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Determination of Amino Sugars in Environmental Samples with High Salt Content by High-Performance Anion-Exchange Chromatography and Pulsed Amperometric Detection

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Amino sugars were determined in natural samples, including seawater, using high-performance anion-exchange chromatography with pulsed amperometric detection and a new off-line sample cleanup procedure. Samples were hydrolyzed with 3 M HCl for 5 h (100 °C) and neutralized with an ion retardation resin. Before injection, salts and organic contaminants were removed with a strong cation exchanger in the Na^+ form. Detection limits for amino sugars were between 1 and 4 nM (signal-to-noise ratio 3), allowing for the first time quantification of amino sugars in seawater without preconcentration. Precision was 2−11% at the 20 nM level. The relatively simple and rapid sample preparation makes it suitable for routine analyses.

Carbohydrates are among the most abundant organic molecules in the biosphere. They play important roles in cellular metabolism and recognition as well as in structural polymers.[1] In addition to being important cellular components, carbohydrates comprise a major fraction of nonliving organic matter (1−30%) in terrestrial and aquatic environments.[2] Molecular characterizations have identified neutral sugars (normal, deoxy, and methylated aldoses), N-acetylated amino sugars, and acidic sugars.[3,4,5] Of these classes of carbohydrates, neutral sugars have been studied extensively and provide important information on the origin of dissolved organic matter and its diagenetic state.[6,7] Much less is known about the abundance and distribution of amino sugars. In part, this situation exists because of the lack of suitable methods for the analysis of amino sugars.

The most common amino sugars are glucosamine (GlcN), galactosamine (GalN), and muramic acid (MA). GlcN and GalN are widely distributed in microorganisms, plants, and animals.[8,9] Chitin, a polymer of *N*-acetyl GlcN, is an important structural polymer in fungi, invertebrates, and algae.[9,10] MA, the lactoyl ether of GlcN, exclusively occurs in bacterial cell walls and has been used to estimate bacterial biomass in natural samples.[9,11,12]

Gas chromatography/mass spectrometry (GC/MS) methods have been successfully applied for the determination of amino sugars in bacterial polysaccharides[13,14] and soils.[15,16] However, GC analysis involves sample vaporization, which can lead to losses of sugars and time-consuming derivatization procedures. Due to these limitations, liquid chromatography using electrochemical[17,18] or fluorometric detection[19,20] has become increasingly popular. A recent method employs microscale LC coupled with MS for trace analysis of muramic acid.[21] High-performance anion-exchange chromatography (HPAEC) separates mono- and oligosaccharides due to subtle differences in the pK_a of the hydroxyl groups on a pellicular strong anion-exchange resin. Coupled with pulsed amperometric detection (PAD) it permits quantification of carbohydrates at the femtomole level.[18] Derivatization is not needed, and the whole analysis avoids the use of organic solvents. Nevertheless, samples with a complex matrix require an efficient purification step prior to chromatography to prevent column and detector fouling and the potential overestimation of individual amino sugars due to coeluting compounds. It is mandatory that inorganic salts are removed prior to sample analysis, which is

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(1) Dwek, R. A. *Chem. Rev.* **1996**, *96*, 683−720.
(2) Romankevich, E. A. *Geochemistry of organic matter in the ocean*; Springer: Berlin, Heidelberg, New York, 1985.
(3) Mopper, K.; Dawson, R.; Liebezeit, G., Ittekot, V. *Mar. Chem.* **1980**, *10*, 55−66.
(4) McCarthy, M. D.; Hedges, J. I.; Benner, R. *Mar. Chem.* **1996**, *55*, 281−298.
(5) Boon, J. J.; Klap, V. A.; Eglinton, T. I. *Org. Geochem.* **1998**, *29* , 1051−1061.
(6) Skoog, A.; Benner R. *Limnol. Oceanogr.* **1997**, *42* (8), 1803−1813.
(7) Cowie, G. L.; Hedges, J. I. *Nature* **1994**, *369*, 304−307.
(8) Pigman, W. W. *The Carbohydrates. Chemistry and Biochemistry*, 2nd ed.; Academic Press: New York, 1970; p 435−438.
(9) Lindberg, B. *Adv. Carbohydr. Chem. Biochem.* **1990**, *48*, 279−318.
(10) McLachlan, J.; McInnes, A. G.; Falk, M. *Can. J. Bot.* **1965**, *43*, 707−713.
(11) Millar, W. N.; Casida, L. E. *Can. J. Microbiol.* **1970**, *18*, 292−304.
(12) Moriarty, D. J. W. *Oecologia* **1975**, *20*, 219−229.
(13) Whiton, R. S.; Lau, P.; Morgan, S. L.; Gilbart, J, Fox, A. *J. Chromatogr.* **1985**, *347*, 109−120.
(14) Neeser, J. R.; Schweizer, T. F. *Anal. Biochem.* **1984**, *142*, 58−67.
(15) Benzing-Purdie, L. *Soil Sci. Soc. Am. J.* **1981**, *45*, 66−70.
(16) Amelung, W.; Cheshire, M. V.; Guggenberger, G. *Soil Biol. Biochem.* **1996**, *28* (12), 1631−1639.
(17) Mopper, K.; Schultz, C. A.; Chevolot, L.; Germain, C.; Revuelta, R.; Dawson, *R. Environ. Sci. Technol.* **1992**, *26*, 133−138.
(18) Lee, Y. C. *Anal. Biochem.* **1990**, *189*, 151−162.
(19) Moriarty, D J. W. *J. Microbiol. Methods* **1983**, *1*, 111−117.
(20) Mimura, T.; Delmas, D. *J. Chromatogr.* **1983**, *280*, 91−98.
(21) Shahgholi, M.; Ohorodnik, S.; Callahan, J. H.; Fox, A. *Anal. Chem.* **1997**, *69*, 1956−1960.

