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Determination of muramic acid by high-performance liquid chromatography–plasma spray mass spectrometry

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ABSTRACT

A high-performance liquid chromatographic–plasma spray mass spectrometric method was developed for the determination of muramic acid, a unique compound present in bacterial peptidoglycan. The method included hydrolysis of bacterial samples by hydrochloric acid, purification using a disposable extraction column, and separation using cation-exchange chromatography with a mobile phase composed of ammonium acetate–trifluoroacetic acid–water. Muramic acid was detected in cells of *Bacillus subtilis*; the presence of yeast cells (*Saccharomyces cerevisiae*) in excess amounts did not interfere with the analyses. In contrast to currently applied liquid and gas chromatographic methods, the described method permits highly selective determination of underivatized muramic acid, and may therefore be useful for detecting bacteria and bacterial cell debris in complex biological matrices.

INTRODUCTION

The bacterial cell wall contains several compounds unique to prokaryotic organisms. A basic structure is the peptidoglycan backbone, which consists of alternating molecules of N-acetylglucosamine and N-acetylmuramic acid linked by β-1,4 glycosidic bonds. As N-acetylmuramic acid is absent in non-bacterial biological matter, including other microorganisms (*e.g.*, viruses and fungi) [1,2], it can be used as a chemical marker for determining bacterial cells or cell debris in various environments. With this in mind, muramic acid has been used as the analyte in the chemical detection of peptidoglycan, *e.g.*, in mammalian tissues [3], soil [4], marine sediments [5] and human septic synovial fluids [6]. As opposed to culturing techniques, which only provide information on the viable portion of bacteria present in a sample, chemical analysis of muramic acid does not discriminate between live and dead cells.

During the past decade, several methods for determining muramic acid by chromatographic and mass spectrometric (MS) techniques have been described. Gas chromatographic (GC) and GC–electron impact (EI) MS analysis of alditol acetate [3,7] and aldononitrile acetate [8,9] derivatives, and of N-heptafluorobutyryl butyl ester derivatives [10,11] have been performed. In addition, some investigators have applied high-performance liquid chromatographic (HPLC) methods using either precolumn fluorescence derivatization with *o*-phthaldialdehyde [4,5,12] or postcolumn redox reaction with bis(1,10-phenanthroline)copper(II) as a mediator

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for amperometric detection [13]. For all of the described methods, derivatization of the analyte is required in order to achieve high sensitivity and selectivity in the detection.

In this study, an HPLC–MS method was developed for the determination of muramic acid after a minimum of sample pretreatment and without derivatization. To obtain high sensitivity, selected ion monitoring (SIM) was applied. The selectivity of this method is illustrated by the determination of muramic acid in pure cultures of *Bacillus subtilis* and in *B. subtilis* cultures in the presence of excess amounts of yeast (*Saccharomyces cerevisiae*).

EXPERIMENTAL

Materials

The following chemicals were used: muramic acid (purity 99%; Sigma, St. Louis, MO, USA), N-methyl-D-glucamine (purity 99%; Aldrich Chemie, Steinheim, Germany), ammonium acetate and acetonitrile (analytical-reagent grade; Merck, Darmstadt, Germany) and trifluoroacetic acid (purity protein-sequencing grade; Fluka, Buchs, Switzerland). Bond Elut C_{18} octadecyl solid-phase 1-ml extraction columns were purchased from Analytichem International (Harbor City, CA, USA). Before use, all glassware was washed in 5% Deconex (Borer Chemie, Zuchwil, Switzerland), rinsed several times with hot tap water and distilled water and then heated for 10 h at 400°C.

Microorganisms

Bacillus subtilis (ATCC 6051) was cultivated overnight on blood-agar plates at 37°C. *Saccharomyces cerevisiae* was isolated from baker's yeast by cultivation on Rogosa agar plates (Oxoid, Basingstoke, UK) overnight at 32°C. The bacterial and yeast cells were transferred into 10-ml test-tubes and washed three times by repeated centrifugations and dispersions in distilled water; after the final centrifugation, the sediments were lyophilized.

