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VOLATILE PRODUCTS FROM ACETYLCHOLINE AS MARKERS IN THE RAPID URINE TEST USING HEAD-SPACE GAS—LIQUID CHROMATOGRAPHY

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SUMMARY

Head-space gas—liquid chromatographic analyses of cultures of all *Proteus* spp. showed that the production of trimethylamine from acetylcholine could be the marker for the detection in 3.5 h of significant numbers of *P. mirabilis*, *P. vulgaris*, *P. rettgeri* and *P. inconstans A* in the rapid test for *Escherichia* and *Klebsiella* spp. in urine specimens. Trimethylamine was not detected in cultures of five other urinary pathogens. Six of fifteen strains of *K. aerogenes* produced trimethylamine from acetylcholine but were distinguished from *Proteus* spp. by ethanol production from arabinose. Ethyl acetate was produced from acetylcholine by *P. mirabilis*, *P. vulgaris*, *P. rettgeri*, *P. inconstans A*, *P. inconstans B*, *E. coli*, *K. aerogenes* and *Streptococcus faecalis*.

INTRODUCTION

Gas—liquid chromatography (GLC) for the detection of bacterial growth is notable for its high information content. A single analysis can detect several volatile compounds and provide information both about the identity and the

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quantity of each compound. These features of GLC have been used to develop a test by head-space GLC (HS-GLC) for the rapid detection of significant numbers, viz. $\geqslant 10^5$ colony-forming units (CFU) per ml [1] of *Escherichia, Klebsiella, Citrobacter* and *Proteus* spp. in urine specimens [2].

The HS-GLC rapid urine test requires two cultures, one with limited and one with ample aeration, from each urine specimen. Limited aeration in unshaken cultures is required for the production from arabinose of ethanol, the volatile compound (marker) detected by HS-GLC that indicates *Escherichia, Klebsiella* and *Citrobacter* spp. Ample aeration in shaken cultures is required for the production from methionine of methyl mercaptan, the marker that indicates *Proteus* spp. The rapid test would be greatly simplified if there were markers for *Escherichia, Klebsiella, Citrobacter* and *Proteus* spp. that were produced in one urine culture, either unshaken or shaken. Materials, labour and total analysis times for the test would be halved.

Trimethylamine is found in unshaken cultures of urine specimens containing *Proteus* spp. [2]. The present investigation was undertaken to determine whether, in the HS-GLC rapid urine test, trimethylamine could be the marker for *Proteus* spp. and ethanol the marker for *Escherichia, Klebsiella* and *Citrobacter* spp. in a single unshaken urine culture.

Substrates were tested for trimethylamine production by *Proteus* spp. and one was selected for further investigation. Trimethylamine production from the substrate was assessed under the conditions of the HS-GLC rapid urine test and its specificity for *Proteus* spp. was determined. A trial of the HS-GLC rapid urine test was made with trimethylamine as the marker for *Proteus* spp. in a single unshaken culture of each urine specimen.

EXPERIMENTAL

Gas chromatography—mass spectrometry

Head-space samples were prepared by adding 2 ml of each liquid for analysis to 3 g potassium carbonate in a 9-ml glass vial which was immediately sealed with a rubber stopper and aluminium cap, shaken on a Vortex mixer to disperse the salt in the liquid and held in a water bath at 60° C for 5 min. While the vial remained in the water bath, 0.3 ml of the supernatant vapour was removed with a gas-tight syringe (Scientific Glass Engineering, No. 500-RN-GSG) which had been heated to 60° C, and immediately injected into the gas chromatograph.

The gas chromatograph was a Varian Aerograph Series 2400 with dual flame ionization detectors and two stainless-steel columns, 2 m × 3 mm, packed with 0.4% Carbowax 1500 on graphite 60—80 mesh. The oven temperature was 110° C and the injector and detector temperatures were 160° C. Nitrogen carrier gas flow-rate was 12 ml/min, hydrogen 30 ml/min and air 300 ml/min. The retention times (t_R) of trimethylamine, ethanol, ethyl acetate and *n*-propanol were 1.00, 0.75, 2.65 and 1.50 min, respectively.

Aqueous solutions of ethanol and trimethylamine hydrochloride were added to potassium carbonate in glass vials and analysed with each day's samples to check t_R for the identification of products from cultures. The concentrations of ethanol and trimethylamine in cultures necessary for a response of twice the

noise level due to volatile compounds in the culture medium were 0.22 m*M* and 0.10 m*M*, respectively.

