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Determination of bacterial muramic acid by gas chromatography–mass spectrometry with negative-ion detection

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ABSTRACT

A gas chromatographic–mass spectrometric method for the determination of trace amounts of muramic acid was developed. Muramic acid, a compound unique to bacterial peptidoglycan, not present elsewhere in nature, was measured as its trifluoroacetylated methyl glycoside, and in that form could be determined at low picogram levels (injected amount) when using negative-ion chemical ionization. The method included methanolysis, extraction, evaporation and acetylation. The presence of excess amounts of yeast in the samples did not interfere with the analysis. The method is rapid and simple and is useful for the determination of small amounts of peptidoglycan in complex environments.

INTRODUCTION

The development of chromatographic and mass spectrometric methods for the analysis of bacterial constituents has considerably facilitated the detection and differentiation of small amounts of bacteria in complex samples. Such methods are of importance when early detection of bacteria is vital, as in medical diagnosis and in the food and biotechnology industries [1]. Chemical markers, that is, cellular components specific to the microorganisms, are used as analytes.

One of the most selective and sensitive chromatographic detectors is the mass spectrometer. In gas chromatography–mass spectrometry (GC–MS), the ionization techniques commonly used are electron impact (EI) and chemical ionization (CI). Compared with EI, negative-ion chemical ionization (NICI) of halogenated derivatives frequently improves the selectivity as underivatized materials are not detected (as they do not contain halogens). Minimum fragmentation of the analyte occurs with NICI because the ionization is gentler, which in turn often results in reduced background noise. In addition, halogenated derivatives are extremely strong electron-capturing compounds, hence the sensitivity is also greatly enhanced.

The bacterial cell wall skeleton, peptidoglycan,

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contains a polysaccharide backbone with the repeating monomers N-acetylglucosamine and N-acetylmuramic acid. As muramic acid (MuAc) is unique to bacteria, it has been used as a chemical marker for detecting both intact bacteria and bacterial debris in, *e.g.*, synovial fluids in animal models of arthritis [2]. In the literature on MuAc analysis, MS methods described include GC–EI-MS of alditol acetate [3], aldononitrile [4,5] and trimethylsilyl (TMS) derivatives [6], and NICI-MS of N-heptafluorobutyryl isobutyl ester derivatives [7]. In addition, an HPLC–MS method for the determination of underivatized MuAc has been reported [8].

In the alditol acetate GC–MS procedure, MuAc is released by hydrolysis and the aldehyde group of the acid is then reduced (to eliminate the anomeric centre) and hydroxyl and amino moieties are subsequently acetylated. The carboxyl group of MuAc forms a lactam [9]; other carboxyl groups, such as those found in amino acids, do not appear to be derivatized. Between the reduction and acetylation steps, the reducing agent (borohydride) is removed (as tetramethylborate gas) by multiple evaporations with methanol–acetic acid, a process that is time consuming. The procedure incorporates prederivatization clean-up steps that remove hydrophobic substances (*e.g.*, fatty acids) before acetylation of hydroxyl or amino groups.

Earlier attempts to develop simpler methods to replace the alditol acetate procedure have not been entirely successful. In the aldononitrile procedure, reaction of the aldehyde moiety with hydroxylamine (to destroy the anomeric centre) replaces reduction with sodium borohydride, and multiple evaporation steps to remove borate are thus eliminated. Unfortunately, with this procedure, extraneous peaks are generated from side-reactions between the hydroxylamine and acetic anhydride [4,5]. The TMS derivative of MuAc is simple to prepare although rather unstable [6]. HPLC–MS analysis, in contrast to other methods, requires a minimum of sample pretreatment as derivatization is not necessary, but at present this technique does not allow the detection of sub-nanogram injected amounts of MuAc [8].

In this work, a procedure for the trace determination of MuAc was developed in which samples were methanolysed and then subjected to trifluoroacetylation. This procedure was adapted from the technique that Pritchard *et al.* [10] used successfully with electron-capture detection. Other NICI-MS procedures for MuAc determination have been developed, but not widely used, however [2,7]. In comparison, highly sensitive and useful GC–NICI-MS methods have been developed for certain other chemical markers of microorganisms, *e.g.*, tuberculostearic acid [11], D-alanine [12,13] and 3-hydroxy-fatty acids [14,15].

EXPERIMENTAL

Chemicals

Acetyl chloride (purity >99%) was obtained from Fluka (Buchs, Switzerland), trifluoroacetic anhydride (TFA) (purity >99%) from Janssen Chimica (Beerse, Belgium), muramic acid (purity 99%), L-rhamnose, D-ribose, D-glucose, D-galactose, D-glucosamine and D-galactosamine from Sigma (St. Louis, MO, USA) and N-methyl-D-glucamine (purity 99%) from Aldrich Chemie (Steinheim, Germany). All solvents were of analytical-reagent grade and used without further purification. Prior to use, glassware was washed in 5% Deconex (Borer Chemie, Zuchwil, Switzerland), rinsed several times with hot tap water and then heated at 400°C for 10 h.