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extremely challenging for charged carbohydrates such as amino sugars.

In this paper, we describe a complete and rapid procedure for the analysis of amino sugars with HPAEC-PAD in natural samples with high salt content. Prior to chromatography, salts and organic contaminants are removed with a new off-line sample preparation protocol that produces exceptionally clean chromatograms and detection limits in the femtomole range. Hydrolysis conditions for releasing amino sugars from natural sample matrixes are also investigated.

Materials. Muramic acid, D(+)-galactosamine hydrochloride, D(+)-glucosamine hydrochloride, D(+)-mannosamine hydrochloride (ManN), *N*-acetyl-3-amino-3-deoxy glucose, α-1-glucose phosphate disodium salt hydrate (Glc-1-P), chitin (purified powder from crab shells), chondroitin sulfate A (sodium salt, from bovine trachea, ~70%), *Pseudomonas flourescens* (ATCC 13430, type II), and *Bacillus subtilis* (ATCC 6633, lyophilized cells) were obtained from Sigma Chemical Co. (St. Louis, MO). Hydrochloric acid (36%), sulfuric acid (98%), sodium chloride, and sodium hydroxide solution (50% w/w) were purchased from Fisher Scientific (Pittsburgh, PA). Sodium acetate was from Fluka (Ronkonkoma, NY). AG50 X8 (100−200 and 200−400 mesh) and AG11 A8 (50−100 mesh) resins were obtained from Bio-Rad (Hercules, CA). Peptidoglycan (1 μg/mL suspension, *Micrococcus luteus*) was from Wako Pure Chemical Industries, Ltd. *Synechococcus bacillaris* was grown in seawater.[22] Open ocean seawater and *Trichodesmium* were collected from the Gulf of Mexico. Seawater samples of dissolved organic matter concentrated with ultrafiltration (UDOM) were from the Pacific Ocean and the Gulf of Mexico.[23]

Preparation of 3-Amino-3-deoxyglucose Hydrochloride (Glc3N), Internal Standard. *N*-Acetyl-3-amino-3-deoxy glucose (4 mg) was treated with 2 mL of 2 M HCl at 100 °C for 3 h in a sealed ampule. After cooling in an ice bath, HCl was evaporated in a rotary evaporator. The residue was dissolved in 2 mL of H_2O and loaded on a column with 2 mL of AG50 X8 resin (H^+ form, 100−200 mesh). Before use, the column was conditioned with 4 mL of 1 M HCl followed by 10 mL of water. The resin was washed three times with 2 mL of H_2O, and Glc3N was eluted with 2 resin volumes (4 mL) of 2 M HCl. The eluate was dried in a rotary evaporator to give pure Glc3N (3.6 mg, 90%).

Hydrolysis. A flowchart for sample preparation is shown in Figure 1**.** Seawater samples (0.3−0.8 mg of C/L) were hydrolyzed with 3 M HCl at 100 °C for 5 h. The sample (1.5 mL) was pipetted into 2-mL ampules and 0.5 mL of 12 M HCl was added. The sealed ampules were placed in a covered boiling water bath. Several naturally occurring polymers (chitin, chondroitin A, peptidoglycan from *M. luteus*) and bacteria (*P. flourescens*, *B. subtilis*, *S. bacillaris*, *Trichmodesmium*) were used to test the efficiency of different hydrolyses in releasing amino sugars. Solid polymers (0.1−0.5 mg) were weighed into ampules and dissolved in 1 mL of 3 M HCl. Liquid peptidoglycan suspension (1.5 mL) was diluted with 0.5 mL of 12 M HCl. The bacteria were hydrolyzed with 3 M HCl (5 h, 100 °C), 6 M HCl (2 h, 100 °C), and 1.2 M H_2SO_4 (3 h, 100 °C) after pretreament with 12 M H_2SO_4 (2 h, 25 °C). Dry bacteria (0.2−1 mg) were weighed into ampules and suspended in 1 mL of 3 M HCl or 1 mL of 6 M HCl. After sealing, the ampules were sonicated to disperse the sample. For the sulfuric acid hydrolysis, the bacterial powders were weighed into ampules and immersed in 100 μL of 12 M H_2SO_4. After 2 h, 900 μL of H_2O was added, the ampules were sealed and sonicated. Ampules were placed in an icebath to terminate the hydrolysis.