Chromatography

Different conditions for the chromatographic separations were evaluated using an LDC Model III Constametric pump and either an LDC Spectro-Monitor III variable-wavelength UV detector (operating at 220 nm) or an LDC RefractoMonitor refractive index detector (LDC, Riviera Beach, FL, USA). A Rheodyne (Cotati, CA, USA) Model 7010 injector equipped with a 10- or 20-μl loop was used to inject samples onto a cation-exchange column (100 × 4.0 mm I.D.) packed with Nucleosil 5-SA (5-μm particles) (Macherey–Nagel, Düren, Germany). The mobile phase was a 0.02 *M* aqueous solution of ammonium acetate with the pH adjusted to 2.5 by addition of trifluoroacetic acid. A flow-rate of 0.7 ml/min was used, and most compounds eluted within 30 min. After analysing biological samples for 1 week, the column was reconditioned with ten column volumes of 1% trifluoroacetic acid to avoid any interference from previously injected, late-eluting compounds.

Separations were also performed using a 100 × 4.6 mm I.D. column packed with Spherisorb aminopropylsilica gel (5-μm particles) (Phase Separations, Queensferry, UK) and with acetonitrile–water (82:18, v/v) as the mobile phase (1 ml/min). Biological samples were not analysed with this column.

Chromatography–mass spectrometry

The HPLC–MS experiments were performed on a Waters Model 600 MS delivery system (Millipore, Milford, MA, USA) equipped with a 60-μl variable-volume loop injector (Rheodyne, Model 7000). The effluent from the column was introduced into a quadrupole mass spectrometer (Model Trio 3; VG, Altrincham, UK) via the standard VG thermospray–plasma spray probe.

Full mass spectra were obtained by scanning from *m/z* 110 to avoid the solvent ions. The temperature of the capillary vaporizer was 220°C and that of the ion source 235°C. In the plasma spray studies the discharge current was 230 μA, whereas in the thermospray studies no discharge current was used. When using thermospray the ammonium acetate concentration of the mobile phase (pH 6.8) was increased to 0.1 *M*. In the selected ion monitoring (SIM) analyses, the ion of *m/z* 234 was used for determining muramic acid and that of *m/z* 196 for determining N-methyl-D-glucamine. All the SIM analyses were performed using the plasma spray interface.

When the aminopropylsilica gel column was used, the vaporizer capillary temperature was 250°C, the ion source temperature 230°C and the discharge current 250 μA.

Sample treatment

Biological samples containing bacteria and/or yeast (0.25–3 mg dry weight) were hydrolysed at 90°C under nitrogen for 2.5 h in 0.5 ml of 6 *M* HCl. After cooling, water (0.5 ml) and the internal standard N-methyl-D-glucamine (25 μg) were added. The hydrolysates were purified by loading them on the solid-phase extraction columns (conditioned with one volume of methanol and three volumes of water prior to use) and eluting them with 4 × 0.5 ml of water; following this procedure, the samples were lyophilized. The residues were dissolved in 0.5–1 ml of the mobile phase before HPLC–plasma spray MS analysis (see above).

Muramic acid reference compound: calibration, recovery, stability

A calibration graph for pure muramic acid was constructed using the HPLC–MS step with plasma spray SIM analysis (*m/z* 234) in the range 6.5–250 ng. A constant amount (50 ng) of N-methyl-D-glucamine added to each sample served as the internal standard.

To evaluate the recovery of muramic acid and the internal standard in the purification, ten standard samples of a mixture of muramic acid and internal standard (5 μg of each) in 1 ml of 3 *M* HCl were prepared. Five of the samples were purified, lyophilized as described under *Sample treatment* and analysed by HPLC–plasma spray MS (SIM mode). The remaining five samples were treated in the same manner, but the purification step was omitted. The recoveries were evaluated by comparing the absolute responses of the purified standard samples with those obtained from the unpurified samples.

On chromatography of muramic acid on the cation-exchange column (UV detection) a fraction containing a rapidly eluting compound (retention time 1.6 min), in addition to the muramic acid fraction (retention time 4 min), were collected. The stability of these compounds after storage at 4°C (for 1 and 3 days and 1 and 8 weeks) was studied using HPLC–MS (scanning mode).

Biological samples

All biological samples were analysed using the plasma spray interface. To study the accuracy of the method, 0–22 μg of muramic acid were added to hydrolysates of 250 μg of *B. subtilis*, after which the samples were subjected to the whole determination procedure (*i.e.*, sample treatment, and HPLC–MS). To determine the recovery of muramic acid, the peak areas were compared with those obtained from reference experiments in which 25 μg of muramic acid (dissolved in the mobile phase) were analysed by HPLC–MS directly (without further sample pre-treatment).