Microorganisms

A strain of group G streptococci isolated from a clinical specimen at the Clinical Microbiology Laboratory, Lund Hospital, was sub-cultured overnight on blood agar plates at 37°C and then inoculated in 10 ml of Todd Hewitt Broth (Difco, MI, USA) and grown overnight at 37°C. The bacteria were then washed and centrifuged (10 000 *g*, 15 min) three times and then resuspended in 0.9% sodium chloride. *Saccharomyces cerevisiae*, isolated from bakers' yeast, was cultured in 100 ml of Lactobacillus Carrying Medium [16] at 32°C for 24 h and then washed and centrifuged and resuspended five times, as described above. Following this procedure, the microorganisms were freeze-dried.

Group A streptococcal cell walls (PG-PS) were prepared as described previously [17]. In brief, streptococcal cells were solubilized by extensive sonication and intact cells and high-molecular-mass debris were removed by centrifugation at 10 000 *g*. The cell wall fragments were then treated sequentially with hyaluronidase, ribonuclease and deoxyribonuclease, papain and pepsin; between the enzyme

treatments, buffers were changed by dialysis. Cell walls were then separated from enzymes and degraded cellular constituents by centrifugation at 110 000 *g*, extracted with chloroform–methanol and freeze-dried.

Gas chromatography–mass spectrometry

The GC–MS analyses were performed on a Hewlett-Packard (Palo Alto, CA, USA) Model 5890 gas chromatograph connected to a VG Trio 1-S mass spectrometer (VG Masslab, Manchester, UK). The mass spectrometer was used in the CI mode with NI detection. A fused-silica capillary column (25 m × 0.25 mm I.D.), containing cross-linked OV-1 as the stationary phase, was interfaced directly to the ion source. Volumes of 1 μl were injected in the splitless mode (injector temperature 260°C) using a Hewlett-Packard Model 7673 autosampler. Helium, at a column head pressure of 10 psi (1 psi = 6894.76 Pa), served as the carrier gas, and isobutane (ionized at an energy of 70 eV) as the reagent gas. The initial column temperature (70°C) was increased at 8°C/min to 200°C and the temperature of the GC–MS interface was 260°C. The ion source temperature was 110°C; for comparisons, fragmentation patterns of the MuAc derivative were also studied at ion source temperatures of 80, 110, 130 and 150°C. Both scan mode and selected ion monitoring (SIM), measuring the ions *m/z* 674, 657, 567 and 480, were used in the experiments.

Sample treatment

Samples (1–2 mg) were methanolysed under a nitrogen atmosphere at 85°C for 18 h in 1 ml of 4 *M* methanolic hydrochloric acid (HCl; prepared by adding acetyl chloride dropwise to methanol while cooling in an ice-bath). After cooling, 1–10 μg of the internal standard N-methyl-D-glucamine, dissolved in 100 μl methanol, were added, and the methanolysate was extracted with 2 ml of hexane. After evaporating the methanolic phase to dryness at 40°C under a stream of nitrogen, 50 μl of TFA and 50 μl of acetonitrile were added; derivatization was performed at 60°C for 5 min. Subsequently, the derivatized samples were allowed to stand for 5 min at room temperature, whereafter 400 μl of acetonitrile were added. Sample portions (50–100 μl) were then transferred to new test-tubes, evaporated to approximately half the volume, diluted with acetonitrile (0.1–1.0 ml) and finally analysed (1-μl aliquots) by GC–MS.

Relative yield and chemical stability

To investigate the influence of methanolysis time and concentration of the HCl on the relative yield of MuAc, duplicate bacterial samples were heated in 4 *M* methanolic HCl for 1, 3, 6, 18, 25, 38 and 48 h. In addition, separate samples were heated for 25 h in 1, 2, 3 and 4 *M* methanolic HCl and treated as described under *Sample treatment*. The area ratio between the molecular ion peak of MuAc and that of the internal standard was calculated.

Different TFA derivatization conditions were also investigated. Bacteria were first heated in 4 *M* methanolic HCl at 85°C for 18 h and then subjected to hexane extraction (see *Sample treatment*), whereafter eight 100-μl aliquots of the methanolic phase were placed in separate tubes. The liquid in the tubes was evaporated and 50 μl of acetonitrile and the same amount of TFA were added. Derivatization of duplicate samples was performed at 80°C for 10 min, 80°C for 5 min, 60°C for 10 min and 60°C for 5 min. Derivatized internal standard was added and the samples were evaporated to approximately half the volume, diluted in acetonitrile (see *Sample treatment*) and analysed in the scan mode. For each of the individual samples the area ratio between the molecular ion peak of MuAc and that of the internal standard was calculated.

