ELSEVIER

Journal of Chromatography A, 776 (1997) 205–219

JOURNAL OF CHROMATOGRAPHY A

Quantitative analysis of neutral and acidic sugars in whole bacterial cell hydrolysates using high-performance anion-exchange liquid chromatography–electrospray ionization tandem mass spectrometry

D.S. Wunschel[a], K.F. Fox[a], A. Fox[a,*], M.L. Nagpal[a], K. Kim[a], G.C. Stewart[b], M. Shahgholi[c]

[a]*University of South Carolina, School of Medicine, Department of Microbiology and Immunology, Columbia SC, 29209, USA*
[b]*Department of Diagnostic Medicine/Pathobiology, Kansas State University, College of Veterinary Medicine, Manhattan, KS 66506-5603, USA*
[c]*Naval Research Laboratory, Chemistry Division, Code 6113, Washington DC, 20375, USA*

Received 30 December 1996; revised 25 March 1997; accepted 25 March 1997

Abstract

A procedure for analysis of a mixture of neutral and acidic sugars in bacterial whole cell hydrolysates using high-performance anion-exchange liquid chromatography–electrospray ionization tandem mass spectrometry (HPAEC–ESI–MS–MS) is described. Certain bacteria (including bacilli), grown under phosphate-limited conditions, switch from producing a teichoic acid (containing ribitol) to a teichuronic acid (characterized by glucuronic acid content). Bacterial cells were hydrolyzed with sulfuric acid to release sugar monomers. The solution was neutralized by extraction with an organic base. Hydrophobic and cationic contaminants (including amino acids) were removed using C_{18} and SCX columns, respectively. HPAEC is well established as a high-resolution chromatographic technique, in conjunction with a pulsed amperometric detector. Alternatively, for more selective detection, sugars (as $M-H^-$ ions) were monitored using ESI–MS. In HPAEC, the mobile phase contains sodium hydroxide and sodium acetate, which are necessary for chromatographic separation of mixtures of neutral and acidic sugars. Elimination of this high ionic content prior to entry into the ESI ion source is vital to avoid compromising sensitivity. This was accomplished using an on-line suppressor and decreasing post-column flow-rates from 1 ml to 50 μl/min. In the selected ion monitoring mode, background (from the complex sample matrix as well as the mobile phase) was eliminated, simplifying chromatograms. Sugar identification was achieved by MS–MS using collision-induced dissociation. © 1997 Elsevier Science B.V.

Keywords: Sugars; Teichoic acids; Teichuronic acids

1. Introduction

Whole cell carbohydrate profiles provide information on characteristic structural polysaccharides as well as allowing taxonomic differentiation among bacterial species. This can be accomplished using gas chromatography separation followed by detection with a non-selective flame ionization detector [1–3]. Using gas chromatography–mass spectrometry (GC–MS), operated in the selected ion monitoring mode, simple chromatograms that are free of background interferences are generated. In total ion mode, MS

*Corresponding author.

0021-9673/97/$17.00 © 1997 Elsevier Science B.V. All rights reserved
PII S0021-9673(97)00356-7

can be used to identify each sugar component [4–7]. Unfortunately, time-consuming derivatization is essential for GC–MS analysis. Several derivatization methods have been used for carbohydrate profiling of bacterial cells [1–3]. Alditol acetates are often preferred because a single stable derivative is produced for each sugar, producing simple chromatograms [8,9]. Both neutral and amino sugars can be analyzed. However, acidic carbohydrates require further derivatization steps to modify the carboxyl group.

Sugars can be analyzed by liquid chromatography (LC) without prior derivatization. High-performance anion-exchange chromatography (HPAEC) allows excellent separation of simple mixtures of neutral, acidic and amino sugars. The sugars form anions in the presence of high concentrations of sodium hydroxide and are detected with pulsed amperometric detection (PAD) [10,11]. The monomeric composition of purified structural components (e.g. polysaccharides or glycopeptides) can be analyzed by this technique [12,13]. However, PAD is relatively non-selective and is unsuitable for complex biological matrices. Post-column derivatization has been employed to allow more selective detection [14,15]. However, identification of sugar components requires the use of the mass spectrometer.

The potential of HPAEC–MS was demonstrated previously for mixtures of sugar standards first with thermospray and then with increased sensitivity using electrospray ionization [16,17]. In HPAEC, the mobile phase contains sodium hydroxide and sodium acetate, which are necessary for chromatographic separation of mixtures of neutral and acidic sugars. Elimination of this high ionic content prior to entry into the electrospray ionization (ESI) source is vital to avoid compromising sensitivity. This was accomplished using an on-line anion suppressor. This device removes Na^+ ions, replacing them with H^+ ions. It has been noted that the suppressor also eliminates amino sugars along with other cations [17]. Carbohydrates have been analyzed by ESI in positive ion mode as metal ion adducts (including sodium, cobalt and lithium) [16–18] and in negative ion mode as anion adducts (including chloride and acetate) [19,20].

In a previous report, the feasibility of analysis of sugars in whole cell hydrolysates by HPAEC–MS was demonstrated [20]. In the current report, the research was focused on maintaining flow-rates and ionic content that were optimal for both chromatography and MS. This involved adjusting the interface conditions between LC and MS. It was found that sugars readily ionize from aqueous solutions as non-adducted molecular ions $[M-H]^-$. Despite the selectivity of the mass spectrometer, sample clean-up is necessary to prevent contamination of the LC column, which affects chromatographic performance [21,22]. Hydrophobic and cation-exchange clean-up columns have been commonly employed prior to HPAEC [23,24]. Peaks were monitored in the selected ion monitoring (SIM) mode, which eliminated background from non-carbohydrate components of the sample matrix as well as the mobile phase, thus simplifying chromatograms. Additionally, sugar identification was achieved in the product ion mode after collision-induced dissociation (CID).

The teichoic acid of Gram-positive bacteria has an essential physiological role. This polymer is composed primarily of alternating units of either glycerol or ribitol phosphate and, therefore, possesses a negative charge. During growth of bacilli under phosphate-limiting conditions, an alternate polymer, teichuronic acid (which contains glucuronic acid) is synthesized [25,26]. However, whether this switch occurs in staphylococci is more controversial [27], although these organisms do synthesize glucuronic acid [28]. Using the HPAEC–ESI–MS–MS method described, the transition from a teichoic acid to a teichuronic acid (by change in carbohydrate composition) was monitored.

2. Experimental

2.1. Growth conditions

The bacterial strains (*Bacillus subtilis*, W23 and a clinical isolate of *Staphylococcus aureus*, DAW) were grown in a minimal medium consisting of 42 m*M* $MgSO_4$, 278 m*M* glucose, 31 m*M* thymine, 10 g/l of gelatone, 7.6 m*M* $(NH_4)_2SO_4$, 1.4 m*M* sodium citrate and 1× final concentration of BME vitamin solution (Gibco BRL, Grand Island, NY, USA). The medium was supplemented with 7.6 m*M* K_2HPO_4, 2.9 m*M* KH_2PO_4 (total phosphate con-

centration of 10.5 m*M*); 0.065 m*M* K_2HPO_4 and 0.025 m*M* KH_2PO_4 (0.09 m*M* total phosphate) or 0.016 m*M* K_2HPO_4 and 0.006 m*M* KH_2PO_4 (0.023 m*M* total phosphate). Cells were grown in 200 ml of liquid culture while being shaken for 24–48 h at 37°C. The cells were harvested by centrifugation at 16 000 *g* and were washed three times in water. After autoclaving, the pellet was then freeze-dried.