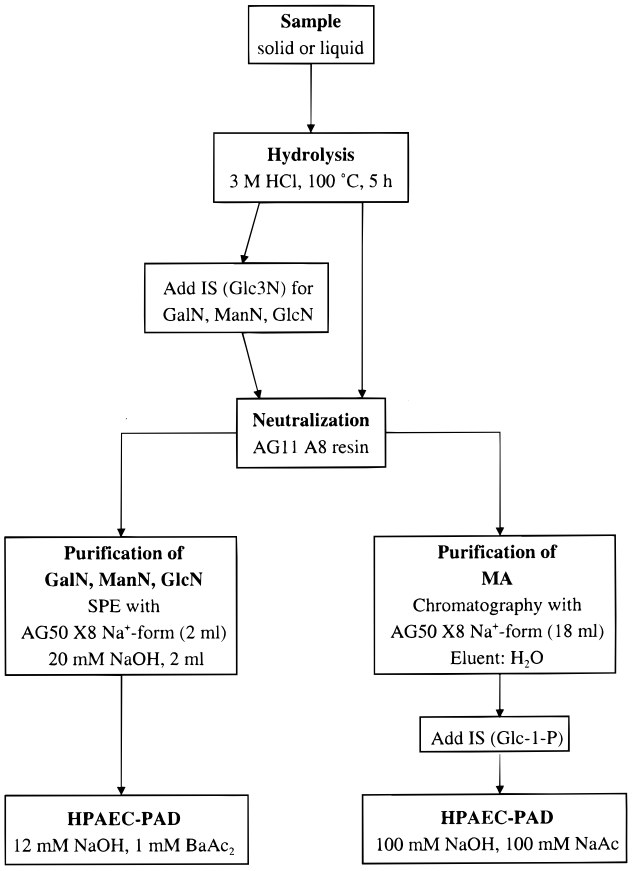

Figure 1. Sample preparation scheme: SPE, solid-phase extraction; $BaAc_2$, barium acetate; NaAc, sodium acetate.

Neutralization. Self-absorbed AG11 A8 resin was utilized to remove the acid. The volume of resin needed for complete neutralization depended on the amount of acid used for hydrolysis. The resin absorbed ~1 mmol of H^+ ions/mL. For 2 mL of 3 M HCl, 7 mL of resin was sufficient for neutralization. The resin was slurried in 0.5 M NaCl and packed into graduated polypropylene columns (12 cm × 1 cm i.d.) with polyethylene frits (Bio-Rad). NaCl was removed by elution with water (20 bed volumes, 4 mL min^{-1}). For neutralization, the flow was adjusted to 2 mL min^{-1}. Glc3N was added as a recovery standard for GalN, ManN, and GlcN, and the hydrolysate was passed through the resin. The ampule was rinsed with 0.5 mL of H_2O, which was also applied to the resin. Finally, the amino sugars were eluted with 4 mL of H_2O. The elution fraction was collected after the first 2 mL (total sample volume after neutralization, 4.5 mL). For 1 mL of 3 M HCl, all volumes were halved. The pH was measured with pH paper after neutralization. AG11 A8 resin was regenerated with 0.5 M NaOH to remove absorbed acid followed by a rinse with 1 M NaCl.

Sample Cleanup Procedure. Prior to HPAEC-PAD analysis, salts and interfering compounds were removed from the hydrolysates. The neutralized sample was divided and two different cleanup procedures were performed for the determination of MA and GalN, ManN, and GlcN.

(22) Biddandah, B.; Benner, R. *Limnol. Oceanogr.* **1997**, *42*, 506−518.
(23) Benner, R.; Biddandah, B.; Black, B.; McCarthy, M. *Mar. Chem.* **1997**, *57*, 243−263.

(1) GalN, ManN, and GlcN Purification. The eluate was treated with 2 mL of AG50 X8 resin (200−400 mesh) in the Na^+ form. The resin was packed into polypropylene columns (4 cm × 0.8 cm i.d.) with polyethylene frits (Bio-Rad) and conditioned with two bed volumes of 50 mM NaOH and deionized water until neutral. Before the samples were applied to the columns, the pH was adjusted to 4 with 100 mM HCl. Solutions were passed through the resin at a flow rate of ∼0.5 mL min^{-1}. Salts and interfering components were washed out with 4 mL (two resin volumes) of water. Amino sugars were eluted with 2 mL of 20 mM NaOH. The eluate was collected under nitrogen and loaded into the HPAEC-PAD system by suction to avoid carbonate contamination of the HPAEC column. The 20 mM NaOH solution was prepared with He-sparged deionized water and 50 wt % NaOH. AG50 X8 resin was regenerated with 1 M NaOH followed by washes with H_2O until neutral and Soxhlet extraction with H_2O (1 h) prior to use.

(2) MA Purification. Cation-exchange chromatography was used for purification of samples for MA analysis. AG50 X8 resin (100−200 mesh, Na^+ form, 18 mL) was packed with deionized water into an acrylic column (15 cm × 0.9 cm i.d., Pharmacia model K9) at a flow rate of 0.5 mL min^{-1} and washed with 1 M NaCl and deionized water until salt free. The flow rate was then adjusted to 0.3 mL min^{-1}. A peristaltic pump (Pharmacia) delivered the mobile phase (H_2O). The pH of the sample was adjusted to 2 with HCl. The sample (1.6 mL) was loaded on the column by suction (0.3 mL min^{-1}) and the chromatography maintained at this flow rate. The elution time of MA was determined with a standard in artificial seawater (36 psu, Sigma). MA eluted right after the inorganic salts in a 12-mL fraction. The fraction was collected in a glass tube and concentrated to ∼1.5 mL in a Savant vacuum centrifuge. Before injection into the HPAEC-PAD system, Glc-1-P was added as an internal standard. Between samples, the column was cleaned with NaCl (1 M, 36 mL, 0.5 mL min^{-1}) and water (0.5 mL min^{-1}) until salt free. The column was repacked with fresh resin after eight samples were processed. AG50 X8 resin was regenerated with 1 M NaOH followed by washes with H_2O until neutral and Soxhlet extraction with H_2O (1 h) prior to use. All solutions were degassed by magnetic stirring under vacuum for 20 min.