To evaluate the chemical stability of the derivatives, two bacterial TFA preparations and one PG-PS TFA preparation were stored in a refrigerator (a) after removal of TFA by evaporation (as described under *Sample treatment*) and (b) in the presence of 10% TFA. The samples were analysed (scan mode) after 1, 3 and 7 days of storage, and the peak areas of the molecular ions of MuAc and the internal standard, selected from the total ion current profile, were integrated. The evaporated samples were compared with those of the corresponding samples stored in 10% TFA.

Linearity, precision, accuracy, quantification and limit of determination

To investigate the linearity and precision of the overall analytical procedure, six bacterial samples were heated in 4 *M* methanolic HCl, then extracted with hexane and evaporated to dryness (as described

under *Sample treatment*), and the resulting residues were dissolved in 2 ml of methanol. Volumes corresponding to 5, 12.5, 25, 50, 125 and 250 μg (dry mass) of bacterial cells were then taken from two of these solutions and placed in separate vials, and the methanol was subsequently evaporated. In addition, volumes corresponding to 50 and 250 μg of bacterial cells were transferred from the remaining four samples into separate vials. Internal standard (2.0 μg) was added to each of these vials and the samples were derivatized with TFA (see *Sample treatment*). After 5 min at room temperature, 100 μl of acetonitrile were added. A 100-μl portion was then transferred into a new test-tube, evaporated to approximately half the volume, diluted with 400 μl of acetonitrile and analysed in the SIM mode. A graph illustrating the area ratios between the molecular ion peak of MuAc and that of the internal standard *versus* the total amount of bacteria in the sample was constructed.

To investigate the limit of determination for bacterial MuAc in the GC–MS part of the method, bacterial samples were heated in 4 *M* methanolic HCl, extracted with hexane and evaporated (see *Sample treatment*). The residues were dissolved in 1 ml of methanol and then diluted tenfold. Portions (100 μl) of the diluted residues were transferred into new tubes for evaporation and TFA derivatization (see *Sample treatment*). After 5 min at room temperature, 400 μl of acetonitrile were added, a 50-μl portion was evaporated to half the volume and diluted with 0.1–2.0 ml acetonitrile and finally analysed in the SIM mode. The limit of determination was defined as the amount of MuAc injected into the GC–MS system that gave a signal-to-noise ratio of about 6 (peak-to-peak noise).

For determination of the amount of MuAc in the bacteria, four samples of 1.2–1.5 mg of PG-PS and four bacterial samples were processed (see *Sample treatment*). The amount of MuAc in the PG-PS preparation (7.7% w/w, dry mass) had previously been determined by the alditol acetate method [17]. The GC–MS analyses were performed in the SIM mode. The amount of MuAc in the bacteria was determined by comparing the bacterial samples with the PG-PS samples with respect to the area ratio between the molecular ion peak of MuAc and that of the internal standard.

Detection of muramic acid in the presence of yeast

Bacterial and yeast samples were heated separately in 4 *M* methanolic HCl, extracted with hexane and evaporated. After this procedure, the yeast preparations were immediately derivatized with TFA and

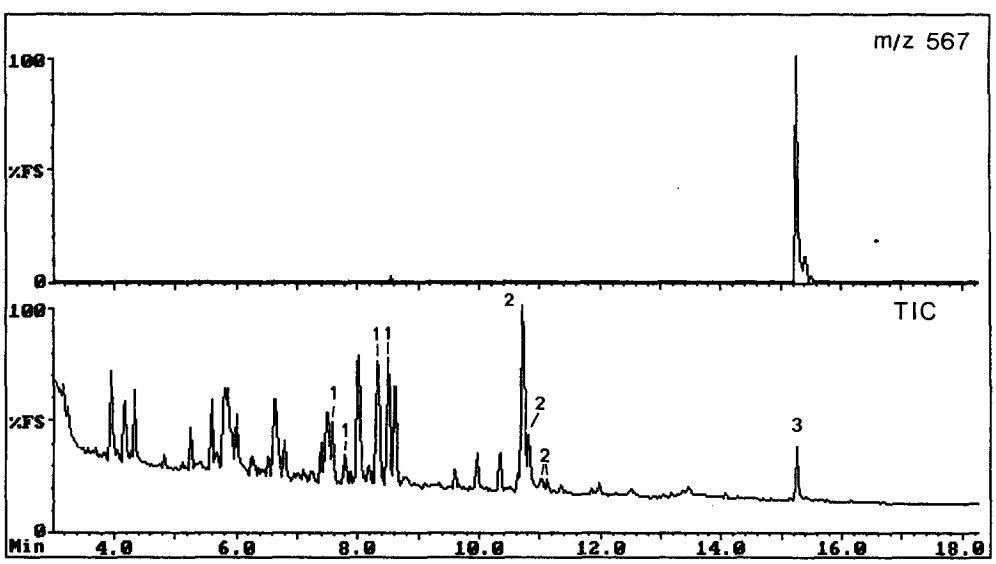

Fig. 1. Bottom trace: total ion current NICI profile of derivatized group G streptococcal carbohydrates analysed in the scan mode; glucose (1), glucosamine (2) and MuAc (3) are indicated. Top trace: ion current profile selected from the same analysis and showing the molecular ion of MuAc (*m/z* 567).