2.2. Sample preparation for analysis by LC–MS–MS

A 10-mg amount of freeze-dried bacteria in 1 ml of 1 *M* H_2SO_4 were heated under vacuum at 100°C for 3 h to liberate sugar monomers. After hydrolysis, 50 μg each of arabinose and galacturonic acid internal standards (Sigma, St. Louis, MO, USA) were added to the reaction mixture. External standards contained 50 μg each of glycerol, ribitol, fucose, quinovose, rhamnose, galactose, glucose, mannose, and glucuronic acid (Sigma), and 50 μg of each internal standard in 1 *M* H_2SO_4. The standards and bacterial samples were then neutralized with 2.5 ml of N,N′-dioctylmethylamine–$CHCl_3$ (50:50, v/v). The aqueous phase was then placed on a C_{18} column (Octadecyl spe, J.T. Baker, Phillipsburg, NJ, USA) to extract hydrophobic contaminants. The cationic species were removed using an SCX column (Aromatic Sulfonic Acid spe, J.T. Baker). The column was conditioned with methanol, water and 2 *M* sulfuric acid, followed by six washes with water [29]. Samples previously acidified with approximately 0.05 *M* H_2SO_4 (51.50 μl of 2 *M* H_2SO_4 added to the 2.0 ml sample volume) were applied to the SCX column and then eluted with another 1 ml of 0.05 *M* H_2SO_4. The samples were then neutralized again using an equal volume of N,N′-dioctylmethylamine–$CHCl_3$ (50:50, v/v). After two chloroform extractions to remove any lingering organic base, the samples were dried under a nitrogen gas flow at 60°C. Finally, all samples were resuspended in 200 μl of water and 40 μl were injected into the LC–MS–MS system.

2.3. Chromatography and mass spectrometry instrumentation

The LC system consisted of a GP40 gradient pump (with Carbopac PA1 column), an AS3500 autosampler and an ASRS 2 m*M* self-regenerating anion suppressor (Dionex, Sunnyvale, CA, USA). The mass spectrometer was a Quattro 1 triple quadrupole (Micromass, Danvers, MA, USA). The Dionex and Micromass equipment were designed to run independently under computer control. An autosampler start signal activates the gradient pump. The Micromass Masslynx software provides for an autocontact closure option, which can also accept a start signal from an external source. The autosampler communication terminal [ground and 'inject out' (pins 1 and 6)] were connected to the Quattro 1 external control panel (A727-200 IS8), I/O port (pins 15 and 16).

2.4. Conditions used for high-performance liquid chromatography

Chromatography was performed on a 4 mm Carbopac PA-1 pellicular anion-exchange column (Dionex) at a flow-rate of 1.0 ml/min using the following program: 0–15 min, isocratic conditions of 20 m*M* NaOH; 15–35 min, gradient from 20 m*M* NaOH, 0 m*M* sodium acetate to 100 m*M* NaOH–120 m*M* sodium acetate; 35–45 min, isocratic conditions of 100 m*M* NaOH–120 m*M* sodium acetate. Column regeneration was performed from 45 to 75 min at 200 m*M* NaOH, followed by a reequilibration phase at the initial 20 m*M* NaOH concentration for 30 min.

2.5. Interfacing of HPAEC system to the electrospray tandem mass spectrometer

The eluent from the 4 mm column was neutralized using a Dionex 2 mm anion suppressor run in the external water mode. The water regenerate (5 ml/min flow-rate) supplied free hydrogen ions through water hydrolysis using 300 mA of current. During the initial isocratic period, NaOH is replaced by water (pH remains neutral) and, later, sodium acetate is also replaced by acetic acid (pH 3–4). Column flow (1 ml/min) was split 1:4 using a low dead volume stainless steel tee (1/16 in. fittings; 1 in.= 2.54 cm) in order to reach the optimal flow-rate for the suppressor. All connections to the column, the tee and the ion suppressor used polyether ether ketone (PEEK) tubing (127 μm I.D.). The distance

between the analytical column/suppressor and the electrospray ion source was minimized to avoid peak broadening.

2.6. Mass spectrometry

The 250 μl/min flow-rate from the anion suppressor was split 1:5 using a second tee and a segment of fused-silica tubing (50 μm I.D.) as a waste line. Flow of column effluent to the ESI needle (also through a 50-μm fused-silica capillary) was adjusted by altering the length of the waste line. The ESI needle voltage was 2.2 kV and the skimmer lens energy was 30 eV. Nitrogen gas was used as the nebulizing gas and the bath gas with flow-rates of 15 and 325 l/h, respectively. The source temperature was maintained at 150°C to aid desolvation.

Chromatographic peaks were monitored by SIM of $[M-H]^-$, except for glycerol where an appreciable amount of acetate adduct $[M+59]^-$ was also monitored. A 0.5 mass unit window was used for each mass, with a scan speed of 0.12 s per ion. For CID, the argon gas was at $5.0 \cdot 10^{-5}$ mbar and the collision energy was 20 eV.

3. Results and discussion

The requirements for ionic content of the mobile phase for HPAEC and nebulization during ESI for MS analysis are quite distinct. HPAEC requires a fixed concentration of NaOH (for neutral sugar separation) followed by a gradient of NaOH and sodium acetate (for acidic sugar analysis). ESI is affected adversely by high ionic content, which influences both the degree of ionic adduction (complicating mass spectral interpretation) and the efficiency of ionization (adversely affecting sensitivity). We chose not to modify the chromatography as conventionally performed by HPAEC–PAD. However, it was vital to decrease the ionic content of the mobile phase prior to electrospray ionization. This was achieved by a combination of on-line anion suppression and reduction of the LC flow-rate from 1 ml to 50 μl/min. It is important to note that ESI is a concentration- (not mass) dependent technique. Thus, no loss in sensitivity was observed from reducing the column flow.

3.1. Electrospray ionization

This is the first report of detecting both neutral and acidic sugar monomers as $[M-H]^-$ ions by ESI without relying on the formation of charged adducts. Although, as noted above, neutral carbohydrates have typically been detected as charged adducts on MS analysis. Given the acetate requirements for HPAEC, large amounts of acetate are introduced into the ESI source. In the presence of acetate, sugar adducts are generated, their abundances influenced by the flow-rate. For example, at a flow-rate of 250 μl/min, both molecular ion and sugar–acetate adducts were formed with nearly equal abundance for many neutral sugars. However, glycerol was unique in that acetate adduction predominated. In contrast, when the solvent flow was decreased to 50 μl/min, the non-adducted molecular ion $[M-H]^-$ was observed almost exclusively. Acidic sugars exist as pre-formed ions in solution and, thus, did not form any significant acetate adducts ions, on desorption, at either flow-rate. Thus, to simplify mass spectral analysis, a flow-rate of 50 μl/min was selected for LC–MS–MS analysis. Fig. 1 demonstrates the production of negatively charged molecular ions from ribitol (a neutral sugar) and glucuronic acid (an acidic sugar) in water at a flow-rate of 250 μl/min. The higher mass ions represent water adducts $[M-H+n\ (18)]^-$, where $n=1-5$. When acetate is present, both $[M-H]^-$ and $[M+59]^-$ are observed for ribitol (m/z 151 and 211, respectively), whereas for glucuronic acid, acetate adduction is not observed.