The recovery was calculated as the slope of the graph of the amount of muramic acid found *versus* the known amount added. The amount found was calculated by comparison with the peak area of the reference experiment. A reference sample was analysed immediately before each sample.

To study the precision of using internal standardization, the same experiments were performed after addition of 25 μg of internal standard to the sample and reference solutions.

To determine the amount of muramic acid in *B. subtilis*, 1.4, 0.6 and 0.7 mg dry weight of bacteria were analysed.

In a separate experiment, aliquots of a mixture of bacteria (1.4 mg/ml) and yeast (1.6 mg/ml), prepared as described under *Sample treatment*, were diluted with different volumes of a likewise prepared yeast sample (2.7 mg/ml) to investigate whether the presence of a 5–50-fold excess concentration of yeast in the bacterial samples interfered with the detection of muramic acid.

RESULTS

Chromatography

When using the cation-exchange column, the retention time of muramic acid was 4 min. The compound appeared as a double peak, with the α- and β-anomers partly separated. Either a UV (220 nm) or a refractive index (RI) detector could be used for muramic acid detection, whereas only an RI detector could be used for detection of the internal standard (retention time 8 min). A higher concentration of ammonium acetate or a lower concentration of trifluoroacetic acid in the mobile phase (higher pH) shortened the retention time of muramic acid. The flow-rate (0.7 ml/min) was chosen to fit the vacuum pump capacity of the mass spectrometer.

Using the aminopropylsilica gel column the α- and β-anomers of muramic acid co-eluted at a retention time of 9 min. However, when the mobile

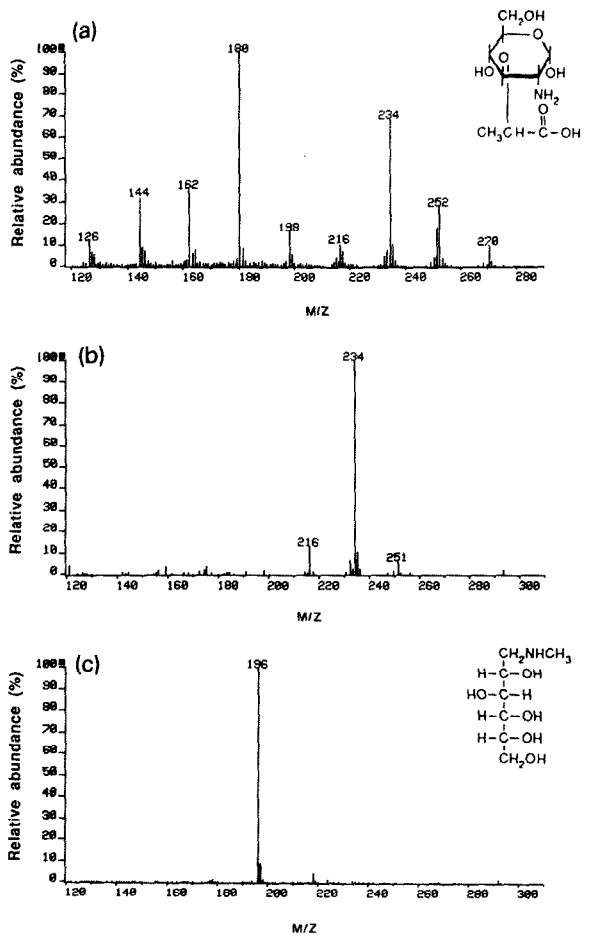

Fig. 1. Mass spectra of (a) muramic acid, (b) a degradation or conversion product of muramic acid and (c) N-methyl-D-glucamine. All three spectra were recorded using HPLC–plasma spray MS.

phase was led from this column into the mass spectrometer, the vaporizer capillary became clogged after a few hours. This phenomenon was not observed when the mobile phase was led directly into the mass spectrometer (*i.e.*, without first passing through the column). Degradation products from the aminopropylsilica gel were considered to be the cause of the problem, and this stationary phase was not used in subsequent analyses.