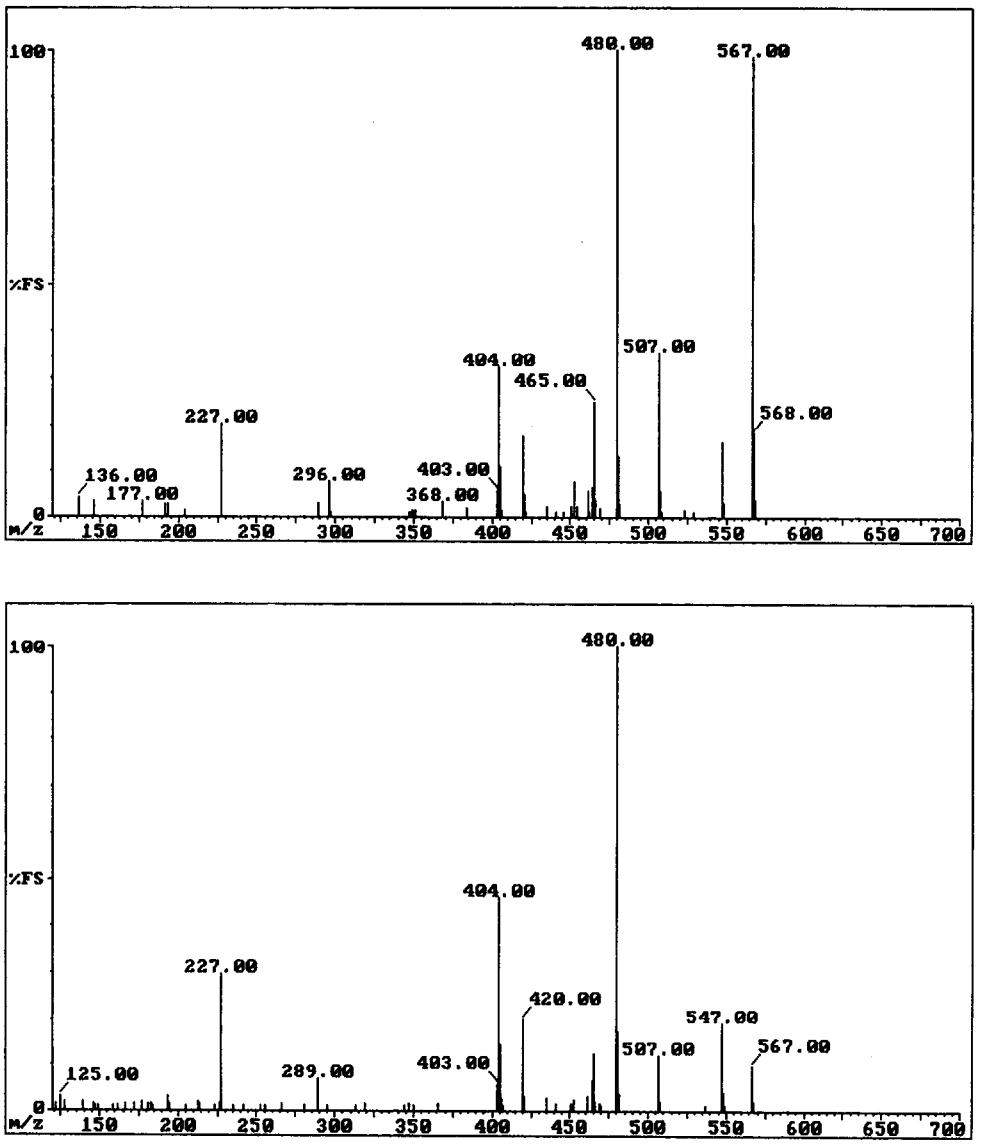

Fig. 2. NICI mass spectra of the MuAc TFA derivative at ion source temperatures of 80 (top) and 110°C (bottom).

then dissolved in 400 μl of acetonitrile, whereas the bacterial preparations were dissolved in 1 ml of methanol, diluted tenfold, transferred (100-μl aliquots) into new tubes and evaporated, and then derivatized with TFA and dissolved in 400 μl of acetonitrile. Portions (25–50 μl) of the derivatized bacteria samples were then mixed with portions (100 μl) of the derivatized yeast, and these mixtures were subsequently evaporated to half the volume, diluted with 1 ml of acetonitrile and subjected to GC–MS analysis in the SIM mode.