The mass spectrometer was a 300-mm radius 60° magnetic deflection instrument coupled to the gas chromatograph by a Watson—Bieman frit [3]. Trimethylamine, ethanol, ethyl acetate and *n*-propanol were identified by comparing their mass spectra with a reference library file [4].

Bacteria, culture media and incubation conditions

The identities of 60 strains of *Proteus* spp. including 10 each of *P. mirabilis*, *P. vulgaris*, *P. morganii*, *P. rettgeri*, *P. inconstans A* and *P. inconstans B* and of 65 strains of other bacteria that may cause urinary tract infections including 10 *E. coli*, 15 *K. aerogenes*, 10 *Streptococcus faecalis*, 10 *Staphylococcus epidermidis*, 10 *S. aureus* and 10 *Pseudomonas aeruginosa* were confirmed by standard tests [5].

A basal yeast-extract peptone medium concentrate was prepared by dissolving 3.3 g Proteose peptone (Difco) and 1 g yeast extract (Oxoid) in 100 ml of 0.33 *M* sodium phosphate buffer pH 7.2, dispensing in sterile screw-capped bottles of 28 ml capacity, autoclaving at 115° C for 15 min and storing at 4° C. Aqueous enrichment solutions (10%) of acetylcholine chloride, choline chloride, phosphatidyl choline, betaine hydrochloride and L-arabinose were sterilized by membrane filtration and stored at 4° C. Within 24 h of use, enriched media were prepared by mixing 1 part of each appropriate enrichment solution with 3 parts of basal medium concentrate and making up the volume to 10 parts with sterile distilled water. The final concentrations of ingredients were the same as those in the medium for the HS-GLC rapid test [2]. Unenriched medium was prepared by mixing 3 parts of basal medium concentrate with 7 parts of sterile distilled water. Media were dispensed in 2.5-ml aliquots in sterile screw-capped bottles of 28 ml capacity.

Media were inoculated, usually with one drop (0.025 ml) of an overnight broth culture of the bacterium under test and incubated at 37° C. Three degrees of aeration were tested. Two degrees of limited aeration were provided in unshaken cultures by incubation either standing upright (minimum aeration) or sloping at an angle of 12° from the horizontal. Ample aeration was provided in cultures incubated standing in a shaker operating at 200 horizontal 20-mm oscillations per min.

Inocula of known numbers of bacteria were counted as CFU per ml by the method of Miles and Misra [6].

Specimens of urine and method of HS-GLC rapid test

Specimens of urine, stored at 4° C, were selected as containing $\geqslant 10^5$ CFU bacteria per ml on the basis of routine laboratory analysis results [2] available on the day after voiding. The urine specimens were then examined by the method of the HS-GLC rapid test and re-examined in parallel by routine laboratory analysis.

Excepting that arabinose acetylcholine medium replaced arabinose methionine medium, urine cultures for the HS-GLC rapid test were inoculated as described previously [2], and incubated standing unshaken for 3.5 h. HS-GLC analysis of urine cultures using a gas chromatograph with an automatic

head-space injector (Perkin-Elmer F45) was carried out as described previously [2]. The t_R values were shorter than in the gas chromatograph used for manual injections viz. trimethylamine 0.45 min, ethanol 0.325 min, ethyl acetate 1.125 min and *n*-propanol 0.60 min.

RESULTS

Substrates for trimethylamine production

Acetylcholine, choline, phosphatidyl choline and betaine were selected for trial on the basis of chemical structure.

In media enriched with acetylcholine chloride or choline chloride, incubated standing unshaken for 3.5 h, trimethylamine was detected by HS-GLC in large amounts in cultures of *P. mirabilis*, *P. vulgaris*, *P. rettgeri* and *P. inconstans A* and detected in only small amounts in uninoculated media incubated in parallel. Trimethylamine was not detected in cultures of *P. morganii* and *P. inconstans B* in larger amounts than in incubated uninoculated media. Analysis of uninoculated media enriched with phosphatidyl choline or betaine hydrochloride incubated standing unshaken for 3.5 h showed large amounts of trimethylamine.

