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DETERMINATION OF AMINOSACCHARIDES BY HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION

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Summary—High-performance anion-exchange chromatography (HPAC) was used for the determination of aminosaccharides in microbial polymers, chitin, animal waste, sewage sludge, plant residues and soil. The aminosaccharides, galactosamine, mannosamine and glucosamine were separated on a strong anion-exchange column with 10m*M* sodium hydroxide as the eluent and determined by pulsed amperometric detection (PAD). The HPAC–PAD methodology was compared with high-performance liquid chromatography (HPLC) with refractive index detection (RI) in terms of selectivity and sensitivity for aminosaccharides. The results indicate that HPAC–PAD required less sample preparation, and was more precise and nearly two orders of magnitude more sensitive than HPLC–RI. HPAC–PAD was not subject to matrix interferences and was highly selective for aminosaccharides. More than 3% of the total nitrogen in alfalfa, and 20% of that in straw, was found to be present as aminosaccharides.

Aminosaccharides are widely distributed in nature and have been reported to occur in plants,[1] micro-organisms,[2] and crustaceans,[3] and have recently been found to be an important constituent of the soil nitrogen pool.[4] They not only serve as a nitrogen source for plant growth, but may also have an important role in the promotion of good soil structure.[5]

It is believed that 5–10% of the total nitrogen in surface soils is present as aminosaccharides.[4] Chitin is a polymer of *N*-acetylglucosamine frequently found in soil, and is present in the cell walls, structural membranes and skeletal components of insects and fungal mycelia.[5] Conditions which favor microbial growth and proliferation in soils tend to increase the hexosamine content of soils. Aminosaccharides are somehow protected from decomposition in soils by being incorporated into the humic acid fraction.[6,7]

Aminosaccharides in soils are normally extracted by hydrolysis with hydrochloric acid and the resulting ammonium ion is determined by the standard colorimetric method of Elson and Morgan[8] or by alkaline distillation. The Elson and Morgan method is based on the chromogen formed when the aminosaccharide is heated with an alkaline solution of acetylacetone and then an acidic ethanolic solution of *p*-dimethylaminobenzaldehyde. However, many substances in soils, including iron and amino acids, produce colors which interfere with this determination of aminosaccharides. The alkaline distillation method involves deamination of aminosaccharides by heating with an alkali. The ammonia released through deamination is then determined by steam distillation, collection in boric acid solution,[9] and titration.

Aminosaccharides have been isolated from soil hydrolysates by both paper chromatography and ion-exchange chromatography. Previous studies have indicated that most of the aminosaccharides occur as D-glucosamine and D-galactosamine, with the former occurring in the greatest amounts.[5] *N*-Acetylglucosamine has also been found in soils.[10]

High-performance liquid chromatography (HPLC) analysis with refractive index (RI) detection is the most common method for quantification of saccharides but has lagged behind in the determination of aminosaccharides because of poor selectivity of the existing stationary phases and insensitivity of the RI detector. The use of high-performance anion-exchange chromatography (HPAC) with pulsed amperometric detection (PAD) has several advantages over RI detection. PAD with a gold electrode is selective only for compounds containing oxidizable functional groups, including hydroxyl, amine and sulfide. Aminosaccharides

are weak acids with pK_a values in the range 9–11 and thus can be separated as anions by control of the pH of the mobile phase.

The objective of this study was to optimize the chromatography in the determination of aminosaccharides in biological materials and to compare HPAC–PAD with HPLC–RI for quantification of aminosaccharides. Chromatographic parameters such as selectivity, resolution and precision were determined for various aminosaccharides to optimize separation and detection.

EXPERIMENTAL

Reagents

All saccharide standards were obtained from Supelco (Bellefonte, PA). Chitin was obtained from Sigma (St. Louis, MO).