3.2. Interfacing the chromatography with the ESI source

Neutral sugars elute during the isocratic portion of the chromatographic run where the concentration of NaOH is 20 m*M*. In contrast, acidic sugars elute after a gradient of NaOH and sodium acetate (with their final concentrations equal to 100 and 120 m*M*, respectively). Before the eluent flow can be introduced into the electrospray source, much of this ionic content must be eliminated, otherwise poor ionization occurs. A 2-mm anion suppressor (operated at 300 mA) was employed to generate H^+ ions to replace the Na^+ ions in the eluent flow prior to MS analysis. Column flow (1 ml/min) was split 1:4

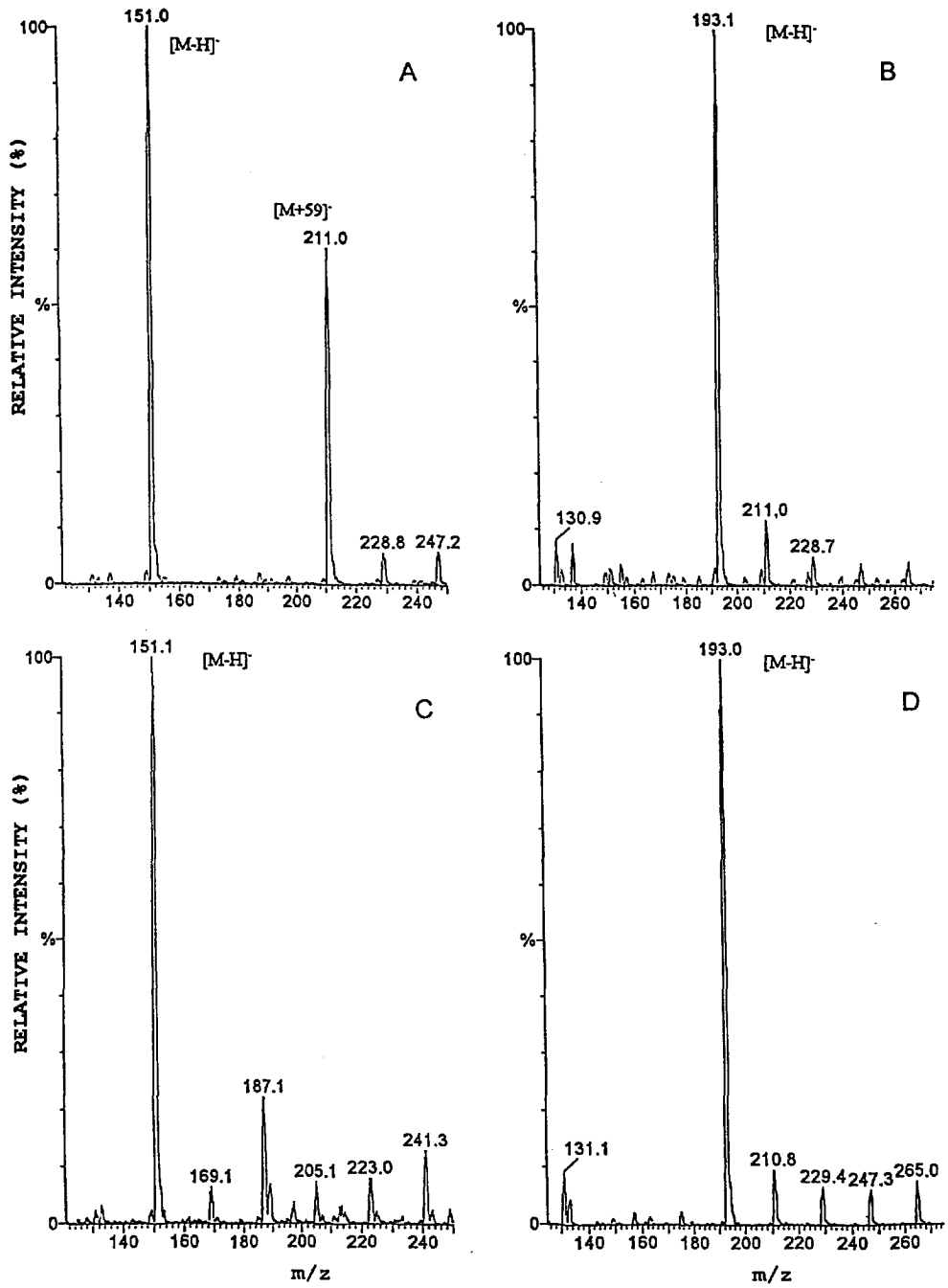

Fig. 1. Mass spectra of the molecular ions for ribitol and glucuronic acid in the presence or absence of acetate. (A) and (B) respectively display spectra obtained for ribitol and glucuronic acid in the presence of 10 m*M* acetic acid. Note the presence of $[M-H]^-$ for each sugar (ribitol, *m/z* 151.0 and glucuronic acid, 193.0) and the acetate adduct (*m/z* 211.0) for ribitol only. (C) and (D) respectively show the spectra for ribitol and glucuronic acid in water alone, displaying only the molecular ions. Water adduction is apparent with $[M-H+nH_2O]^-$ where $n=1-5$.

to reach the optimal inlet flow-rate for the suppressor. The suppressor outlet flow (250 μl/min) was split 1:5, resulting in 50 μl/min flow into the ESI source. Chromatograms were generated in the negative-ion mode using SIM monitoring of the masses corresponding to the molecular ions $[M-H]^-$. Fig. 2 demonstrates detection of a mixture of sugars after chromatographic separation. All sugar components were detected with similar efficiency. When the operating current was dropped to 100 mA, the intensities of the neutral sugar peaks were affected minimally. However, a dramatic decrease in the acidic sugar signal was observed; acidic sugars elute when the ionic concentration is highest. Ion suppression, preferentially occurring in the acidic sugar region, is readily apparent on comparing Fig. 3 with Fig. 2. A similar reduction of signal was observed when the flow-rate into the MS system was increased from 50 to 250 μl/min.

3.3. Chromatography

Despite the steps required to interface the LC and MS systems, chromatographic resolution was maintained and was similar to that observed with an HPAEC–PAD system. Sugars selected for analysis corresponded to those known to be found in whole cell hydrolysates of bacteria. The sugar alcohols (glycerol and ribitol, which are found in teichoic acid) eluted first, followed by methylpentoses (rhamnose, fucose and quinovose, which are found in spores of bacilli). Hexoses (mannose, galactose and glucose) are widely distributed in bacteria. The pentoses selected were arabinose and ribose, which elute prior and just after the three hexoses, respectively. Arabinose was chosen as an internal standard for neutral sugars since it is not commonly found in bacteria. Ribose is a component of ribonucleic acid. Glucuronic acid is an acidic sugar of particular interest because it is found in teichuronic acid. Galacturonic acid was selected as an internal standard because it is not generally found in teichuronic acid. All sugars were almost baseline resolved, with the exception of the hexoses. Others have also noted that (under isocratic conditions) this is a difficult separation. Although, using certain gradients, it is possible to baseline resolve these sugars on a PA-1 column [30]. It should be noted that glycerol was exceptional in its tendency to form acetate adducts that appeared at m/z 151, the same as non-adducted ribitol. It was therefore important to have chromatographic separation of these two compounds to eliminate any contribution of glycerol acetate to the ribitol signal.

3.4. Sample preparation

The bacterial samples were subjected to acid hydrolysis to release sugar monomers. Using a selective detector minimizes the need for sample clean-up. However, it was found (in agreement with others) that HPAEC is adversely affected by the presence of extraneous components [21–24]. C_{18} columns were employed to remove hydrophobic contaminants and SCX columns were used to remove cations, including amino acids. After analysis of bacterial hydrolysates, it was observed that retention times shifted in subsequent runs. Accordingly, standard mixtures of sugars were chromatographed twice after each hydrolysate to verify optimal column performance and avoid column fouling. When necessary, the column was cleaned by washing it with 1 *M* HCl for 1–3 h.