The spontaneous decomposition of phosphatidyl choline and betaine made them unsuitable for use in the HS-GLC rapid urine test. Acetylcholine and choline appeared satisfactory and acetylcholine was selected for further investigation.

Media enriched with 0.1%, 0.5%, 1%, 2.5% and 5% acetylcholine chloride were inoculated with all species of *Proteus*, incubated standing unshaken for 3.5 h and analysed by HS-GLC. Medium enriched with 1% acetylcholine chloride gave the highest yields of trimethylamine from *P. mirabilis*, *P. vulgaris*, *P. rettgeri* and *P. inconstans A*. Even 5% acetylcholine chloride did not stimulate the production of trimethylamine by *P. morganii* and *P. inconstans B*. Medium containing 1% acetylcholine chloride was used in all subsequent tests.

Trimethylamine production under the conditions of the HS-GLC rapid urine test

Experiments were done to determine whether trimethylamine was produced by *Proteus* spp. from acetylcholine in the same medium incubated under the same conditions as used for the production from arabinose of ethanol, the marker for *Escherichia*, *Klebsiella* and *Citrobacter* spp.

In unshaken cultures, either standing or sloping, of three strains of *P. mirabilis* in acetylcholine medium incubated for 3.5 h the yields of trimethylamine were 20—30 times the yields in similar shaken cultures. Standing cultures (minimum aeration) gave the highest yields. Therefore, as with ethanol, limited aeration was best for the production of trimethylamine.

The period of incubation of cultures in the rapid urine test is chosen to be long enough for the detection of markers if there are significant numbers of bacteria in the urine specimen, viz. 10^5 CFU per ml urine [1], but not long enough for the detection of markers if there are fewer bacteria in the urine specimen. Acetylcholine media were inoculated with 10^4, 10^5 and 10^6 CFU *P. mirabilis* per ml medium, incubated standing unshaken and analysed by

HS-GLC at hourly intervals up to 6 h. Trimethylamine was detected in cultures with inocula of 10^6 CFU per ml after 3 h, with inocula of 10^5 CFU per ml between 3 and 4 h and with inocula of 10^4 CFU per ml after 4 h. Therefore, as with *Escherichia*, *Klebsiella* and *Citrobacter* spp., an incubation period of 3.5 h appeared to be suitable for the detection of the equivalent of significant numbers of *P. mirabilis* in urine specimens.

Specificity of trimethylamine production from acetylcholine

The distribution of the production of trimethylamine and/or other volatile compounds among *Proteus* spp. and among other species of bacteria that may cause urinary tract infections was determined.

Volatile compounds produced by all species of Proteus. Cultures of 10 strains of each species of *Proteus* in unenriched medium and in acetylcholine medium were incubated standing unshaken for 3.5 h and analysed by HS-GLC. Results from inoculated media were corrected by subtraction of trace amounts of ethanol and trimethylamine detected in uninoculated media incubated in parallel with cultures.

The results are shown in Table I. Trimethylamine and ethanol were produced by all strains of *P. mirabilis*, *P. vulgaris* and *P. rettgeri* in unenriched medium and in larger amounts in acetylcholine medium. Ethyl acetate was produced by all strains of the same three species in acetylcholine medium but not in unenriched medium. Trimethylamine, ethanol and ethyl acetate were produced similarly by *P. inconstans A* but in smaller amounts than by the other three species, an 8-times more sensitive setting of the gas chromatograph amplifier being required for their detection. These smaller amounts would be sufficient to detect *P. inconstans A* in urine specimens because the inocula gave concentrations of approximately 10^5 CFU per ml culture medium, the lowest limit of a significant count. The results showed that trimethylamine could be the primary marker for *P. mirabilis*, *P. vulgaris*, *P. rettgeri* and *P. inconstans A* with ethanol and ethyl acetate as secondary markers under the conditions of the HS-GLC rapid urine test.

Ethyl acetate was produced by all strains of *P. inconstans B* in amounts similar to *P. inconstans A* in acetylcholine medium and not in unenriched medium.

No volatile product was detected in cultures of all strains of *P. morganii*.

Volatile compounds produced by other bacteria that may cause urinary tract infections. Cultures of at least 10 strains of *E. coli*, *K. aerogenes*, *S. faecalis*, *S. epidermidis*, *S. aureus* and *P. aeruginosa* in arabinose medium and arabinose acetylcholine medium were incubated standing unshaken for 3.5 h and analysed by HS-GLC. Cultures of 10 strains of *P. mirabilis* were included for comparison. The results were corrected by subtraction of trace amounts of ethanol and trimethylamine in incubated uninoculated media.