Chromatographic instrumentation

HPAC–PAD detection. The HPAC–PAD analysis was performed with a Dionex (Sunnyvale, CA) LC gradient pump module and Model PAD2 detector. Sample injection was by a Dionex autosampler equipped with a 200-μl sample loop. Aminosaccharides were separated with a CarboPac PA1 pellicular anion-exchange resin (250 × 4 mm) and a CarboPac PA guard column (25 × 3 mm) at a flow-rate of 0.8 ml/min at ambient temperature with a 5–200m*M* sodium hydroxide gradient system. The aminosaccharides were eluted isocratically with 5m*M* sodium hydroxide for 15 min, then the eluent concentration was ramped to 200m*M* in 5 min, and then maintained at this level for a further 5 min. With the instrumentation used, this was achieved with three solutions: A was 18-MΩ water filtered through a 0.22-μm membrane, B was 100m*M* sodium hydroxide and C 200m*M* sodium hydroxide. These were combined in the following proportions.

	0–15 min	15–20 min	20–25 min
A, %	95	95 → 0	0
B, %	5	5 → 0	0
C, %	0	0 → 100	100

The 200m*M* sodium hydroxide was used to elute interfering species that may act as displacing ions and shorten the retention times in subsequent runs. The mobile phase was degassed to prevent sorption of carbon dioxide and subsequent production of carbonate which would act as a displacing ion and shorten retention times. Sodium hydroxide (300m*M*) was used as a post-column addition to reduce baseline shifts that occur with the 5–200m*M* sodium hydroxide gradient and also to increase the PAD sensitivity. Detection was by triple-pulsed amperometry with a gold working electrode.[11] The following working pulse potentials and durations were used for detection of aminosaccharides: $E_1 = 0.05$ V ($t_1 = 720$ msec); $E_2 = 0.60$ V ($t_2 = 120$ msec); $E_3 = -0.60$ V ($t_3 = 120$ msec). The CHOH groups are oxidized at E_1, E_2 removes the reaction products and E_3 cleans the electrode. Cyclic voltammetry was used in selection of the three potentials. The response time of the PAD was set to 1 sec. Chromatographic data were collected and plotted with the Dionex AutoIon 300 software.

HPLC–RI. The HPLC–RI analysis was performed with a Beckman (Fullerton, CA) System Gold™ liquid chromatograph equipped with a Beckman 110B solvent pump and a Rheodyne Model 7000 sample injector (Berkeley, CA) fitted with a 20-μl stainless-steel injection loop. The system was composed of the following: a Vydac (Separations Group, Hesperia, CA), 1630 anion-exchange column (250 × 4.1 mm i.d.), a Bio-rad (Richmond, CA) carbohydrate microguard column (30 × 4.6 mm), a Beckman μ-Spherogel 300 × 7.5 mm carbohydrate column (300 × 7.5 mm i.d.), an Eldex Model III (Menlo Park, CA) thermostatic column heater, an Altex (Fullerton, CA) 156 refractive index detector, and a Hewlett–Packard Model 3390A printer–plotter integrator with variable input voltage. The mobile phase was HPLC-grade water (heated to 85°) at a flow-rate of 0.5 ml/min.

Gas chromatography

Samples were reduced and the acetylated derivatives analyzed by gas chromatography[1] with a Hewlett–Packard 5890A gas chromatograph with a flame-ionization detector. The column used was a 0.2-mm i.d. Chrom Q fused silica capillary column (Alltech, Deerfield, IL). The operating conditions with helium as a carrier gas were injection temperature, 250°; detector temperature, 250° and column temperature, 210°; gas flow, 20.0 ml/min.

Soil treatment

Field plots (2 m × 2 m) were established by incorporating 25 metric tons per hectare levels of straw (*Hordeum vulgare*) [carbon/nitrogen ratio (C/N), 48.0], poultry manure (C/N, 5.0), sewage sludge (C/N, 5.0) (Riverside, CA), and

alfalfa (*Medicago sativa*) (C/N, 7.0) into an Arlington soil (coarse-loamy Haplic Durixeralf) (pH 7.9; C/N, 12.0) at the Citrus Field Station of the University of California, Riverside. The additives were mixed into the upper 15 cm of soil and irrigated (12 cm water/day) once a week for three months. Field-moist soil samples were sieved through a 1-mm mesh sieve to remove large organic debris. The carbon content was determined by a modified Mebius method[12] and the nitrogen content by a micro-Kjeldahl method.[13]