RESULTS

Gas chromatography–mass spectrometry

A total ion current (TIC) profile of the carbo-

hydrates in the group G streptococcal strain (analysed in the scan mode) is presented in Fig. 1 (bottom trace). The top trace shows an ion current profile that was selected from the TIC profile and focused on the molecular ion of the MuAc derivative (*m/z* 567). Glucosamine, galactosamine, glucose, galactose, ribose and rhamnose were identified by comparing their spectra and retention times with those of corresponding reference substances. The MuAc derivative eluted after 15.3 min, *i.e.*, about 5 min after the other amino sugars and 7 min after the internal standard derivative.

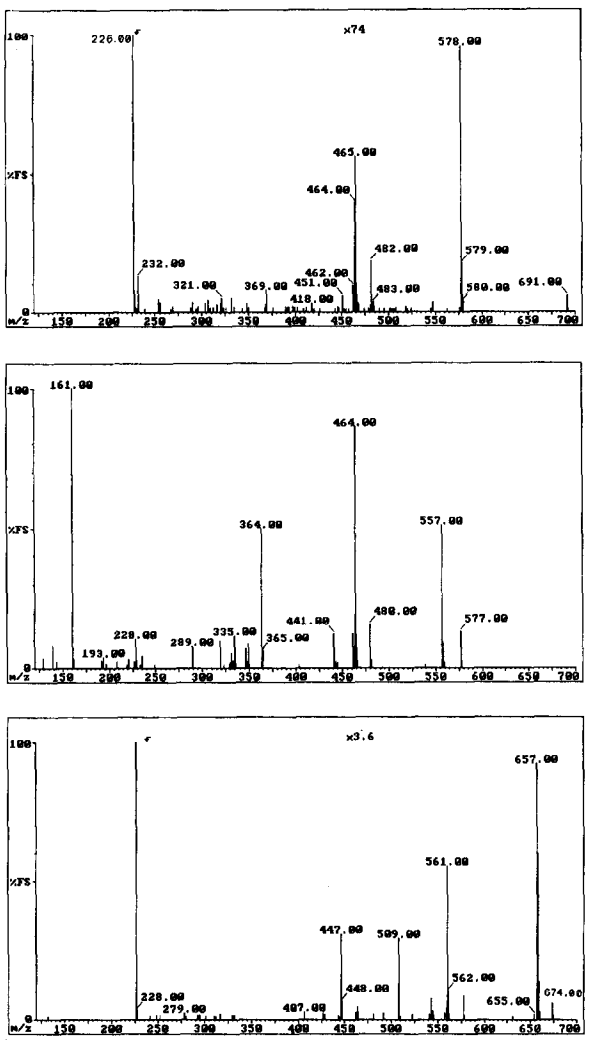

Fig. 3. NICI mass spectra of TFA-derivatized glucose (top), glucosamine (centre) and N-methyl-D-glucamine (internal standard) (bottom) at an ion source temperature of 110°C.

The relative abundance of the fragments in the mass spectrum of the MuAc derivative varied with the ion source temperature, and for the molecular ion of *m/z* 567, $[M]^-$, a significant decrease was seen at higher temperatures. Mass spectra produced at ion source temperatures of 80 and 110°C are shown in Fig. 2. The fragments found in the higher *m/z* range were interpreted as $[M - HF]^-$ (*m/z* 547), $[M - CH_3OCH = O]^-$ (*m/z* 507) and $[M - CH_3CHCOOCH_3]^-$ (*m/z* 480).

For comparison, mass spectra of the TFA derivatives of glucose, glucosamine (peaks marked 1 and 2 in Fig. 1) and N-methyl-D-glucamine are shown with the molecular ions of *m/z* 578, 577 and 674, respectively in Fig. 3. In the glucose spectrum additional prominent ions were found and interpreted as $[M + O = COCF_3]^-$ (*m/z* 691), $[M - O = CCF_3]^-$ (*m/z* 482), and $[M - O = COCF_3]^-$ (*m/z* 465); in the glucosamine spectrum the ions $[M - HF]^-$ (*m/z* 557) and $[M - O = COCF_3]^-$ (*m/z* 464) were found. Corresponding mass spectra were found for galactosamine, galactose, ribose and rhamnose.

Relative yield and chemical stability

The relative yield of MuAc achieved under different methanolysis conditions is illustrated in Fig. 4. When using 4 *M* methanolic HCl, the maximum

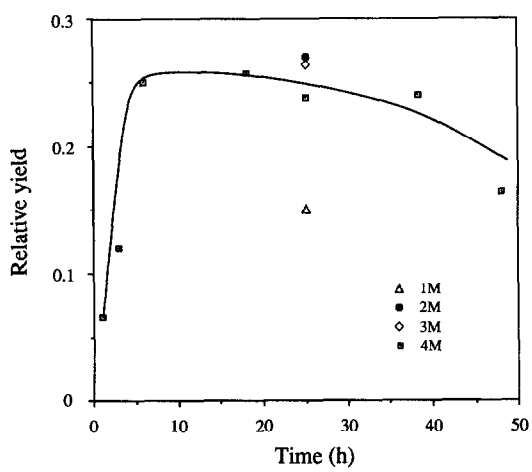

Fig. 4. Relative yield of MuAc obtained after heating streptococci in (□) 4 *M* methanolic HCl at 85°C for 1–48 h and in (△) 1, (●) 2 and (◇) 3 *M* methanolic HCl at 85°C for 25 h. Relative yields are expressed as bacterial response relative to the internal standard, *i.e.*, the area ratio between *m/z* 567 and *m/z* 674 ions. Values represent means for duplicate samples.

yield was reached after heating for 6 h. Yields obtained using 2, 3 and 4 *M* methanolic HCl were similar, whereas the yield using 1 *M* HCl was comparatively lower (methanolysis time 25 h).