The results are shown in Table II. The substrates for markers, acetylcholine and arabinose, were mutually compatible in one culture medium. In arabinose acetylcholine medium, arabinose did not interfere with the production of trimethylamine by all strains of *P. mirabilis*, and acetylcholine did not interfere with the production of ethanol by all strains of *K. aerogenes* and *E. coli*.

Trimethylamine was produced by 6 of the 15 strains of *K. aerogenes* but the

TABLE I

VOLATILE COMPOUNDS PRODUCED BY *PROTEUS* SPP. IN UNENRICHED MEDIUM AND ACETYLCHOLINE MEDIUM

Results of HS-GLC analysis after incubation standing unshaken for 3.5 h.

Proteus species (No. of strains)	Amplifier attenuation	Trimethylamine		Ethanol		Ethyl acetate	
		Unenriched medium	Acetylcholine medium	Unenriched medium	Acetylcholine medium	Unenriched medium	Acetylcholine medium
P. mirabilis (10)	× 8	28*	150	5	20	0	7.2
		12—30**	93—254	2—7	11—24		2.1—9
P. vulgaris (10)	× 8	8	73	3	10	0	2.6
		2.5—25	16—142	2—5	6—14		0.8—4.9
P. rettgeri (10)	× 8	2.5	16	0.8	2.3	0	0.5
		0.2—12	5—53	0.5—1.5	1.4—4.8		0.2—1.2
P. inconstans A (10)	× 1	3	13	0.7	2.1	0	0.5
		0.2—10	4.3—45	0.3—2	2.1—4.7		0.2—1.9
P. inconstans B (10)	× 1	0	0	0	0	0	0.8
							0.1—1.4
P. morganii (10)	× 1	0	0	0	0	0	0

*Median of peak areas in mm^2.
**Range of peak areas in mm^2.

TABLE II

VOLATILE COMPOUNDS PRODUCED BY URINARY PATHOGENS IN ARABINOSE MEDIUM (AM) AND ARABINOSE ACETYLCHOLINE MEDIUM (AAM)

Results of HS-GLC analysis after incubation standing unshaken for 3.5 h.

Bacterial species (No. of strains)	Amplifier attenuation	Trimethylamine		Ethanol		Ethyl acetate		*n*-Propanol	
		AM	AAM	AM	AAM	AM	AAM	AM	AAM
P. mirabilis (10)	× 8	6*	108	10	20	0	6	0	0
		2—12**	73—150	4—18	16—33		4.5—13		
K. aerogenes (6)	× 8	2	48	49	48	0	15	0	0
		0—3	5—173	9—155	9—152		3—79		
K. aerogenes (9)	× 8	0	0	100	101	0	32	0	0
				35—130	34—126		9—43		
S. faecalis (10)	× 1	0	0	23	15	0	1.8	0	0
				19—24	7—17		0.8—2.2		
E. coli (10)	× 8	0	0	90	83	0	24	7	4.5
				66—135	56—110		14—30	1—12	1—8
S. epidermidis (10)	× 1	0	0	0	0	0	0	0	0
S. aureus (10)	× 1	0	0	0	0	0	0	0	0
P. aeruginosa (10)	× 1	0	0	0	0	0	0	0	0

*Median of peak areas in mm^2.
**Range of peak areas in mm^2.

amount of ethanol relative to trimethylamine was larger than for *Proteus* spp. Trimethylamine-producing strains of *K. aerogenes*, with ethanol peak areas of size similar to trimethylamine peak areas, were readily distinguishable from *P. mirabilis* with small ethanol and large trimethylamine peak areas.

Trimethylamine was not produced by 9 of the 15 strains of *K. aerogenes*, nor by *S. faecalis*, *E. coli*, *S. epidermidis*, *S. aureus* or *P. aeruginosa*.

Ethanol was produced by all strains of *S. faecalis* in both arabinose medium and arabinose acetylcholine medium. The amounts were small relative to *E. coli* and *K. aerogenes*, requiring an 8-times more sensitive setting of the gas chromatograph amplifier for their detection.