HPAEC with PAD. Amino sugar analysis was performed with a Dionex 500 ion chromatography system equipped with a PAD detector (model ED40) and a Rheodyne rotary injection valve (model 9125, Cotati) with a 280-μL PEEK sample loop. The detector cell incorporated a 1-mm-diameter gold working electrode and a pH Ag/AgCl reference electrode. Chromatographic data were recorded with a personal computer equipped with Hewlett-Packard ChemStation software. Separation was achieved using a Dionex CarboPac PA1 column (250 mm × 4 mm i.d.) coupled with a CarboPac PA1 guard column (50 mm × 4 mm i.d.) or AminoTrap guard column (50 mm × 4 mm i.d.). GalN, ManN, and GlcN were separated using isocratic conditions with 12 mM NaOH and 1 mM barium acetate as the eluent. Typically, barium acetate was dissolved in deionized water, and after sparging with He for 15 min, the required amount of NaOH (50% w/w solution) was added. Sparging was continued for 2 min to mix the mobile phase. The plastic reservoir bottles (DX 500 2 l bottles, Dionex) were closed and pressurized with He to 0.8 MPa. The eluent was prepared at least 2 h before use. After six runs, the column was cleaned with 200 mM NaOH. MA was measured in a separate chromatographic analysis. The mobile phase was 100 mM NaOH with 100 mM sodium acetate for the isocratic elution. Sodium acetate was dissolved in deionized water and sparged in the bottle with He for 15 min before NaOH was added. Again the eluent was mixed through sparging and thereafter pressurized to 0.8 MPa. Chromatography of all amino sugars was carried out at a flow rate of 1 mL min^{-1} under standard conditions. The following detector settings were used: $E_{DET} = 0.05$ V ($t_{DEL} = 200$ ms, $t_{INT} = 200$ ms), $E_{OX} = 0.75$ V ($t_{OX} = 200$ ms), and $E_{RED} = -0.15$ V ($t_{RED} = 400$ ms).

GC/MS Identification of MA. MA was identified as the trimethylsilyl derivative according to the method of York et al.[24] with the modifications of the Complex Carbohydrate Research Center (CCRC Athens, GA). Samples were hydrolyzed in 3 M HCl (100 °C, 5 h) and neutralized with AG11 A8 resin. Prior to derivatization, aqueous hydrolysates were purified with AG50 X8 resin (H^+ form).[25] Samples were evaporated to dryness and redissolved in 500 μL of methanolic 1 M HCl to convert MA into the methyl ester methyl glucoside. The vial was sealed and heated at 80 °C for ∼16 h. Methanolic HCl was evaporated with a stream of nitrogen at 40 °C. The evaporation step was repeated twice with 250 μL of methanol to remove all HCl. N-Acetylation was done by adding 200 μL of methanol, 20 μL of pyridine, and 20 μL of acetic anhydride. After 6 h at room temperature, the reagents were evaporated with nitrogen. MA trimethylsilyl derivatives were formed by adding 200 μL of Sylon BFT (99% bis(trimethylsilyl trifluoroacetamide + 1% trimethylchlorosilane, Supelco) reagent. Vials were closed and heated at 80 °C for 20 min. The reagent was evaporated with nitrogen until just dry. Hexane (1 mL) was added, and after centrifugation, the hexane was transferred to a clean tube and evaporated to dryness with nitrogen. The residue was redissolved in hexane and injected onto the GC. Inositol was used as an internal standard and added to the hydrolysate after evaporation. A Hewlett-Packard GC 5890 with a 95% methyl−5% phenyl-silicone capillary column (30 m × 0.25 mm i.d., DB5, J&W Scientific Inc.) and a quadrupole mass detector (5972) were used for analysis of MA. The temperature was programmed from 160 °C increasing at 3 °C min^{-1} after an initial delay of 3 min. At 260 °C the temperature was held for 15 min. The α- and β-anomers of MA were separated on the column, and elution was complete after 24 min.

RESULTS AND DISCUSSION

The objective of this work was to develop a method for measuring amino sugars with HPAEC-PAD in samples from natural environments, including seawater. Since concentrations in open ocean seawater were expected to be in the low-nanomolar range, limits of detection comparable to those achieved for neutral sugars (2−10 nM[17]) were required. High salt concentrations severely compromised sensitivity and interfered with the separation of amino sugars on the anion-exchange column. Another problem was the lack of specificity of the PAD for amino sugars, which could lead to overestimation of amino sugar concentrations due to coeluting compounds. Therefore it was critical to remove

(24) York, W. S.; Darvill, A. G.; McNeill, M.; Stevenson, T. T.; Albersheim, P. *Methods Enzymol.* **1985**, *118*, 29−30.

salts and coeluting compounds prior to chromatography and PAD. By using ion-exchange resins, we were able to simplify the complex sample matrix and desalt the samples for reliable quantification of GalN, ManN, GlcN, and MA with detection limits at the low-nanomolar levels.

Sample Preparation. Neutralization is the first critical step following hydrolysis. Monomeric amino sugars were unstable in concentrated acids. Acid removal by evaporation would destroy some of the sugars. Sulfuric acid is normally removed with $Ba(OH)_2$[17] or $CaCO_3$,[6,17] involving precipitation of sulfate. However, amino sugars were lost by coprecipitation using this neutralization. We used AG11 A8 resin to avoid those problems. It allowed fast and efficient removal of HCl or H_2SO_4 and recoveries were usually 95−100%. In addition, this neutralization method partially desalted the sample and the styrene divinylbenzene polymer matrix retained some hydrophilic compounds. The resin contained paired anion- and cation-exchange sites, which countered each other when in the self-absorbed form. Hydrogen ions were held so strongly that they were not eluted with water. Salts and organic compounds were more or less retarded and separated by elution with water.