Isolation of bacterial polymers

The bacterial polymers were isolated as described by Anderson *et al.*[14] and Martin and Richards.[15]

Aminosaccharide extraction

Samples (1 g) of air-dried soil, 0.5-g samples of sewage sludge and poultry manure, 0.3-g samples of straw and alfalfa, 0.1 g of chitin, and 1 mg of bacterial polymer carbon [*Chromobacterium violaceum*, American Type Culture Collection (ATCC) 9544 and *Hansenula holstii*, ATCC 2448] were treated with 1.25 ml of 12*N* sulfuric acid for 2 hr at room temperature, then after addition of 1.25 ml of water (to give 6*N* sulfuric acid) were heated at 90° under a reflux condenser for 16 hr.[4] The mixtures were cooled, then treated with 4–5 ml of water and 1 ml of 0.1*M* EDTA, titrated to pH 4 with 5*M* potassium hydroxide, and centrifuged at 10^4 rpm. The supernatant solution was diluted to 12 ml.

Purification of acidic extracts

A 1-ml aliquot of acid extract of organic additive, microbial polymer or soil was diluted to 5 ml with water and passed through a Supelco™ strong-acid cation-exchanger (3-propylsulfonic acid, H^+ form) 3-ml solid-phase extraction column (SCX) (Bellefonte, PA). The SCX column was then rinsed with 3 ml of water to elute all non-retained compounds. The aminosaccharides were eluted with 5 ml of 0.3*M* hydrochloric acid. Quantification by HPLC–RI required further purification of the extracts. An aliquot to be analyzed by HPLC–RI and containing 10–25 mg of aminosaccharides was applied to a column of Bio-Gel™ P-2 (100–200 mesh; 12 × 2.8 cm; Bio-Rad, Richmond, CA). The aminosaccharides were eluted with water.

All samples were filtered through GS 0.22-μm filters (Millipore, Bedford, MA) before analysis.

RESULTS AND DISCUSSION

Extraction

Numerous reports have shown that aminosaccharides can be extracted from biological materials by treatment with hot mineral acids,[1,4,16] but the use of these acids at 6*N* concentration results in a high salt concentration in the extracts. The high levels of salts and other possible interference present in the 6*N* acid extracts cause few difficulties for the HPAC–PAD analysis, because of the high level of sodium hydroxide (300m*M*) used as a post column treatment. On the other hand, the HPLC–RI method is subject to many interferences in the analysis of plant and soil extracts. The non-saccharide impurities were selectively removed from the extracts. EDTA (disodium salt) was added before neutralization to prevent co-precipitation of the bi- and tervalent cations with the saccharides extracted. Strong-acid cation-exchange columns were used to retain the aminosaccharides, and elution with water removed most of the impurities. Tests conducted with the SCX resins and the aminosaccharide standards galactosamine, mannosamine, glucosamine, acetyl-D-galactosamine, acetyl-D-glucosamine and acetyl-D-mannosamine showed 98–100% recovery. Acid extracts analyzed by HPLC–RI required an additional pretreatment with the Bio-Rad P-2 gel for removal of salts and other neutral low molecular-weight compounds. Tests conducted with the same aminosaccharide standards showed 100% recovery with the gel. HPAC–PAD required only the treatment with SCX resin before the analysis. Neutral (non-ionic) and basic compounds were eluted in the void volume during the HPAC–PAD analysis.