Ethyl acetate was produced by all strains of *P. mirabilis*, *K. aerogenes* and *E. coli* and, in trace amounts, by *S. faecalis* in arabinose acetylcholine medium but not in arabinose medium.

n-Propanol was produced by all strains of *E. coli* in both arabinose medium and arabinose acetylcholine medium as previously reported [7].

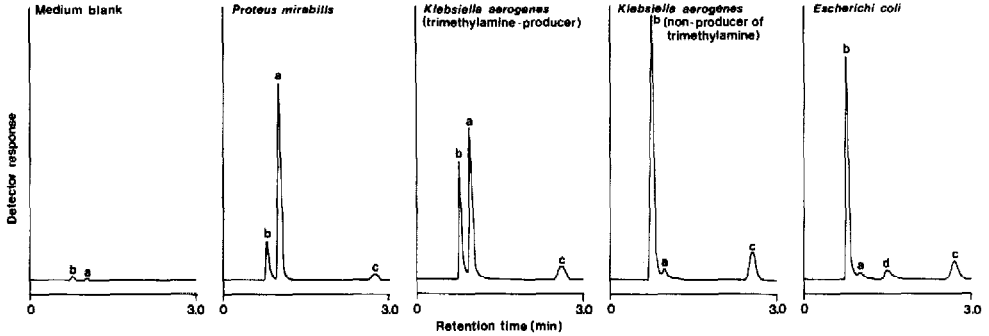

Fig. 1. Chromatograms of medium blank and of cultures of *P. mirabilis*, *K. aerogenes* (trimethylamine-producer), *K. aerogenes* (non-producer of trimethylamine) and *E. coli* in arabinose acetylcholine medium incubated standing unshaken for 3.5 h. Amplifier attenuation × 8, i.e. one-eighth the sensitivity in Fig. 2. Peaks: a = trimethylamine, b = ethanol, c = ethyl acetate, d = *n*-propanol.

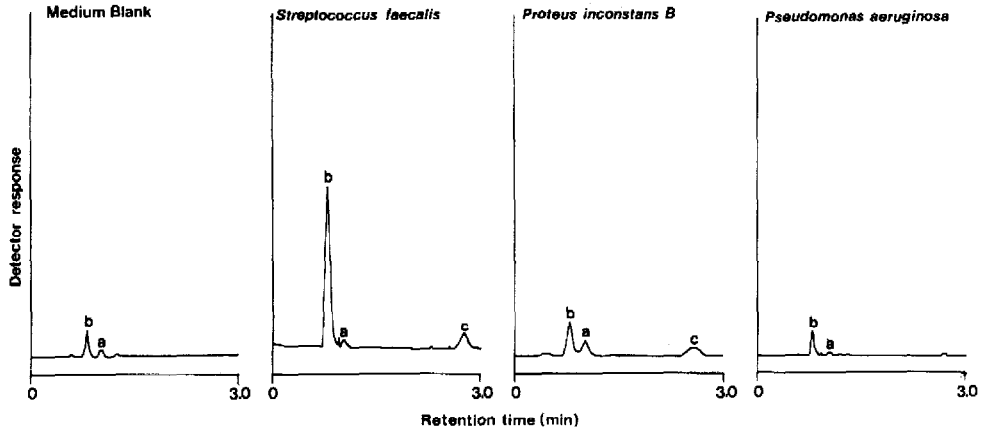

Fig. 2. Chromatograms of medium blank and of cultures of *S. faecalis*, *P. inconstans B* and *P. aeruginosa* in arabinose acetylcholine medium incubated standing unshaken for 3.5 h. Amplifier attenuation × 1, i.e. eight times the sensitivity in Fig. 1. Peaks: a = trimethylamine, b = ethanol, c = ethyl acetate.

It was possible to distinguish six different types of chromatogram from cultures in arabinose acetylcholine medium compared with incubated uninoculated medium (Medium blanks). The chromatograms illustrated in the figures, are characteristic of

(A) *P. mirabilis*, *P. vulgaris*, *P. rettgeri* and *P. inconstans A* (Fig. 1) with trimethylamine, ethanol and ethyl acetate peaks, the ethanol peak being only a fraction of the trimethylamine peak,

(B) *K. aerogenes*, trimethylamine-producer (Fig. 1) with trimethylamine, ethanol and ethyl acetate peaks, the ethanol and trimethylamine peaks being of similar size,

(C) *K. aerogenes*, non-producer of trimethylamine (Fig. 1) and *S. faecalis* (Fig. 2) with ethanol and ethyl acetate peaks,

(D) *E. coli* (Fig. 1) with ethanol, ethyl acetate and *n*-propanol peaks,

(E) *P. inconstans B* (Fig. 2) with an ethyl acetate peak and

(F) *P. morganii*, *S. epidermidis*, *S. aureus* and *P. aeruginosa* (Fig. 2) with no volatile products other than those in the medium blank.