Chromatography–mass spectrometry

A plasma spray mass spectrum of pure muramic acid after cation-exchange chromatography is shown in Fig. 1a. The MS ionization conditions were chosen to give a maximum of high-mass fragments. The molecular adduct ion of m/z 252 (M + 1) was detected, as were other prominent ions presumably resulting from addition or successive losses of water molecules resulting in fragments of m/z 270 (+ 1 H_2O), 234 (− 1 H_2O), 216 (− 2 H_2O), 198 (− 3 H_2O), 180 (− 4 H_2O) and 162 (− 5 H_2O). The fragment of m/z 162 may also originate from the loss of the carboxylic acid-containing group COOH–CHOH–CH_3 (m/z 90) and the fragment of m/z 144 from an additional loss of one molecule of water. The fragmentation patterns of the two anomers of muramic acid were virtually identical.

A rapidly eluting compound (retention time 1.6 min) was detected on injection of the muramic acid reference compound onto the cation-exchange column. Total ion current (TIC) analyses of the collected muramic acid fraction revealed that after 3 days of storage more than 99% of muramic acid remained, after 1 week 97% and after 8 weeks 42%; the amounts of the rapidly eluting compound increased correspondingly. As ions of m/z 216, 234 and 251 were present in its plasma spray mass spectrum (Fig. 1b), the compound was assumed to be a degradation or conversion product of muramic acid. When analysing the fraction containing this compound after storage for up to 8 weeks, muramic acid did not appear, indicating that the degradation or conversion reaction was irreversible.

The molecular adduct ion of m/z 196 (M + 1) was seen in the plasma spray mass spectrum of N-methyl-D-glucamine (Fig. 1c).

The fragment of m/z 234 was abundant in the mass spectrum of muramic acid and was therefore used for the muramic acid measurements in the SIM analyses. Analogously, the fragment of m/z 196 was selected for N-methyl-D-glucamine. Fig. 2 shows selected ion current profiles of muramic acid reference compound (13 ng) and N-methyl-D-glucamine (50 ng).

The detection limit for muramic acid was 3 ng at a signal-to-noise ratio of 3:1, and a linear calibration graph was found in the measured interval (Fig. 3). In the purification, the recovery of muramic acid was 96% (s_{n-1} = 3%, n = 5) and that of N-methyl-D-glucamine was 108% (s_{n-1} = 8%, n = 5).

A thermospray MS fragmentation pattern of muramic acid is shown in Fig. 4. As in the plasma

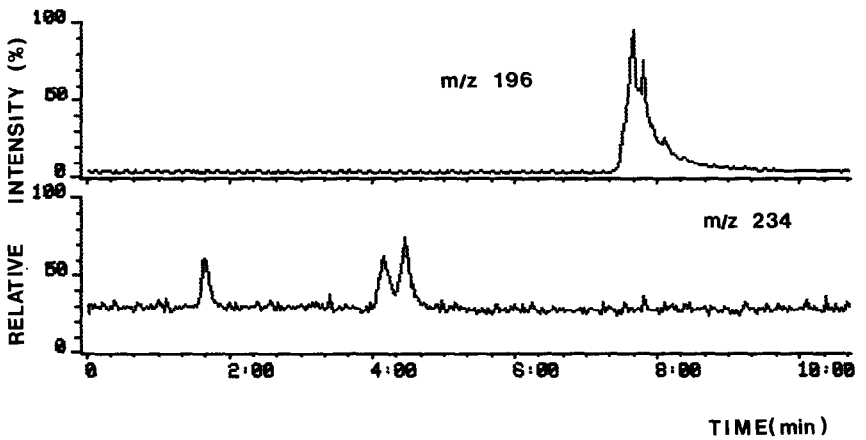

Fig. 2. HPLC–plasma spray MS (SIM) analysis of 13 ng of muramic acid (bottom trace, retention time 4.2–4.4 min, focused at *m/z* 234) and 50 ng of the internal standard N-methyl-D-glucamine (top trace, retention time 7.6 min, focused at *m/z* 196).

spray mass spectrum, the molecular adduct ion of *m/z* 252 (M + 1) and also ions presumably representing addition or losses of water molecules were found. The ion of *m/z* 234 was less abundant than when using plasma spray ionization.