The final TFA derivatization conditions chosen were heating for 5 min at 60°C. An increased temperature or derivatization time did not increase the yield.

The MuAc derivative was stable for 7 days, even when TFA had been evaporated. After 1 day of storage, the TFA-derivatized internal standard (in the absence of TFA) was not degraded, whereas after 3 days only 78% remained and after 1 week 44% (mean of three values).

Linearity, precision, accuracy, quantification and limit of determination

The relative response, *i.e.*, the area ratio between the molecular ion peak of MuAc (*m/z* 567) and the internal standard (*m/z* 674), *versus* the total amount of bacteria in the prepared samples is shown in Fig. 5. The straight line, fitted to the individual data using the method of least squares, followed the equation $y = 3.0 \cdot 10^{-3}x - 4.4 \cdot 10^{-3}$, where y is the relative response and x is amount of bacteria. The relative standard deviation of the relative response was 10% for the six 50-μg samples (mean 0.143, $s_{n-1} = 0.016$) and 5.3% for the six 250-μg samples (mean 0.739, $s_{n-1} = 0.039$). The amount of MuAc in the bacteria was found to be 0.49% (dry mass). The limit of determination of the overall method, defined as the amount of MuAc which gave a relative standard deviation of 10%, was found to be 250 ng in the original sample, whereas the limit of determination of MuAc in the GC–MS part of the method was found to be 3 pg (injected amount).

Detection of muramic acid in the presence of yeast

SIM chromatograms focused on the ion of *m/z* 567 (MuAc) in the analysis of a preparation of 0.6 ng of bacterial cells (corresponding to 3 pg of MuAc) and 120 ng of yeast cells (injected amounts) and a corresponding chromatogram for 120 ng pure yeast are shown in Fig. 6. The noise was of equal magnitude to that when pure bacterial samples were analysed and no interfering responses from pure yeast were detected.

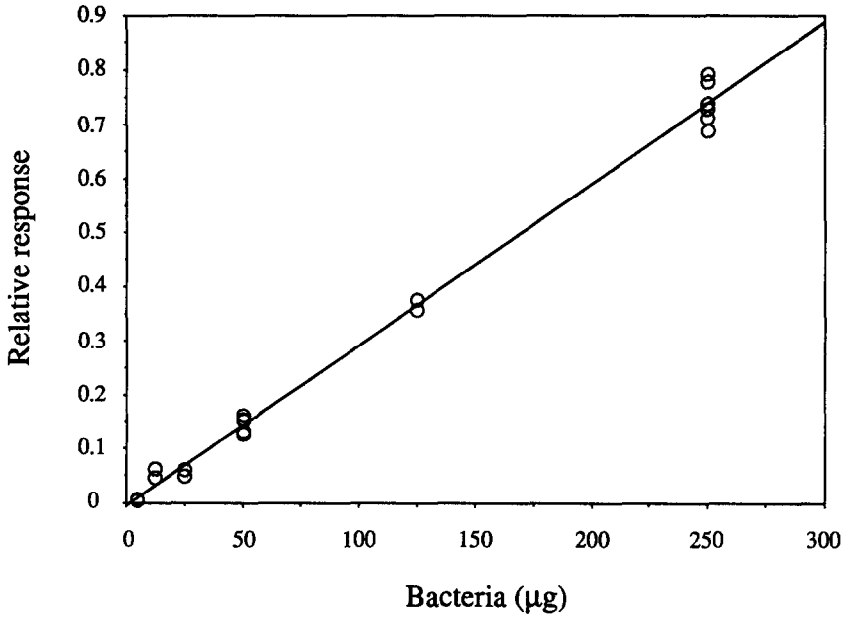

Fig. 5. Test of linearity, precision and accuracy. Relative response, *i.e.*, the area ratio between the molecular ion peak area of MuAc (*m/z* 567) and that of the internal standard (*m/z* 674), *versus* the total amount of bacteria in the samples, are shown. The method of least squares was used to fit a straight line to the individual data.