After neutralization, different purification steps were performed for MA and GalN, ManN, and GlcN to achieve high recoveries (Figure 1). Samples for GalN, ManN, and GlcN analysis were prepared using AG50 X8 resin in the Na^+ form. Acidification to pH 4 was important to retain the amino sugars. Washes with water removed salts and neutral and anionic components, and 20 mM NaOH eluted the amino sugars. Base-catalyzed epimerization of amino sugars between elution and injection could not be detected. The eluate was collected under He and loaded into the sample loop by suction to exclude atmospheric CO_2, which fouled the anion-exchange column with carbonate causing shifting retention times. Amino sugar monomers were unstable, and neutral hydrolysates were stored frozen not longer than 1 day.

Samples for MA determination were purified on a column packed with AG50 X8 resin in the Na^+ form. AG50 X8 resin in the Na^+ form strongly excluded NaCl while retaining MA slightly. The best separation from salts and neutral compounds was achieved with a flow rate of 0.3 mL min^{-1} (one bed volume/hour) and by loading the sample at pH 2. A pH of 2 increased the retention of MA, leading to nearly complete separation from inorganic salts. Soxhlet extraction strongly affected the capacity of the resin. It is possible that swelling of the polymer lattice at elevated temperatures exposed buried cation-exchange groups and changed the resin properties. Optimal performance was obtained by extracting the resin for 1 h. Longer Soxhlet extraction resulted in slightly better retention of MA, but washing with 1 M NaCl returned the resin to its normal capacity. Sample load was ∼8% of the bed volume. Between samples, compounds retained in the column were removed with two bed volumes of 1 M NaCl. Since MA eluted in a 12-mL fraction, the diluted sample was concentrated in a vacuum centrifuge before injection. No adsorptive losses or destruction was observed during the concentration step. For samples with a higher MA concentration, an aliquot of the elution fraction was directly injected into the HPAEC. Bio-Rad's AG50 X8 resin was the resin of choice because it produced the cleanest chromatograms and did not introduce interfering contaminants.

During the development of the purification procedure for MA, we observed an unidentified peak in the chromatogram that potentially interfered with the quantification of MA. The MA content in UDOM was confirmed by GC/MS analysis using the derivatization method described by York et al.

HPAEC-PAD of Amino Sugars. Glc3N, GalN, ManN, and GlcN were resolved with 12 mM NaOH and 1 mM barium acetate as the mobile phase. The barium acetate was added for two reasons: first, to improve the separation of GalN and ManN and, second, to avoid shifting retention times.[26] Incomplete separation of GalN and ManN and decreasing retention times resulted from carbonate contamination of the column. Carbonate is introduced through atmospheric CO_2 absorption and it strongly binds to anionic-exchange groups in the resin, which inactivates the column and interferes with the amino sugar binding. Ba(II) effectively removed carbonate by complexation, allowing nearly baseline separation with 12 mM NaOH and leading to only minimal shifts in retention times (2% after six injections). The internal standard, Glc3N, was completely resolved from GalN and no coelution with any unidentified compound was observed. The PA 1 guard column was replaced with an AminoTrap guard column to evaluate the potential interference of amino acids in seawater and bacterial samples.[27] The AminoTrap column caused hydrophobic amino acids to elute after the amino sugars, but no differences were found in any of the samples when switching guard columns, so it appears that interferences with amino acids were negligible. Because a different sample preparation step was needed for MA, a separate chromatographic analysis was performed with 100 mM NaOH and 100 mM NaAc as the mobile phase.

Internal Standards. Two different internal standards were used for quantification and to account for potential interferences from other matrix components. Glc3N served as an internal standard for GalN, ManN, and GlcN and was added after hydrolysis. It corrected for losses during the purification step and for changing detector response in the chromatographic analysis. Glc3N was not detected in any natural sample and did not coelute with amino sugars or unidentified peaks in the chromatogram.

Glc-1-P was used as an internal standard for MA quantification. It was added prior to chromatographic analysis and accounted only for changing detector response. Losses of MA during sample processing were determined by carrying out recovery tests on MA standard solutions (Table 1). These recovery values were used to correct measured MA concentrations in samples.

Recoveries, Limit of Detection, Linearity, and Precision. Recoveries for all amino sugars through the neutralization and purification procedures were determined with 100 nM standards spiked into 3 M HCl in deionized water and 3 M HCl in artificial seawater (Table 1). Recoveries were generally lower from high-salt solutions. Competitive interactions of Na^+, Ca^{2+}, and Mg^{2+} ions with exchange sites in the resin clearly affected retention of Glc3N, GalN, ManN, and GlcN. It was important to establish a pH of 4 before extraction to accomplish optimal recoveries for Glc3N, GalN, ManN, and GlcN. Glc3N was an excellent internal recovery standard for GalN, ManN, and GlcN because recovery

(25) Hicks, R. E.; Newell, S. E. *Anal. Biochem.* **1983**, *128*, 438−445.
(26) Cataldi, T. R. I.; Campa, C.; Margiotta, G.; Bufo, S. *Anal. Chem.* **1998**, *70*, 3940−3945.
(27) Weitzhandler, M.; Pohl, C.; Rohrer, J.; Narayanan, L.; Slingsby, R.; Avdalovic, N. *Anal. Biochem.* **1996**, *241*, 128−134.

Table 1. Recoveries of Amino Sugars from Different Matrixes and Limits of Detection (LOD)

	% recovery[a]				
	H_2O	seawater (20 psu)[b]	seawater (36 psu)[b]	spiked UDOM[c]	LOD[e] (pmol)
Glc3N[d]	92 ± 2	82 ± 3	81 ± 2	nd[f]	nd
GalN	91 ± 2	80 ± 2	79 ± 2	nd	0.25
ManN	89 ± 4	78 ± 5	78 ± 4	nd	0.34
GlcN	95 ± 3	83 ± 2	82 ± 3	nd	0.22
MA	86 ± 1	73 ± 2	73 ± 2	82 ± 3	1.21

[a] Neutralization step is included. Relative standard deviations were calculated from triplicates except for MA (n = 5). [b] Sigma artificial seawater. [c] 50 nM standard spiked to UDOM sample after hydrolysis. [d] Internal standard. [e] S/N = 3. LOD represents a corrected concentration detection limit which includes losses during sample preparation. [f] nd, not determined.