3.5. Quantitation

The standard curves were generated for each of ten sugars (glycerol, ribitol, rhamnose, fucose, quinovose, mannose, galactose, glucose, ribose and glucuronic acid). Duplicate samples (seven levels ranging from 5 to 320 μg of each compound) and two blank samples were analyzed. To each sample, 50 μg of arabinose (internal standard for neutral sugars) and galacturonic acid (internal standard for glucuronic acid) were added. All samples, in 1 *M* sulfuric acid, were taken through the entire analytical procedure. The ratio of peak area for each sugar compared to its internal standard was plotted against the total amount of that sugar in the sample. $M-H^-$ were detected in the SIM mode for all sugars, except glycerol. For glycerol, as noted above, there was a significant amount of acetate adduction. Thus, the sum of $[M-H]^-$ and $[M+59]^-$ was used in the calculation.

Table 1 lists the R^2 values for each plot. With the exception of glycerol, R^2 values were greater than

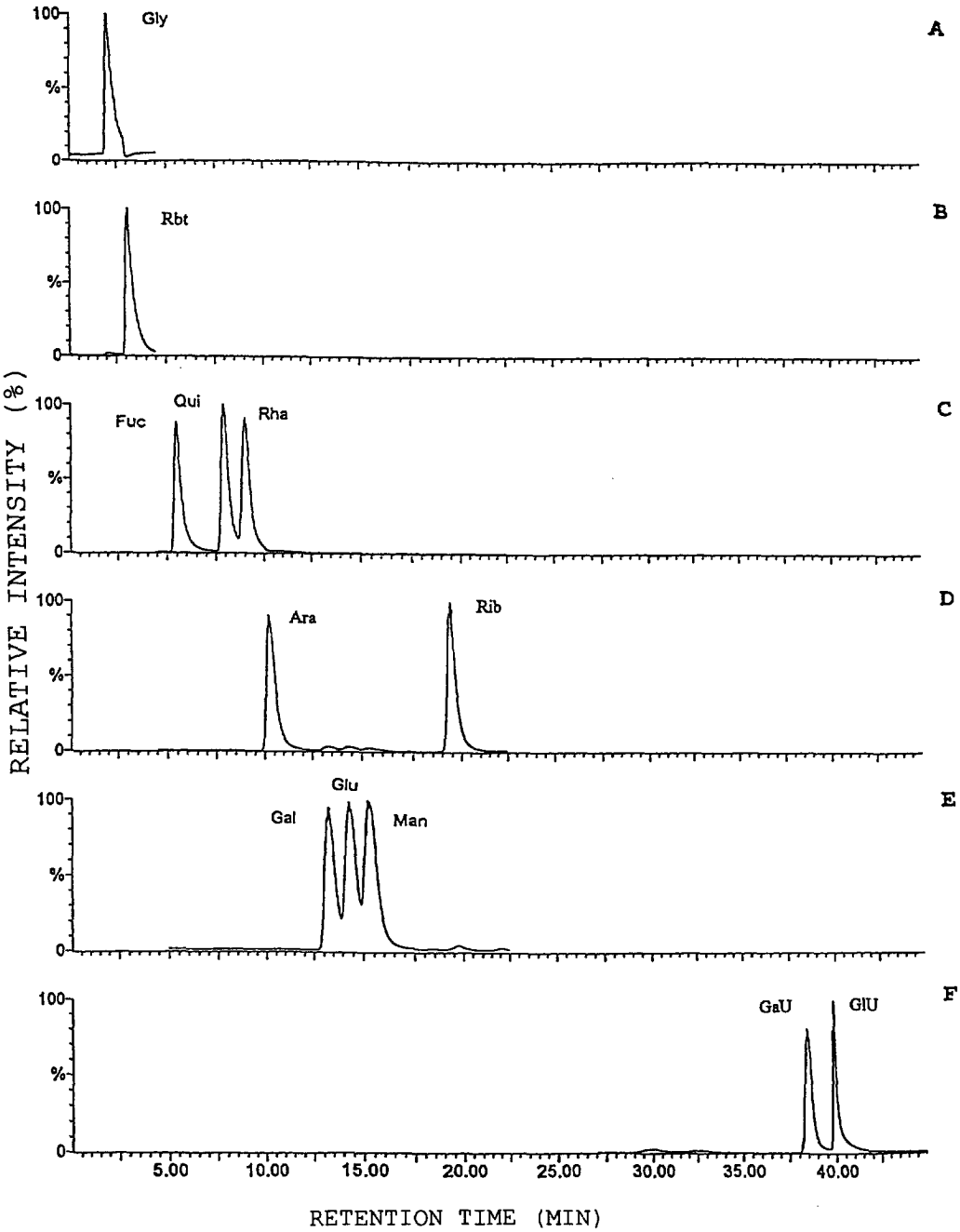

Fig. 2. Single ion monitoring chromatogram of a standard mixture of sugars (4 μg of each sugar injected from a 50 μg/ml solution). The suppressor was operated at 300 mA. $[M-H]^-$ was monitored for each. (A) Glycerol (*m/z* 91), (B) ribitol (*m/z* 151), (C) fucose, quinovose and rhamnose (*m/z* 163), (D) arabinose and ribose (*m/z* 149), (E) galactose, glucose and mannose (*m/z* 179), (F) galacturonic and glucuronic acids (*m/z* 193). The abbreviations used are as follows: Gly=glycerol, Rbt=ribitol, Fuc=fucose, Qui=quinovose, Rha=rhamnose, Ara=arabinose, Gal=galactose, Glu=glucose, Man=mannose, Rib=ribose, GaU=galacturonic acid and GlU=Glucuronic acid.