Biological samples

The identity of muramic acid in the SIM analyses of the bacteria was ascertained by monitoring three ions: *m/z* 270, 252 and 234 (Fig. 5). Fig. 6 shows the results of adding increasing amounts of muramic acid to *B. subtilis* samples. The data in Fig. 6a are equivalent to quantification by external standardization. A straight line ($y = 0.84x + 7.9$) was fitted to the data; the slope, corresponding to the absolute recovery of muramic acid, was 84 ± 28% (95% confidence interval); the intercept corresponded to the amount of muramic acid in the bacteria. In the plot in Fig. 6b, internal standardization was used in the quantification; a straight line ($y = 1.5x + 8.9$) was fitted to the data.

The mean amount of muramic acid in dried cells of *B. subtilis* was found to be 2.2% (w/w) (2.1, 2.2, 2.4%).

Fig. 7 illustrates the correlation between the bacterial concentration and the concentration of muramic acid. Notably, the presence of yeast cells in the samples, even in amounts 5–50 times larger compared with bacteria (2.3–2.6 and 0–0.49 mg/ml, respectively), did not interfere with the detection of muramic acid. Muramic acid was not found in pure yeast samples. The superior selectivity of MS over

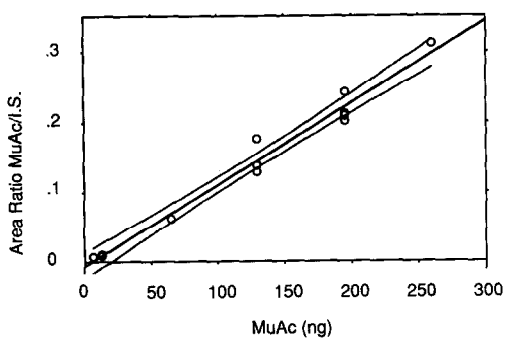

Fig. 3. Calibration graph based on muramic acid and the internal standard (MuAc/I.S.) from HPLC–plasma spray MS (SIM) determinations. The method of least squares was used to fit straight lines to the individual data. A 95% confidence interval of the true area ratio is included.

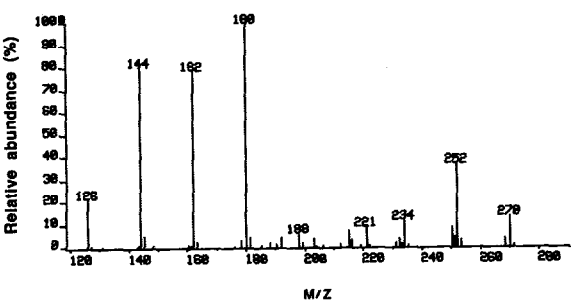

Fig. 4. Mass spectrum of muramic acid obtained using HPLC–thermospray MS.

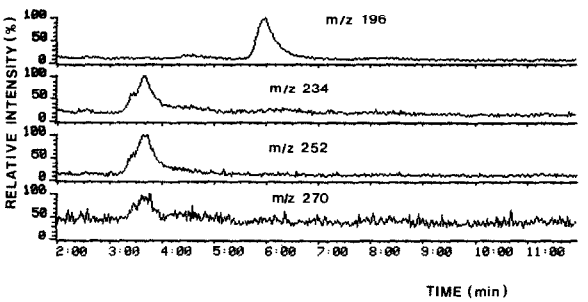

Fig. 5. HPLC–plasma spray MS (SIM) analysis of *Bacillus subtilis* cells monitoring the ions of *m/z* 196 (N-methyl-D-glucamine) and 234, 252 and 270 (muramic acid). The injected amount corresponded to 50 μg (dry weight) of bacteria.

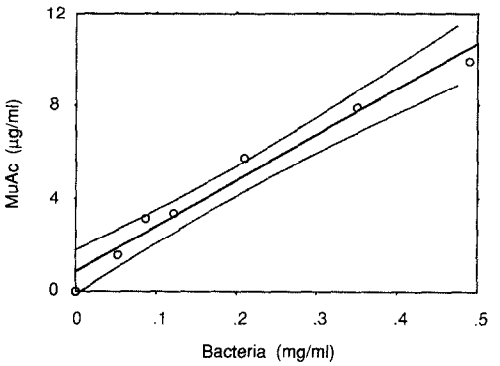

Fig. 7. Muramic acid (MuAc) concentrations in *Bacillus subtilis* preparations in the presence of excess amounts (2.3–2.6 mg/ml, dry weight) of yeast cells (*Saccharomyces cerevisiae*). The method of least squares was used to fit a straight line to the data. A 95% confidence interval of the true amounts of muramic acid is included.