Table 2. Amino Sugar Recoveries from Freeze-Dried Bacteria under Different Hydrolysis Conditions[a]

	hydrolysis	GalN	GlcN	MA
B. subtilis	1.2 M H_2SO_4[b]	1.08	0.98	0.49
	6 M HCl[c]	0.69	0.84	1.02
	3 M HCl[d]	1.00	1.00	1.00
P. flourescens	1.2 M H_2SO_4	0.85	1.05	0.91
	6 M HCl	0.85	0.93	1.01
	3 M HCl	1.00	1.00	1.00
S. bacillaris	1.2 M H_2SO_4	0.85	0.71	0.64
	6 M HCl	0.98	1.25	1.17
	3 M HCl	1.00	1.00	1.00
Trichodesmium	1.2 M H_2SO_4	0.93	0.91	0.67
	6 M HCl	0.53	1.22	0.96
	3 M HCl	1.00	1.00	1.00

[a] Yields are normalized to 3 M HCl hydrolysis (n = 2). [b] Dry bacteria were pretreated with 12 M H_2SO_4 for 2 h at 25 °C, then diluted to 1.2 M, and hydrolyzed at 100 °C for 3 h. [c] 6 M HCl at 100 °C for 2 h. [d] 3 M HCl at 100 °C for 5 h.

efficiences were similar. Lower recoveries for MA resulted from incomplete separation from salts. The influence of matrix effects on the recovery of MA was investigated by spiking known amounts of MA (50 nM) into UDOM hydrolysates. The recovery efficiency of MA was 82%, which was higher than the recovery efficiency from high-salt solutions (Table 1). Limits of detection (signal-to-noise ratio 3) for individual amino sugars are shown in Table 1. The detection limits represent corrected concentration detection limits and include the losses during the sample preparation steps. The working electrode was polished prior to this test, and fresh eluents were used. Effective sample cleanup allowed minimum detection limits of 220−340 fmol for GalN, ManN, and GlcN. The detection limit for MA was ~3 times higher (1200 fmol) because of a lower detector response and increased baseline noise due to acetate in the mobile phase.

The detector response was linear for all amino sugars over a concentration range of 1−500 pmol. The precision of the method was evaluated with replicates of seawater, UDOM, and bacterial samples (n = 4). At the 20 nM, level the relative standard deviation for GalN, ManN, and GlcN was 3−6%. MA had a relative standard deviation of 11%. At the 200 nM level, the relative standard deviation for GalN, ManN, and GlcN was 2−4% and 6% for MA. Day-to-day reproducibility was determined by running the same UDOM sample on four different days. Values were within 3−5% for GalN, ManN, and GlcN and 12% for MA. High recoveries with low relative standard deviations and excellent reproducibility showed the suitability of the method for analysis of amino sugars in complex samples.

Hydrolysis. Several different hydrolyses have been described for efficient extraction of amino sugars from natural samples. The sample matrix and the type of amino sugar polymer generally determined acid concentrations and hydrolysis times. A 3 M HCl solution was found to efficiently recover GalN and GlcN in mucins (100 °C, 3 h)[29] and MA in dry bacteria (100 °C, 15 h).[30] A 6 M HCl solution was used to extract GlcN and MA from marsh litter grass (100 °C, 4.5 h)[25] and MA from dry bacteria (100 °C, 2 h).[30] For sediments and soils, proposed hydrolysis times with 3 and 6 M HCl were usually longer than for glycoproteins, polysaccharides, and bacteria and ranged from 4.5 h[31] to 18 h.[15] It is possible that protective properties of the matrix reduced the efficiency of hydrolysis. Trifluoroacetic acid hydrolysis (4 M, 125 °C, 1 h) was recommended for complete release of amino sugars with minimum destruction of neutral sugars in glycoproteins and plant cell-wall polysaccharides.[14]

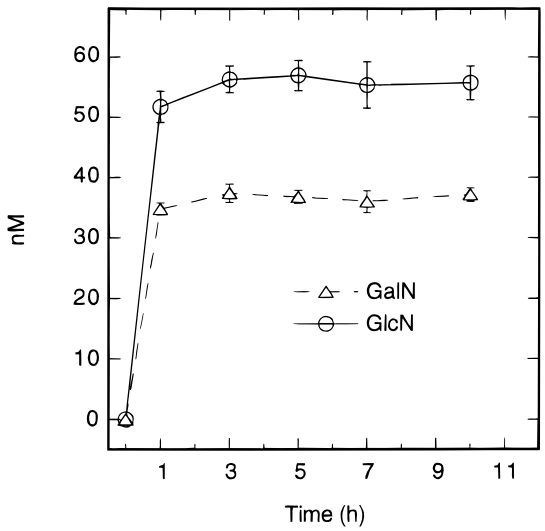

Figure 2. Recovery of GalN and GlcN as a function of hydrolysis time for seawater. Hydrolysis in 3 M HCl at 100 °C. ManN and MA were below the detection limit. The relativ standard deviation was calculated from triplicates.