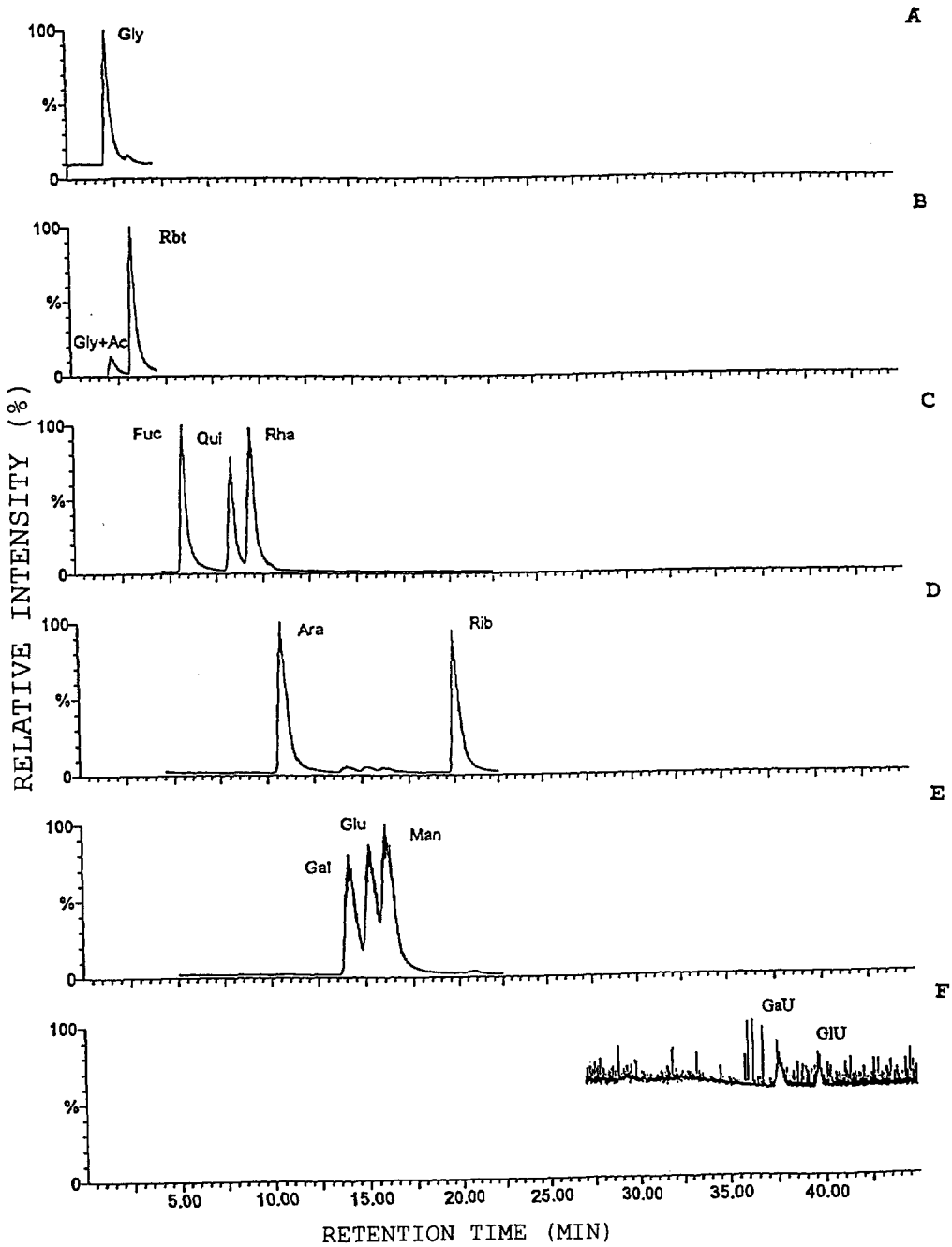

Fig. 3. Single ion monitoring chromatogram of a standard mixture of sugars (4 μg of each sugar injected from a 50 μg/ml solution). The suppressor was operated at 100 mA. $[M-H]^-$ was monitored for each. (A) Glycerol (*m/z* 91), (B) ribitol (*m/z* 151), (C) fucose, quinovose and rhamnose (*m/z* 163), (D) arabinose and ribose (*m/z* 149), (E) galactose, glucose and mannose (*m/z* 179), (F) galacturonic and glucuronic acids (*m/z* 193). See Fig. 2 for abbreviations. All panels are shown with the same magnification, with the exception of (F), which is magnified 100×. This demonstrates the dramatic suppression of the glucuronic acid signal due to the high ionic content of the mobile phase. Gly+Ac refers to the acetate adduct peak for glycerol, which is seen at *m/z* 151.

Table 1
Reproducibility, linearity and sensitivity of the procedure

Compound	R^2	Relative S.D.	Signal-to-noise ratio
Glycerol	0.873	33.1	15.6
Ribitol	0.989	3.6	106.8
Fucose	0.964	10.0	33.8
Quinovose	0.984	4.2	34.5
Rhamnose	0.978	6.3	30.3
Galactose	0.993	7.9	11.7
Glucose	0.994	4.5	12.8
Mannose	0.983	4.0	13.1
Ribose	0.979	20.7	18.5
Glucuronic acid	0.996	3.5	10.9

0.964. The relative standard deviation for each sugar was determined using five replicates containing 40 μg of each sugar and 50 μg of internal standard. The relative standard deviation was less than 10%, with the exceptions of glycerol and ribose. Glycerol is more volatile than other sugars and may have been partially lost during the evaporation steps. Ribose elutes during the sodium hydroxide–sodium acetate gradient step, with its internal standard eluting during the isocratic portion of the run. Thus, they are ionized under different ESI conditions. With the exception of ribitol, the signal-to-noise ratio for the 5 μg samples was in the range of 10.9 to 34.5. This indicates that the detection limit was in the low microgram to high nanogram level for each sugar.

3.6. Applications

B. subtilis W23 synthesizes a ribitol containing teichoic acid in the presence of phosphate excess. Under conditions of phosphate limitation, teichoic acid production is replaced by teichuronic acid (a glucuronic acid-containing polymer) [25,26,31]. In this report, markers for each of these polymers (along with ions for pentoses and methylpentoses) were analyzed in cells grown under both normal and phosphate-limiting conditions: m/z 151.0 (glycerol+ acetate, and ribitol), m/z 163.0 (methylpentoses), m/z 149.0 (pentoses) and m/z 193.0 (hexuronic acids). Figs. 4 and 5 represent the sum of the SIM channels (of all ionic species monitored), with the intensities normalized to the largest peak. Ribitol was readily detected in cells grown under conditions of phosphate excess (0.59% on a dry weight basis), while the peak for glucuronic acid was too low to quantitate. For cells grown under phosphate-limiting conditions, ribitol production was dramatically decreased (0.03%) and glucuronic acid was a major component of chromatograms (0.31%). In neither case were methylpentoses characteristic of the spores detected. Ribose, from RNA, was detected in each instance.

The presence of teichoic acids in *S. aureus* is well documented, but the presence of a teichuronic acid is more controversial [27]. Glucuronic acid (a marker for teichuronic acid) has been reported to be synthesized by certain strains of *S. aureus* [28]. *S. aureus* strain DAW was grown under the same conditions as those used for *B. subtilis* W23. Staphylococci that were cultured under normal phosphate conditions produced a large amount of ribitol (0.42%). Ribitol production did not decrease under phosphate-limiting conditions (0.49%). Trace levels of a peak at the retention time for glucuronic acid were observed under normal phosphate conditions. There was a slight increase in the amount of glucuronic acid under the phosphate-limiting conditions (0.06%) (see Fig. 4). Our results agree with those of Dobson and Archibald [27] and we can provide no strong evidence for a teichoic acid to teichuronic acid switch in *S. aureus*.

3.7. MS–MS identification

Further steps were taken to confirm the identity of sugars present in bacterial hydrolysates based on their product spectra. Product ions are generated from the molecular ion by covalent bond cleavage