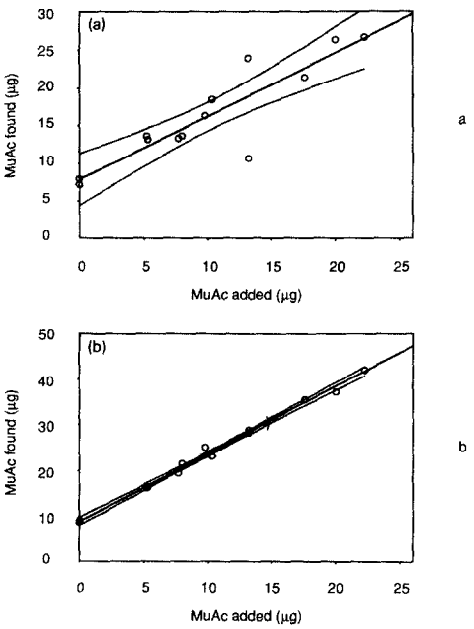

Fig. 6. (a) Test of accuracy of HPLC–plasma spray MS (SIM) analysis of muramic acid added in increasing amounts to *Bacillus subtilis* samples. (b) Comparison of found and added amounts of muramic acid using determination by HPLC–plasma spray MS (SIM) with internal standardization. The method of least squares was used to fit straight lines to the data. 95% confidence intervals of the true amounts of muramic acid amounts are included.

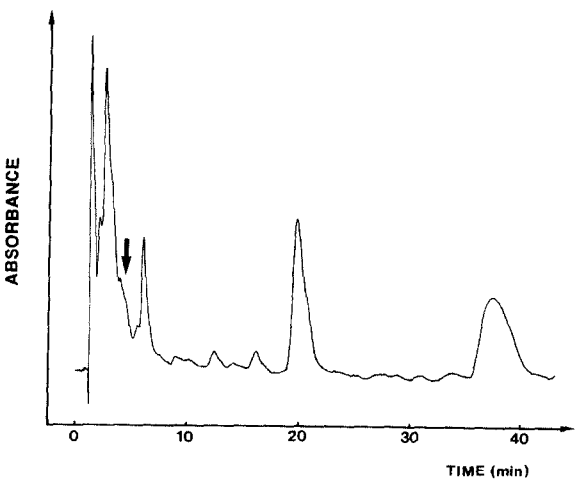

Fig. 8. HPLC–UV detection (220 nm) of a 1:1 (w/w) mixture of cells of *Bacillus subtilis* and yeast (*Saccharomyces cerevisiae*). The arrow indicates the retention time for muramic acid.

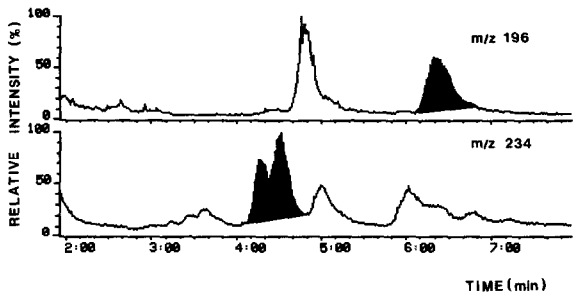

Fig. 9. HPLC–plasma spray MS (SIM) analysis of muramic acid (*m/z* 234, bottom trace, black peak) and N-methyl-D-glucamine (*m/z* 196, top trace, black peak) in a 1:5 (w/w) mixture of cells of *Bacillus subtilis* and *Saccharomyces cerevisiae*.

UV detection is illustrated in Figs. 8 and 9, which show analyses of bacteria–yeast hydrolysates. Muramic acid co-eluted with other compounds and could only be detected when the HPLC–MS technique was used. Notably, the sample analysed by HPLC–MS contained a fivefold lower bacteria-to-yeast ratio than the sample analysed with UV detection; the total amount of yeast cells was identical.