Liquid chromatographic analyses

The chromatographic characteristics, including t_R, k' and number of theoretical plates are listed in Table 1. The retention times of selected aminosaccharides detected by HPLC–RI indicate co-elution problems with galactosamine, mannosamine and glucosamine (6.30–6.50 min) and *N*,*N*′-diacetylchitobiose, acetylgalactosamine, acetylmannosamine and acetylglucosamine (10.60–10.72 min). Chromatograms of a mixture of 7 aminosaccharides analyzed by HPLC–RI and HPAC–PAD are shown in Figs. 1 and 2, respectively. All 7 aminosaccharides were detected by HPAC–PAD, but only 3 peaks were evident with HPLC–RI. 2-Deoxy-

Table 1. Chromatographic characteristics in detection of aminosaccharides by HPAC-PAD and HPAC-RI*

	HPAC–PAD			HPLC–RI		
Aminosaccharide	t_R, min	k'†	N‡	t_R, min	k'†	N‡
D-Galactosamine	11.24	4.50	3300	6.45	1.1	1160
D-Mannosamine	12.44	5.20	2800	6.50	1.1	2601
D-Glucosamine	13.69	5.50	3100	6.30	1.1	2804
N,N'-Diacetylchitobiose	15.01	6.30	5500	10.60	2.6	2978
Acetyl-D-galactosamine	17.65	6.80	5800	10.68	2.6	2873
Acetyl-D-manosamine	18.67	6.85	6200	10.72	2.7	2468
Acetyl-D-glucosamine	19.90	7.05	6600	10.63	2.6	2970

*HPAC–PAD; CarboPac PA1 (250 × 4.6 mm); eluent, 5mM NaOH for 15 min then ramped to 200mM NaOH in 5 min.

HPLC–RI: column, Beckman μ-Spherogel (300 × 7.5 mm); eluent, HPLC grade H_2O (85°).

†$k' = (t_R - t_M)/t_M$, where t_R = retention time of solute and t_M = retention time of solvent front.

‡N, number of theoretical plates = $16[t_R/W]^2$ where t_R is the retention time and W the peak width.

ribose was used as an internal standard for both analyses. The time of analysis for detection of all 7 solutes by HPAC–PAD was < 20 min.

Column efficiency, expressed as number of theoretical plates (N), ranged from 2800 (D-mannosamine) to 6600 (acetyl-D-glucosamine) for the HPAC–PAD analysis and 1160 (D-galactosamine) to 2978 (N,N'-diacetylchitobiose) for the HPLC–RI analysis (Table 1). When the N values were pooled and averaged for all the solutes, the mean N was 4757 for HPAC–PAD and 2550 for HLPC–RI, indicating that the HPAC–PAD analysis was almost twice as efficient for separation of the aminosaccharides.

The precisions and limits of detection (LODs) for HPAC–PAD and HPLC–RI quantification of the selected saccharides are given in Table 2. Precision was determined from the results of ten 200-μl (HPAC–PAD) or 20-μl (HPLC–RI) injections of combined standards at 1 mg/l. level for HPAC–PAD and 20 mg/l. for HPLC–RI. The relative standard deviations for detection of various saccharides ranged from 0.1 to 0.25% (HPAC–PAD) and from 0.5 to 0.9% (HPLC–RI). The LODs were examined by spiking extracts with a known amount of the saccharide to be determined. The results show that the LODs for the saccharides tested ranged from 0.15 to 0.35 mg/l. with HPAC–PAD (200-μl injection), and 30 to 50 mg/l. with HPLC–RI (20-μl injection), based on a 3-fold signal-to-noise ratio for the baseline ($S/N = 3$). The HPAC–PAD response was linear from the LOD to 5 mg/l. Samples were diluted when necessary. HPAC–PAD gave detection limits nearly two orders of magnitude lower than those given by HPLC–RI. Increasing the injection size beyond 20 μl in the HPLC–RI analysis

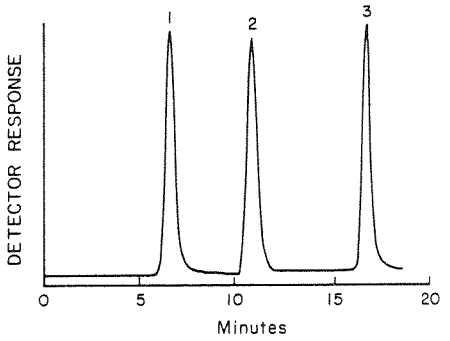

Fig. 1. Chromatogram of aminosaccharides detected by HPLC–RI [1 = galactosamine, glucosamine and mannosamine; 2 = N,N'-acetylchitobiose, acetylgalactosamine, acetylmannosamine, and acetylglucosamine; 3 = 2-deoxyribose (internal standard)].