Four bacteria (*B. subtilis*, *P. flourescens*, *S. bacillaris*, and *Trichodesmium*) were subjected to acid hydrolysis using 3 M HCl (5 h, 100 °C), H_2SO_4 (12 M H_2SO_4 1 h, 25 °C, then dilution to 1.2 M, 3 h, 100 °C), and 6 M HCl (2 h, 100 °C) to identify the appropriate hydrolysis conditions for amino sugars. The H_2SO_4 hydrolysis was chosen because it effectively cleaved neutral sugar polymers,[7,17,28] and it would be advantageous to do a single hydrolysis for amino sugars and neutral sugars. A 6 M HCl solution (2 h, 100 °C) was shown to efficiently extract MA from dry bacteria.[30] Table 2 shows recoveries of amino sugars normalized to results from the 3 M HCl hydrolysis. Sulfuric acid

(28) Borch, N. H.; Kirchmann, D. L. *Mar. Chem.* **1997**, *57*, 85−95.
(29) Griggs, L. J.; Post, A.; White, E. R.; Finkelstein, J. A.; Moeckel, K. G. H.; Zarembo, J. E.; Weisbach J. A.; *Anal. Biochem.* **1971**, *43*, 369−381.
(30) Mimura, T.; Romano, J.-C.; *Appl. Environ. Microbiol.* **1985**, *50*, 229−237.
(31) King, J. D.; White, D. C. *Appl. Environ. Microbiol.* **1977**, *33*, 777−783.

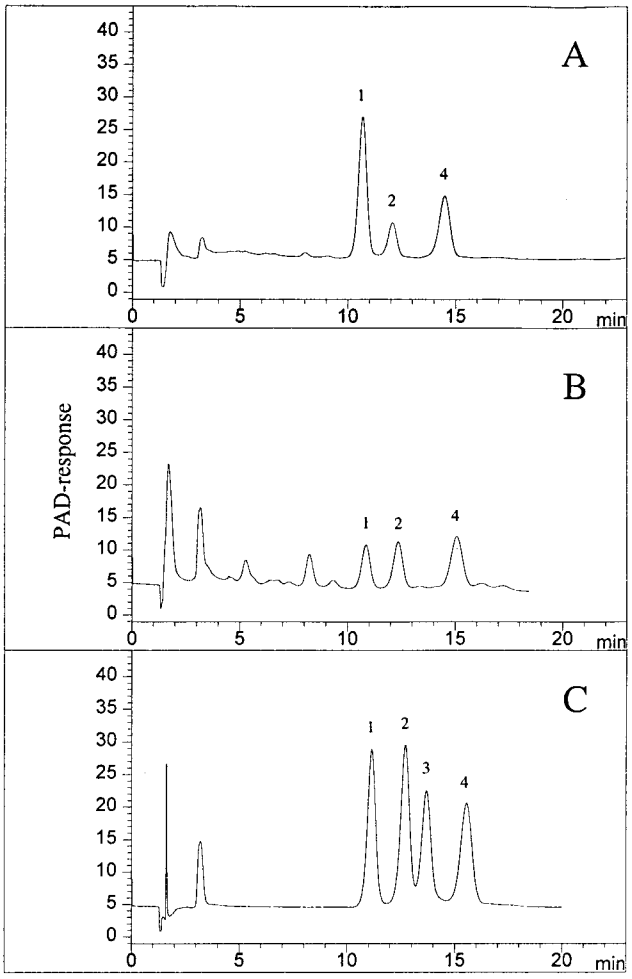

Figure 3. Selected chromatograms of (A) a sediment from the Arabian Sea ([GalN] = 6.4 μg/mg of OC, [GlcN] = 15.2 μg/mg of OC), (B) seawater from the Gulf of Mexico (100-m depth, [GalN] = 18 nM, [GlcN] = 24 nM) obtained after acid hydrolysis and purification, and (C) a 50 nM standard mixture. Chromatographic conditions: 12 mM NaOH with 1 mM barium acetate at 1 mL/min; Dionex PA1 column with PA1 guard column; PAD (scales of *y*-axes are not the same). Retention times vary slightly from (A) to (C) due to changing column characteristics over time (6 month). Identification: 1, Glc3N (IS); 2, GalN; 3, ManN; 4, GlcN.