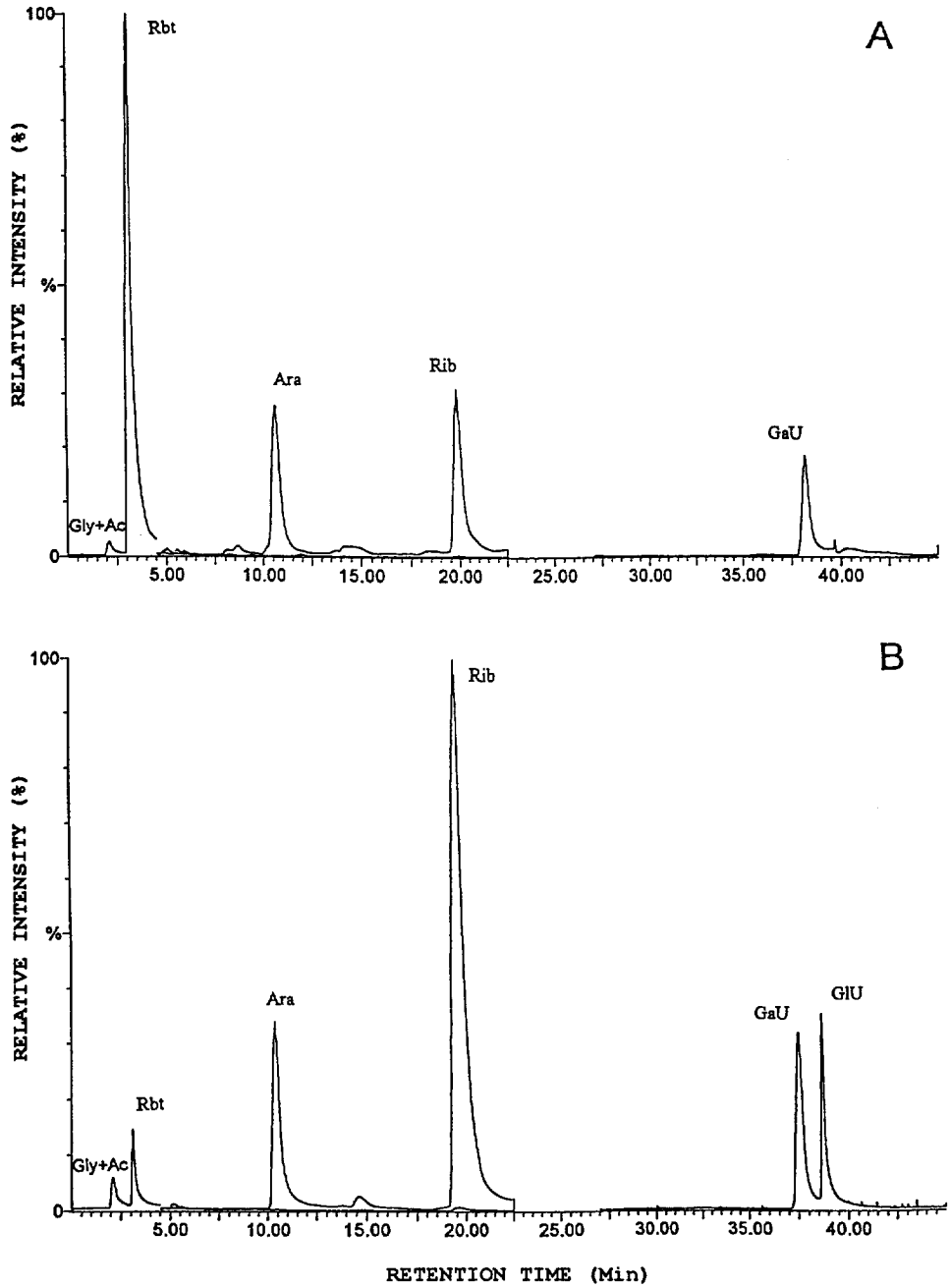

Fig. 4. SIM traces for four ions [m/z 151.0 (0–4.5 min), 163.0 and 149.0 (4.5–22.5 min) and 193.0 (27.0–45.0 min)]. All peaks are normalized to the peak of highest abundance in the chromatograms. Sugars present in *B. subtilis* W23 (A) grown under normal (10.5 m*M*) phosphate conditions and (B) under (0.09 m*M*) low phosphate conditions. Abbreviations are defined in Fig. 2. Note the large amount of ribitol and the absence of glucuronic acid in (A). There is a significant decrease in ribitol and the appearance of glucuronic acid in (B). No spore-specific methylpentoses are noted in either chromatogram.

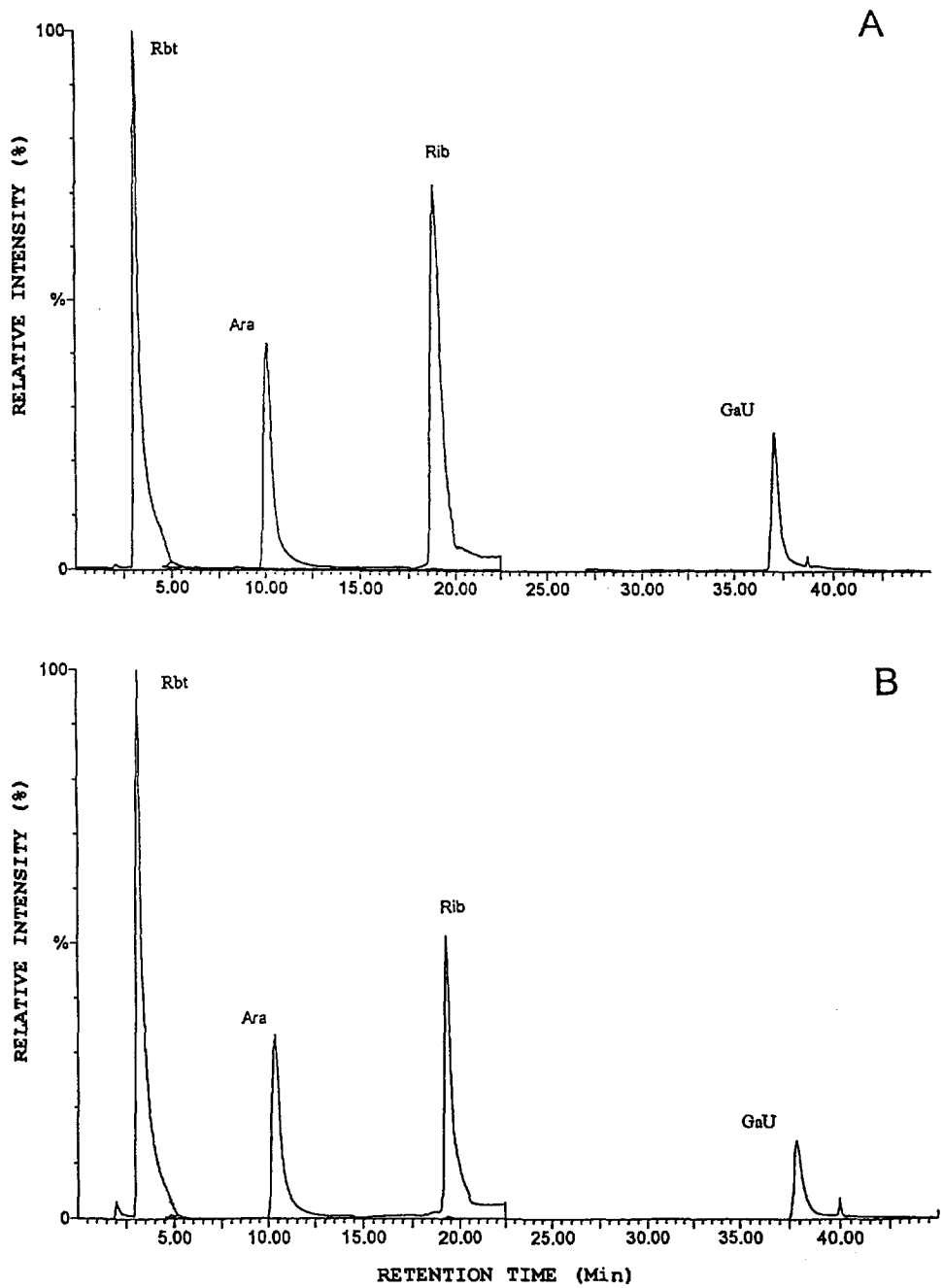

Fig. 5. SIM traces for four ions [m/z 151.0 (0–4.5 min), 163.0 and 149.0 (4.5–22.5 min) and 193.0 (27.0–45.0 min)]. All peaks are normalized to the peak of highest abundance in the chromatograms. Sugars present in *S. aureus* DAW (A) grown under normal (10.5 mM) phosphate conditions and (B) under (0.09 mM) low phosphate conditions. Abbreviations are defined in Fig. 2. Note the large amount of ribitol and the absence of glucuronic acid in (A). Note that (B) has a similar appearance to (A).

within the sugar backbone and/or losses of water. For example, for the neutral sugar, ribitol, product ions *m/z* 119, 101, 89, 85, 83, 73, 71, 59 and 55 resulted from fragmentation of the *m/z* 151 parent ion. For the acidic sugar, glucuronic acid, *m/z* 131, 113, 103, 101, 95, 89, 85, 73, 71 and 59 were generated from *m/z* 193. As can be seen in Fig. 6, product ion spectra for pure sugar standards are identical to the corresponding sugars found in bacterial cell hydrolysates. An interpretation of the major ions present in product spectra for ribitol and glucuronic acid are given in Fig. 7.