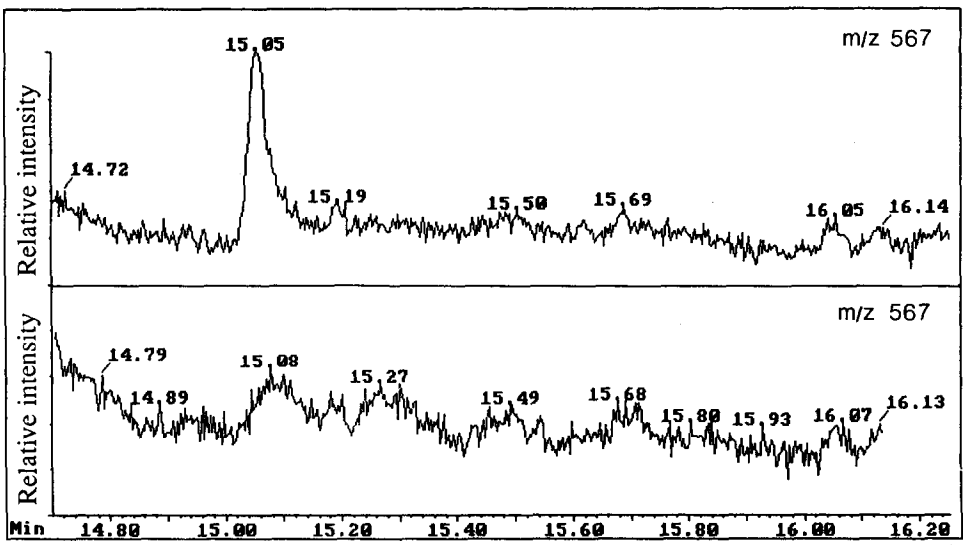

Fig. 6. Selected ion monitoring chromatograms (*m/z* 567) from analysis of MuAc. The top trace represents the injection of an amount corresponding to 0.6 ng of bacterial cells and 120 ng of yeast cells and the bottom trace the injection an amount corresponding to 120 ng of pure yeast. The higher noise apparent in the bottom trace is due to automatic normalization of the total ion current.

DISCUSSIONS

Chromatographic analysis of bacterial constituents is a useful method for the detection of bacteria or bacterial debris in biological samples and, in contrast to classical microbiological and biochemical procedures, culturing is not required. By coupling GC separation with MS detection, a high level of sensitivity is achieved that permits the determination of very small amounts of bacteria. The selectivity can be deliberately chosen by using analytes that are specific for particular species or genera or for bacteria in general. MuAc exemplifies the latter type of analyte, or chemical marker, as it is unique for bacteria. A highly sensitive and selective method for the determination of MuAc would allow the detection of trace amounts of peptidoglycan in, for example, environmental samples (*e.g.*, soil, ground water and air), foods, pharmacological products, biotechnical processes or body fluids (*e.g.*, samples for clinical diagnosis of infectious diseases).

Compared with GC–EI-MS analysis of the MuAc alditol acetate derivative, which provides a limit of detection of 2.5 ng (injected amount) [3], the NICI TFA method offers a considerable improvement in sensitivity, as MuAc can be determined at low picogram levels. However, the alditol acetate method is more attractive for carbohydrate profiling than the NICI TFA method, as alditol acetates elute as single peaks, whereas multiple peaks appear upon TFA derivatization. The TFA derivative of MuAc eluted as one dominating and one minor peak (not baseline separated), with only the major peak being considered in the quantification.

Even under mild conditions TFA reacts with the hydroxyl groups of carbohydrates. As TFA derivatives are known to be unstable in the presence of moisture, it has been recommended that preparations should be stored at −20°C in a 10% excess of TFA [18]. We followed this recommendation and then evaporated the excess of TFA prior to analysis in order to prolong the life of the GC column. On storing the evaporated samples, the MuAc derivative was found to be stable for at least 1 week, whereas degradation of the N-methyl-D-glucamine derivative was observed after 3 days. We therefore recommend that samples devoid of an excess of TFA be analysed on the same day they are prepared to avoid problems in the quantification.

Compared with previously reported techniques, the NICI TFA method is simple, as it includes only one "clean-up" step. The hexane extraction of

methanolysates is effective in separating fatty acid methyl esters from methyl glycosides [19]. All water-soluble methanolysis products are present in the final sample, however. Despite this, the presence of a 200-fold excess of yeast did not interfere with analysis for trace amounts of MuAc derivative. To ascertain the identity of the MuAc and the internal standard derivative, we monitored four ions in the SIM analyses.

Methanolysis was the most time-consuming step in the TFA procedure. In addition to the production of methyl glycosides from the hydroxyl groups involved in linkage of monomeric units, the carboxyl group of muramic acid is methylated. The maximum yield was achieved after 6 h of heating.