Trial of HS-GLC rapid urine test using arabinose acetylcholine medium

Specimens of urine (35) were examined by routine laboratory analysis, which was regarded as the standard of correct diagnosis, and by the HS-GLC rapid urine test using a single culture in arabinose acetylcholine medium.

The results of HS-GLC analysis of urine cultures were corrected for volatile

TABLE III

TRIAL OF HS-GLC RAPID URINE TEST USING SINGLE CULTURES OF URINE SPECIMENS IN ARABINOSE ACETYLCHOLINE MEDIUM

Results of HS-GLC analysis after incubation standing unshaken for 3.5 h.

Routine laboratory analysis result*	No. of specimens	HS-GLC rapid test result				
		Trimethyl-amine	Ethanol	Ethyl acetate	*n*-Propanol	Chromatogram type
P. mirabilis	6	113** 23—254***	12 6—17	13 1.5—32	0	A
K. aerogenes	6	4.5 0.5—6	16 2.7—32	2.4 0.3—5.3	0	B
K. aerogenes	2	0	0.4, 4.6	0.1, 0.3	0	C
S. faecalis	4	0	1.0 0.5—2.5	0.5 0.3—4	0	C
E. coli	4	0	100 25—122	17 3—22	3.5 1—7	D
P. inconstans B	2	0	0	1.5, 12	0	E
S. epidermidis	2	0	0	0	0	F
P. aeruginosa	3	0	0	0	0	F
Nil	6	0	0	0	0	F

*Species detected in significant numbers viz. $\geqslant 10^5$ CFU per ml urine.
**Median of peak areas in mm^2.
***Range of peak areas in mm^2.

compounds in the culture medium or urine specimens [2]. All six types of chromatogram were represented in the results (Table III). *P. mirabilis*, *K. aerogenes* (producers and nonproducers of trimethylamine), *S. faecalis*, *E. coli* and *P. inconstans B* were detected in significant numbers in 24 urine specimens and the findings were confirmed the following day by the routine laboratory analysis result. *S. epidermidis* and *P. aeruginosa* showed no volatile products. Six of the urine specimens did not yield significant numbers of bacteria and markers were undetected in all six of them, confirming previous experience that the HS-GLC rapid urine test does not give false positive results [2].

DISCUSSION

The present investigation has shown that trimethylamine could be the marker for *P. mirabilis*, *P. vulgaris*, *P. rettgeri* and *P. inconstans A* and ethanol the marker for *Escherichia* and *Klebsiella* spp. in single unshaken cultures of urine specimens in arabinose acetylcholine medium in the HS-GLC rapid urine test. Materials, labour and total analysis times would be half those in the present test in which methyl mercaptan is the marker for *Proteus* spp. and two cultures, one unshaken and the other shaken, are required.

Two species of *Proteus*, *P. inconstans B* and *P. morganii* did not produce trimethylamine. However, *P. inconstans B* was detected both in pure cultures and in cultures of urine specimens in acetylcholine medium by the production of ethyl acetate, a marker not previously reported in the HS-GLC rapid test. In addition, *P. morganii* differs from other *Proteus* spp. in producing methyl mercaptan and its oxidation product dimethyl disulphide from methionine in cultures incubated with limited aeration [8]. It is probable that if methionine, in addition to arabinose and acetylcholine, were added to medium used for unshaken cultures of urine specimens in the HS-GLC rapid test, six volatile products would be detected and quantitated, viz. trimethylamine, ethanol, ethyl acetate, *n*-propanol, methyl mercaptan and dimethyl disulphide. In this way the high information content of a single HS-GLC analysis would detect and suggest the identity of *Escherichia*, *Klebsiella* and *Citrobacter* spp., all six *Proteus* spp. and *S. faecalis* in significant numbers in specimens of urine.

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