4. Conclusions

Neutral and acidic sugars present in whole bacterial cell hydrolysates have been analyzed by HPAEC–ESI–MS–MS. A simple analytical procedure was developed. Samples were hydrolyzed in sulfuric acid and neutralized in organic base. Despite the selectivity of MS detection, it was necessary to utilize a clean-up scheme involving C_{18} and SCX columns for removal of hydrophobic and cationic contaminants (including amino acids), to avoid column fouling. Sugars were detected as $[M-H]^-$ ions in the negative-ion mode from aqueous solution, eliminating the need for post-column addition of adduct forming anions or cations.

For efficient ionization and detection, as noted by others [16,17], the use of an anion suppressor between the analytical column and the MS ion source is vital to reduce the ionic content of the mobile phase. Furthermore, post-chromatographic flow splitting improves the sensitivity of ESI analysis and reduces the complexity of mass spectra, by limiting adduction. In this fashion, optimal flow-rates were maintained for both chromatography and mass spectrometry.

Using appropriate internal standards, excellent reproducibility and linearity was observed in the ESI analysis of a mixture of ten neutral sugars and two acidic sugars. SIM was used to improve the sensitivity of analysis and to eliminate background from the mobile phase and the sample matrix. Product ion spectra allowed absolute identification of sugars. The transition from teichoic to teichuronic acid for *B. subtilis* strain W23, but not for *S. aureus* strain DAW (when grown under conditions of phosphate deprivation), was documented. This work was accomplished using a state-of-the-art triple quadrupole mass spectrometer. Recently, more modestly priced ion trap instruments (with tandem MS capability) have become available. This may help to popularize the approach developed here.

A recent development in LC–MS technology is the trend towards microscale LC (micro-LC) in place of the more traditional analytical-scale LC. As noted above, when using analytical columns, a large portion of the sample is discarded (by splitting flow) before entry into the MS system. When using microcolumns, higher concentrations of the analyte can be placed on a column and the whole eluent can pass into the MS system. The low flow-rates (of the order of 1 μl/min) of microcolumns, on the other hand, are compatible with ESI–MS, requiring no column splitting following chromatography. Furthermore, by focussing the entire sample into a narrow band and passing the entire eluent into the mass spectrometer, the sensitivity for carbohydrate analysis is dramatically increased. Micro-LC has only been reported with packing materials that use ESI-compatible mobile phases. This eliminates the use of ion suppressors, making them more compatible with ESI–MS, however, chromatographic resolution is compromised [32,33]. For example, native chitobiose, in phytoplankton cellular hydrolyzates, has been analyzed using capillary columns packed with porous graphitic carbon [32]. Muramic acid, a sugar marker for bacterial cell walls, has been analyzed in hydrolyzates of organic dust using a custom-made LiChrospher-diol column [33–35].

In conclusion, analysis of sugar mixtures in complex hydrolyzates of bacterial cells was achieved using HPAEC coupled with ESI–MS–MS. Sugars were readily detected in the selected ion monitoring mode for $[M-H]^-$ and were identified by their product ion spectra after collision-induced dissociation.

Acknowledgments

This research was supported by the US Department of Agriculture (94-37204-1088) and ARO/ERDEC DAAH04-95-1-0359. An equipment grant

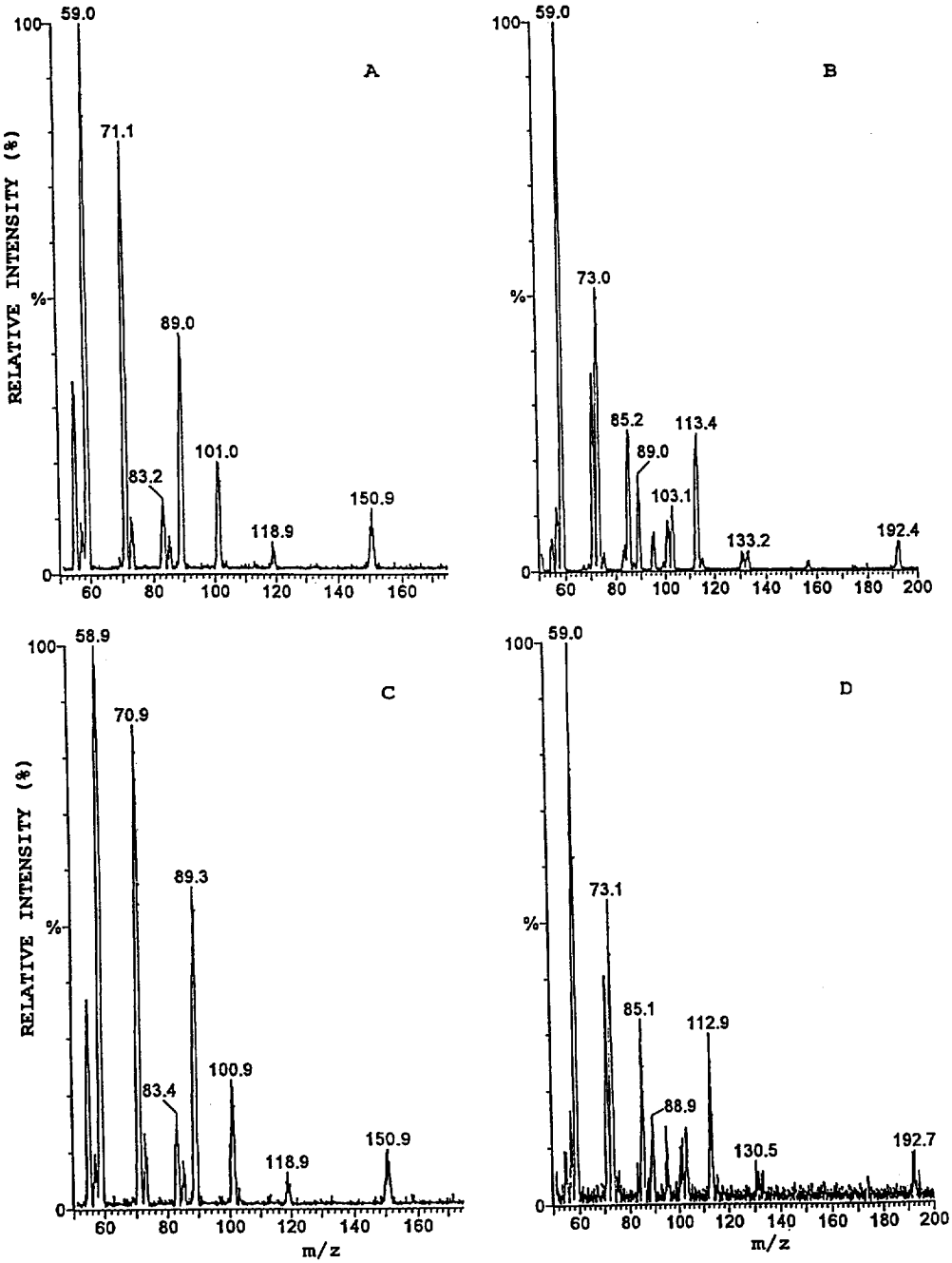

Fig. 6. Daughter ion spectra of (A) ribitol standard, (B) glucuronic acid standard, (C) ribitol produced by *B. subtilis* W23 grown in the normal phosphate (10.55 m*M*) media and (D) glucuronic acid produced by *B. subtilis* W23 grown in low (0.023 m*M*) phosphate media. Each daughter ion spectrum of the sugar present in the bacterial hydrolysate is identical to the corresponding standard spectrum.

