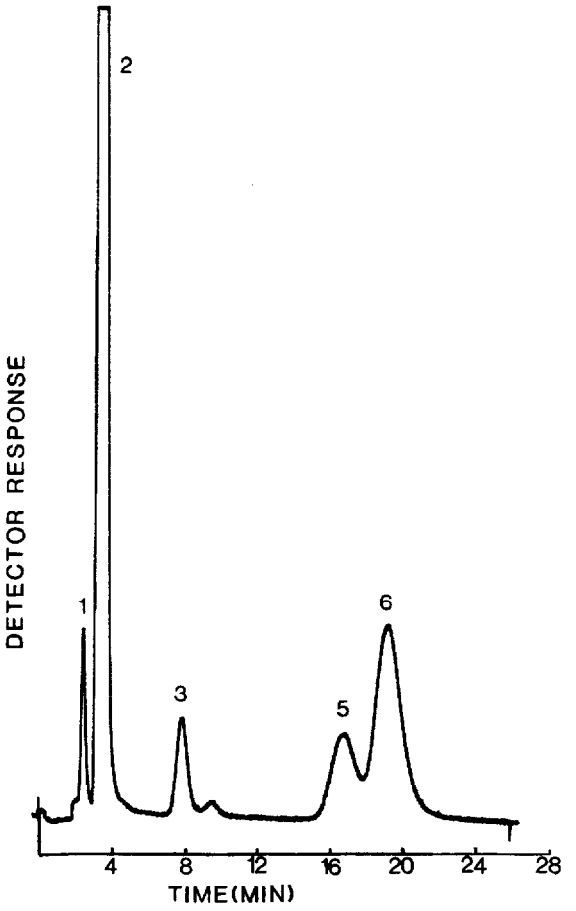

Fig. 3. Standard gas chromatogram. Ethanol and free amines analysed on Chromosorb 103 at 165°C. Peaks: 1 = ethylamine; 2 = ethanol; 3 = isobutylamine; 5 = isoamylamine; 6 = *n*-amylamine.

leaving gaps in the column upon cooling. This effect was minimised in the present investigation by paying scrupulous attention to packing of columns. Whereas Amine 220 plus potassium hydroxide was excellent for separating primary, secondary and tertiary amines in the Dunn et al. [17] study, it proved less satisfactory for the purpose of separating a large number of bacterial amines. Direct analysis of free amines in aqueous samples offers the advantages of simplicity and rapidity of analysis since it obviates the need for complex and time-consuming extraction and derivatization procedures. Results of the present study indicate that although the tailing of amines is largely reduced on the packings tested, the number of amines separated remains relatively low. The ghost peaks observed with alkaline supports have been described by other workers [18]. The phenomenon of ghosting complicated peak identification. The major disadvantage of alkali-washed packings lies in the thermal instability of the liquid phases tested which prevents the temperature programmed analysis that is required for the complete separation of complex mixtures of bacterial amines. Nevertheless, alkaline packings seemed useful for the separa-

tion of homologues of simple aliphatic amines, in agreement with other studies [19]. When amines of *Proteus mirabilis* were analysed isothermally on Chromosorb 103, ethylamine was found in addition to the two amines previously reported [13] when a head space gas sample was analysed. The separation of putrescine and cadaverine from the other amines tested is of particular significance. The diamines are commonly found in cultures of Gram-negative bacteria where they are believed to be synthesized by direct decarboxylation of ornithine and lysine respectively [20]. Putrescine may also be formed by decarboxylation of L-arginine to agmatine, followed by the cleavage of the resultant agmatine by the enzyme urea hydrolase [21]. It has been suggested that putrescine along with other polyamines may participate in regulatory mechanisms involved in transcription and cell division [22]. The diamines are reportedly present in dental plaque [23] and the urine of cancer patients [24]. The techniques described in this study permit the direct analysis of bacterial cultures and body fluids for thirteen amines including putrescine, cadaverine and other urinary monoamines.

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