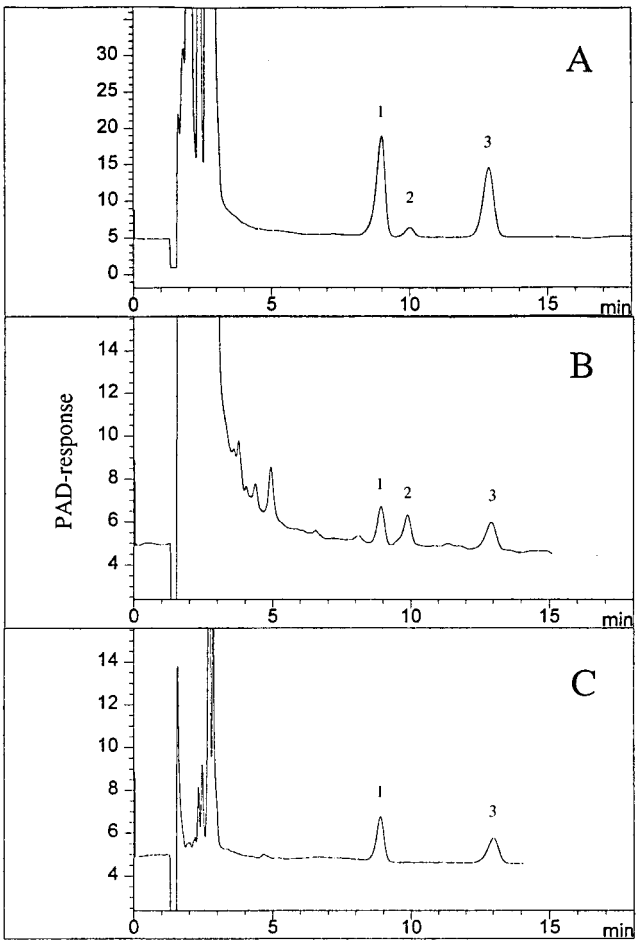

Figure 4. Selected chromatograms of (A) freeze-dried *B. subtilis* ([MA] = 166.7 μg/mg of OC), (B) particulate material (0.1−0.8 μM) from the Otsuchi Bay, Japan ([MA] = 7.7 μg/mg of OC, salinity 36 psu)[34] obtained after acid hydrolysis and purification, and (C) a 30 nM standard. Chromatographic conditions: 100 mM NaOH with 100 mM sodium acetate at 1 mL min^{-1}; Dionex PA1 column with PA1 guard column; PAD (scales of *y*-axes are not the same). Identification: 1, MA; 2, resin peak; 3, α-1-glucose phosphate (IS).

hydrolysis resulted in relatively high yields of GalN and GlcN, but typically it resulted in low yields of MA. In comparison to both HCl hydrolyses, the yield of MA was ~50% lower with sulfuric acid, suggesting that cleavage of the amide linkage required stronger conditions. The 3 and 6 M HCl hydrolyses yielded similar results, although GalN yields were typically higher with 3 M HCl. Concentrations of MA in *B. subtilis* (Gm^+) and *P. flourescens* (Gm^-) were slightly higher than expected values for these bacteria, indicating quantitative release of MA with both HCl hydrolyses.[12]

A time series was carried out with 3 M HCl (100 °C) to determine appropriate hydrolysis times for seawater. The results of this test are shown in Figure 2 and indicate that ~90% of GalN and GlcN are released within 1 h. Maximum recoveries were obtained after 3 h. ManN and MA concentrations were below the detection limit.

The efficiency of 3 M HCl (5 h, 100 °C) hydrolysis was tested with chitin, chondroitin A and a peptidoglycan preparation from *M. luteus*. These polymers were chosen because they represent typical natural sources of amino sugars. About 62% of the chitin sample was recovered as GlcN, and 78% of the chondroitin was released as GalN. Glycosidic linkages in polysaccharides containing uronic acids, such as chondroitin, are stabilized by the carboxyl group and are more difficult to cleave than linkages in polysaccharides with only neutral or amino sugars.[32] The good recovery of GalN from chondroitin, a copolymer of sulfated GalN with glucuronic acid, indicated efficient release of amino sugars bonded in such polymers. Recovery of GlcN and MA from the peptidoglycan preparation was 74 and 63%, respectively. For calculation of the recovery values we assumed a pure peptidoglycan polymer typical for *M. luteus* (MW = 1592).[33] The ratio of GlcN/MA was 1.18, which is close to the theoretical ratio of 1.0 expected for bacterial peptidoglycan.

Neutral sugar yields from seawater samples were compared with yields with sulfuric acid hydrolysis to evaluate the efficiency of the 3 M HCl treatment in hydrolyzing neutral sugar polymers.

(32) Wolfrom, M. L.; Franks, N. E. *General Polysaccharides, Methods in Carbohydrate Chemistry*; Academic Press: New York, 1965; Vol. V, p 269−275.
(33) Schleifer, K. H.; Kandler, O. *Bacteriol. Rev.* **1972**, *36*, 407−477.

Highly variable neutral sugar yields among samples suggested a strong influence of sample matrix on hydrolysis with HCl (data not shown). The HCl hydrolysis is therefore not recommended for neutral sugar determinations.

Additionally, *S. bacillaris* and *Trichodesmium* were hydrolyzed under conditions optimized for amino acid hydrolysis in natural samples (6 M HCl, 140 °C, 70 min[34]). Nearly complete destruction of all amino sugars was observed in both bacterial samples using this hydrolysis. These results suggest that hydrolyses optimized for amino acids are likely destructive for amino sugars.

In conclusion, the results of these hydrolysis tests suggest that 3 M HCl (100 °C, 5 h) is satisfactory for the analysis of amino sugars in bacteria and seawater samples. No hydrolysis studies were performed on sediments, and appropriate conditions need to be identified for optimal release of amino sugars. However, a chromatogram of amino sugars released from a sediment is presented in Figure 3A to show the suitability of the sample cleanup procedure for this sample matrix.

Natural Samples. The effectiveness of the method was tested on a variety of natural samples. Figure 3 shows chromatograms of amino sugars in a sediment (A) and in unfiltered seawater (B). Concentrations for GalN and GlcN in seawater ranged between 15 and 30 nM at 10-m depth and 2−4 nM at 4000-m depth. The concentrations of ManN and MA were below the detection limit.

In Figure 4, chromatograms of MA in *B. subtilis* (A) and in marine particulate material containing a natural assemblage of marine bacteria[35] (B, salinity ∼36 psu) are presented. Concentrations were 41 nmol/mg of OC for *B. subtilis* and 7.7 nmol/mg of OC for the particulate material.

Concentrations of amino sugars in UDOM samples ranged from 1 to 140 nmol/mg of OC. Although these samples showed a high matrix complexity, the off-line sample purification was extremely effective in producing clean chromatograms.

(34) Cowie, G. L.; Hedges, J. I. *Mar. Chem*. **1992**, *37*, 223−238.
(35) Fukuda, R.; Ogawa, H.; Nagata, T.; Koike, I. *Appl. Environ. Microbiol*. **1998**, *64* (9), 3352−3358.

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