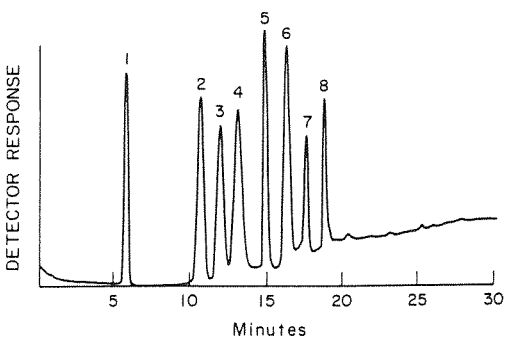

Fig. 2. Chromatogram of aminosaccharides detected by HPAC–PAD [1 = 2-deoxyribose (internal standard); 2 = galactosamine; 3 = mannosamine; 4 = glucosamine; 5 = N,N'-diacetylchitobiose; 6 = acetylgalactosamine; 7 = acetylmannosamine; 8 = acetylglucosamine].

Table 2. Precision and detection limits of HPAC–PAD and HPLC–RI analyses for selected aminosaccharides*

Aminosaccharide	Relative standard deviation, %†		Detection limit, *mg/l.*‡	
	HPAC–PAD	HPLC–RI	HPAC–PAD	HPLC–RI
D-Galactosamine	0.15	0.90	0.15	50
D-Mannosamine	0.15	0.70	0.15	50
D-Glucosamine	0.10	0.90	0.20	50
N,N'-Diacetylchitobiose	0.15	0.50	0.35	30
Acetyl-D-galactosamine	0.25	0.60	0.20	35
Acetyl-D-mannosamine	0.20	0.50	0.25	30
Acetyl-D-glucosamine	0.25	0.50	0.30	30

*HPAC–PAD: CarboPac PA1 (250 × 4.6 mm); eluent, 5m*M* NaOH for 15 min, then ramped to 200m*M* NaOH in 5 min. HPLC-RI: column, Beckman μ-Spherogel (300 × 7.5 mm); eluent, HPLC grade H_2O (85°).

†Based on ten injections of a standard, concentration 1 mg/l. (HPAC-PAD) and 20 mg/l. (HPLC–RI).

‡Assumed to be three times the signal-to-noise ratio at the baseline ($S/N = 3$).

resulted in decreased resolution, owing to increased peak distortion and overlap.

Table 3 shows the resolution (R_s) of selected aminosaccharides detected by HPAC–PAD and HPLC–RI. Overall, HPAC–PAD provided much better separation, with R_s values ranging from 1.05 to 6.10, indicating > 96% resolution of Gaussian peaks.[17]

Detection of aminosaccharides in chitin, plants and soil

Acetyl-D-glucosamine is a major constituent in the hard shells of crustaceans. Crab shell chitin was used to evaluate the hydrolysis procedure, preliminary purification, and detection of aminosaccharides by HPAC–PAD. Figure 3 is a chromatogram showing the detection of D-glucosamine and acetylglucosamine in a 6*N* sulfuric acid extract of crab shell chitin. Approximately 65% of the chitin sample solubilized by 6*N* sulfuric acid was recovered as glucosamine and acetylglucosamine.