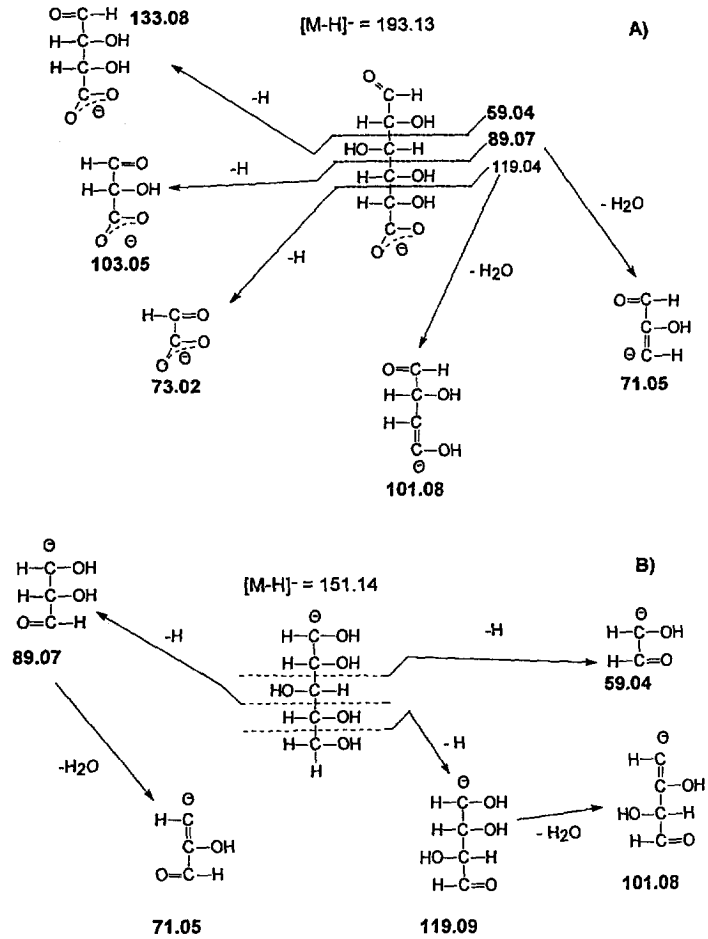

Fig. 7. Interpretations of daughter ion spectra of (A) glucuronic acid and (B) ribitol, respectively. The numbers refer to m/z.

(ARO DAAH0493-G-0506) provided support for MS instrumentation.

References

[1] A. Fox, P.Y. Lau, A. Brown, S.L. Morgan, Z.-T. Zhu, M. Lema, M.D. Walla, J. Clin Microbiol. 19 (1984) 326.
[2] D.J. Pritchard, J.E. Coligan, S.E. Speed, B.M. Gray, J. Clin Microbiol. 13 (1981) 89.
[3] K. Bryn, E. Jantzen, J. Chromatogr. 370 (1986) 103.
[4] M.D. Walla, P.Y. Lau, S.L. Morgan, A. Fox, A. Brown, J. Chromatogr. 288 (1984) 399.
[5] A. Fox, J.C. Rogers, K.F. Fox, G. Schnitzer, S.L. Morgan, A. Brown, R. Aono, J. Clin Microbiol. 28 (1990) 546.
[6] A. Fox, G.E. Black, K.F. Fox, S. Rostovtseva, J. Clin Microbiol. 31 (1993) 887.
[7] D.S. Wunschel, K.F. Fox, G.E. Black, A. Fox, Syst. Appl. Microbiol. 17 (1994) 625.
[8] A. Fox, S.L. Morgan and J. Gilbart, in C.J. Biermann and G.D. McGinnis (Editors), Analysis of Carbohydrates by GLC and MS, CRC Press, Boca Raton, FL, 1989, p. 87.
[9] A. Fox and G.E. Black, in C. Fenseleau (Editor), Mass Spectrometry for the Characterization of Microorganisms (ACS Symposium Series), American Chemical Society, Washington, DC, 1994, p. 107.
[10] R.D. Rocklin, C.A. Pohl, J. Liq. Chromatogr. 6 (1983) 1577.
[11] M.R. Hardy, R.R. Townsend, Y.C. Lee, Anal. Biochem. 170 (1988) 54.
[12] A.J. Clarke, V. Sarabia, W. Keenleyside, P.R. MacLachlan, C. Whitfield, Anal. Biochem. 199 (1991) 68.
[13] N.G. Karlsson, G.C. Hansson, Anal. Biochem. 224 (1995) 538.
[14] R. Anumula, Anal. Biochem. 220 (1994) 275.
[15] G.O.H. Peelen, J.G.N. de Jong, R.A. Wevers, Anal. Biochem. 198 (1991) 334.

[16] R.C. Simpson, C.C. Fenselau, M.R. Hardy, R.R. Townsend, Y.C. Lee, R.J. Cotter, Anal. Chem. 62 (1990) 248.
[17] J.J. Conboy, J. Henion, Biol. Mass Spectrom. 21 (1992) 397.
[18] M. Kohler, J.A. Leary, Anal. Chem. 67 (1995) 3501.
[19] T. Niwa, L. Dewald, J. Sone, T. Miyazaki, M. Kajita, Clin. Chem. 40 (1994) 260.
[20] G.E. Black and A. Fox, in A.P. Snyder (Editor), Biochemical and Biotechnological Applications of Electrospray Ionization Mass Spectrometry (ACS Symposium Series), American Chemical Society, Washington, DC, 1995, p. 81.
[21] C. Corradini, A. Cristalli, D. Corradini, Ital. J. Food Sci. 1 (1994) 103.
[22] J. Sullivan, M. Douek, J. Chromatogr. A 671 (1994) 339.
[23] N.G. Karlsson, G.C. Hansson, Anal. Biochem. 224 (1995) 538.
[24] N. Tomiya, T. Suzuki, J. Awaya, K. Mizuno, A. Matsubara, K. Nakano, M. Kurono, Anal. Biochem. 206 (1992) 98.
[25] D.C. Ellwood, D.W. Tempest, Biochem. J. 111 (1969) 1.
[26] J. Wright, J.E. Heckels, Biochem. J. 147 (1975) 187.
[27] B.C. Dobson, A.R. Archibald, Arch. Microbiol. 119 (1978) 295.
[28] R. Ekstedt, J. Bernhard, Proc. Soc. Exp. Biol. Med. 142 (1973) 86.
[29] K. Ueda, S.L. Morgan, A. Fox, J. Gilbart, A. Sonesson, L. Larsson, G. Odham, Anal. Chem. 61 (1989) 265.
[30] W.K. Herber, R.S.R. Robinett, J. Chromatogr. A 676 (1994) 287.
[31] K.F. Fox, D.S. Wunschel, G.C. Stewart, K.K. Kim and A. Fox, Microbiol., manuscript submitted.
[32] M. Shahgholi, M. Ross, J.H. Callahan, R.A. Smucker, Anal. Chem. 68 (1996) 1335.
[33] M. Shahgholi, S. Ohorodnik, J. Callahan, A. Fox, Anal. Chem. 69 (1997) 1956.
[34] G.E. Black, A. Fox, J. Chromatogr. A 720 (1996) 51.
[35] G.E. Black, A. Fox, K.F. Fox, A.P. Snyder, P.B.W. Smith, Anal. Chem. 66 (1994) 4171.