The influence of the ion source temperature on the NICI mass spectra of halogenated derivatives has been described previously [20]. For the MuAc derivative, there was a major difference between the relative abundance of the fragments obtained at 80°C and those obtained at 110°C, whereas the mass spectrum at 150°C was almost identical with that at 110°C. To ensure optimum selectivity in the analysis, a high abundance of the molecular ion is desirable. A temperature of 110°C was used because it is the lowest stable ion source temperature permitted by our GC–MS system when using standard filaments. Previously reported data on fragmentation patterns of carbohydrate TFA derivatives [21] and N-heptafluorobutyryl-2-butyl ester derivatives of (*R*)-alanine [22] were used in the interpretation of spectra.

Methanolysis of the free MuAc standard produced a mixture of compounds containing methyl and TFA groups (data not shown), whereas when bacteria were processed only the MuAc methyl glycoside was detected. A MuAc standard therefore cannot be used in the quantification. Instead, a peptidoglycan-containing specimen with a known amount of MuAc can be used; in our study, a PG-PS preparation with a MuAc content previously determined by the alditol acetate method was employed. In other investigations, N-methyl-D-glucamine has been used as an internal standard in determinations of bacterial carbohydrates [3,8]. The amount of bacterial MuAc found (0.49% of the dry mass) corresponded well with the amount found in *S. pyogenes* (0.53%) when using the alditol acetate method [5], and the complete carbohydrate composition of the group G streptococci analysed agreed well with previously reported results [10].

So far, the alditol acetate method has been used most often when determining MuAc. The NICI TFA method described here is superior in sensitivity, but its selectivity for different sample matrices remains to be evaluated. In addition, it should be investigated whether the use of a different internal standard would improve the precision in the overall method. Experiments are now being planned to assess the technique with regard to its applicability for the determination of peptidoglycan in organic dust and different kinds of clinical samples and for the detection of bacterial contamination of biotechnical processes.

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REFERENCES

1 S. L. Morgan, A. Fox and J. Gilbart, *J. Microbiol. Methods*, 9 (1989) 57.
2 B. Christensson, J. Gilbart, A. Fox and S. L. Morgan, *Arthritis Rheum.*, 32 (1989) 1268.
3 J. Gilbart, A. Fox, R. S. Whiton and S. L. Morgan, *J. Microbiol. Methods*, 5 (1986) 271.
4 R. H. Findlay, D. J. W. Moriarty and D. C. White, *Geomicrobiology*, 3 (1983) 135.
5 W. L. Eudy, M. D. Walla, S. L. Morgan and A. Fox, *Analyst (London)*, 110 (1985) 381.
6 D. G. Pritchard, R. L. Settine and J. C. Bennett, *Arthritis Rheum.*, 23 (1980) 608.
7 A. Tunlid and G. Odham, *J. Microbiol. Methods*, 1 (1983) 63.
8 I. Elmroth, L. Larsson, G. Westerdahl and G. Odham, *J. Chromatogr.*, 598 (1992) 43.
9 A. Fox, S. L. Morgan and J. Gilbart, in J. Bierman and G. McGinnis (Editors), *Analysis of Carbohydrates by GLC and MS*, CRC Press, Boca Raton, FL, 1989, pp. 87–117.
10 D. G. Pritchard, J. E. Coligan, S. E. Speed and B. M. Gray *J. Clin. Microbiol.*, 13 (1981) 89.
11 L. Larsson, G. Odham, G. Westerdahl and B. Olsson, *J. Clin. Microbiol.*, 25 (1987) 893.
12 A. Sonesson, L. Larsson, A. Fox, G. Westerdahl and G. Odham, *J. Chromatogr.*, 431 (1988) 1.
13 K. Ueda, S. L. Morgan, A. Fox, J. Gilbart, A. Sonesson, L. Larsson and G. Odham, *Anal. Chem.*, 61 (1989) 265.
14 G. Odham, A. Tunlid, G. Westerdahl, L. Larsson, J. B. Guckert and D. C. White, *J. Microbiol. Methods*, 3 (1985) 331.

15 I. Elmroth, A. Valeur, G. Odham and L. Larsson, *Biotechnol. Bioeng.*, 35 (1990) 787.
16 L. Axelsson, T. C. Chung, W. J. Dobrogosz and S. E. Lindgren, *Microbiol. Ecol. Health Dis.*, 2 (1989) 131.
17 J. Gilbart, J. Harrison, C. Parks and A. Fox, *J. Chromatogr.*, 441 (1988) 323.
18 K. Bryn and E. Jantzen, *J. Chromatogr.*, 240 (1982) 405.
19 E. Jantzen and K. Bryn, in M. Goodfellow and D. Minnikin (Editors), *Chemical Methods in Bacterial Systematics (Society for Applied Bacteriology Series*, No. 20), Academic Press, London, 1985, pp. 145–171.
20 D. V. Crabtree, A. J. Adler and G. J. Handelman, *J. Chromatogr.*, 466 (1989) 251.
21 W. A. König, H. Bauer, W. Voelter and E. Bayer, *Chem. Ber.*, 106 (1973) 1905.
22 A. Tunlid and G. Odham, *Biomed. Mass Spectrom.*, 11 (1984) 428.