DISCUSSION

Muramic acid is a unique compound, as it is present in bacterial peptidoglycan but absent elsewhere in nature. The amount of muramic acid in bacteria varies between different strains and genera, and there is usually more in Gram-positive than in Gram-negative species [9]. Muramic acid has been used as a chemical marker for the determination of bacteria, *e.g.*, in soil [4], and surface microlayers and sediments in marine environments [5]. This acid has also been used for the detection of bacterial cell debris in mammalian tissues in animal models of arthritis [14] and for the detection of bacteria in joint fluids from patients with septic arthritis [6]. Another possible use for muramic acid determination is in the detection of bacterial infections in eukaryotic cell cultures used in biotechnical processes. We have previously reported the application of GC–MS analysis of 3-hydroxymyristic acid in industrial fermentations as a means of detecting trace levels of Gram-negative bacteria [15]. In contrast to GC–MS methods, HPLC–MS allows several microbial constituents, such as amino acids and carbohydrates, to be analysed underivatized. This means that sample treatment will be simplified considerably and that there will be a decrease in the overall analysis time.

Throughout this study, the samples were dissolved in the mobile phase to ensure constant chromatographic conditions. To avoid long retention times and to obtain good peak shapes, a comparatively short column (100 mm × 4.0 mm I.D.) was used. After prolonged use, the ability of the column to separate the α- and β-anomers of muramic acid decreased and the retention times were reduced (Fig. 5).

When determining the recovery of muramic acid from bacterial samples (Fig. 6a), the data points were greatly scattered, resulting in a wide confidence interval; this shows the disadvantage of using external standardization where variations in injection volumes and, perhaps of more importance, ionization conditions are not compensated for. The precision was significantly improved by internal standardization (Fig. 6b), although the amounts found were significantly higher than the amounts added, which may indicate some imperfections in the behaviour of the internal standard.

An advantage of using MS as the HPLC detector when analysing complex samples is the high selectivity offered. Because of this selectivity, the muramic acid determinations in this study were possible, even though appreciable amounts of water-soluble cellular hydrolysis products (*e.g.*, carbohydrates, amino acids and peptides) were co-injected owing to the minimum of sample purification. The measurement of three ions typical of muramic acid (*m/z* 234, 252 and 270), at identical retention times and with similar peak shapes, ascertained the identity of muramic acid in the sample (Fig. 5). There was no indication that co-eluting substances interfered with the determination of muramic acid in *B. subtilis* cells in the presence of high concentrations of *S. cerevisiae* (Figs. 7 and 9). In contrast, UV detection in HPLC analysis of a corresponding sample was insufficient, despite a more favourable bacteria-to-yeast ratio (Fig. 8).

Degradation of muramic acid in water solution has been reported previously [11]. As fragments of *m/z* 216, 234 and 251 were found in the mass spectrum of the degradation product, it might represent a lactam compound formed by conversion of muramic acid [8,16]. Clearly, the rapid elution of the

product from the cation-exchange column indicates that no interaction occurs. This degradation of muramic acid should be taken into account in quantitative studies of biological samples.

A major advantage of plasma spray over thermospray MS is the wide choice of mobile phases that can be used. This means that whereas thermospray MS requires a volatile ionic buffer with high ionic strength for the ionization, effluents from both normal- and reversed-phase columns can be introduced directly into the MS system under plasma spray conditions. In the thermospray analyses of muramic acid, the ammonium acetate concentration had to be increased from 0.02 to 0.1 *M* to achieve sufficient ionic strength; this also resulted in a higher pH. Under these conditions, muramic acid was not retarded on the stationary phase. Therefore, to be able to use thermospray analysis of muramic acid in complex matrices, the ammonium acetate has to be added postcolumn.

The development of HPLC–MS techniques has been intensified during recent years, resulting in improvements in reproducibility and sensitivity. The detection limit (3 ng) of the described assay for muramic acid corresponds to about $3 \cdot 10^5$ bacterial cells, provided that muramic acid accounts for 1% of the total cell mass and that the dry weight per cell is $1 \cdot 10^{-12}$ g. HPLC methods utilizing fluorescence detection may offer lower detection limits [4,5], but these methods also entail much lower selectivity than mass spectrometric detection. GC–MS is a sensitive and very useful method for the determination of muramic acid, but the need for derivatization leads to extensive sample handling. At present, GC–MS cannot be used in applications where a short analysis time is required [3,7–9].

The HPLC–MS technique described here may represent an interesting alternative to traditional microbiological methods, *e.g.*, to detect bacterial contamination of eukaryotic cultures in industrial fermentations. For this specific application, rapid and specific detection of small amounts of the contaminating bacteria is desirable. Owing to the extreme complexity of sample matrices containing eukaryotic cells, cultivation medium and various metabolites, the selectivity of the proposed method should be very valuable.

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