Figure 4 shows the HPAC–PAD chromatograms of sulfuric acid extracts of alfalfa, soil amended with alfalfa (after three months of decomposition), and an unamended soil. In the alfalfa and alfalfa-amended soil, higher levels of galactosamine, mannosamine, glucosamine, *N,N'*-diacetylchitobiose, acetylgalactosamine, acetylglucosamine and acetylmannosamine were detected than in the unamended soil. Gas chromatographic analysis confirmed the

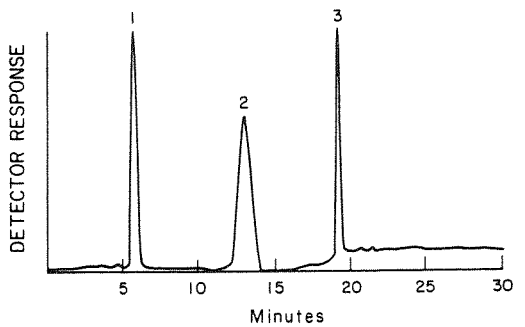

Fig. 3. HPAC–PAD chromatogram of an acidic extract of chitin. Chromatographic conditions as described in Table 1 [1 = 2-deoxyribose (internal standard); 2 = glucosamine; 3 = acetylglucosamine].

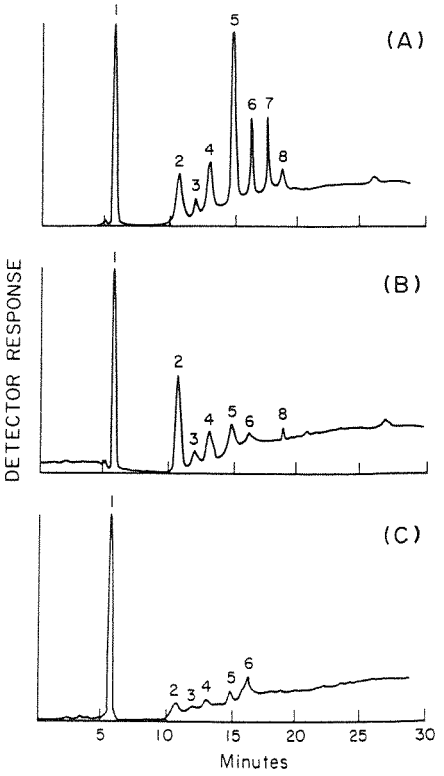

Fig. 4. HPAC–PAD chromatograms of the acidic extracts of (A) alfalfa, (B) alfalfa-amended soil after three months, (C) unamended soil. Chromatographic conditions as described in Table 1 [1 = 2-deoxyribose (internal standard); 2 = galactosamine; 3 = mannosamine; 4 = glucosamine; 5 = *N,N'*-diacetylchitobiose; 6 = acetylgalactosamine; 7 = acetylmannosamine; 8 = acetylglucosamine].

Table 3. Resolution (R_s)* of aminosacharides detected in acid extracts† by HPAC–PAD and HPLC–RI

	D-Galactosamine	D-Mannosamine	D-Glucosamine	*N,N'*-Diacetyl-chitobiose	Acetyl-D-galactosamine	Acetyl-D-mannosamine	Acetyl-D-glucosamine
D-Galactosamine	—	1.30 (0.01)†	2.96 (0.01)	4.33 (3.61)	5.20 (3.60)	5.50 (3.60)	6.10 (3.60)
D-Mannosamine		—	1.46 (0.01)	3.00 (3.60)	4.42 (3.60)	5.16 (3.60)	5.72 (3.60)
D-Glucosamine			—	1.57 (3.60)	3.81 (3.60)	4.52 (3.60)	4.80 (3.60)
N,N'-Diacetylchitobiose				—	1.20 (0.01)	2.00 (0.01)	2.40 (0.01)
Acetyl-D-galactosamine					—	1.25 (0.01)	1.80 (0.01)
Acetyl-D-mannosamine						—	1.05 (0.01)
Acetyl-D-glucosamine							—

*$R_s = 2(\{t_R\}_y - \{t_R\}_x)/W_x + W_y$, where x and y denote two solutes and W is the width of the peak at baseline, expressed in time units.
†HPAC–PAD: column; CarboPac PA1 (250 × 4.5 mm.); eluant: 5% 100m*M* NaOH for 15 min, then ramped to 200m*M* in 5 min; detection: pulsed amperometric detection. HPLC–RI: column; Beckman μ-Spherogel carbohydrate (300 × 7.5 mm); eluant: HPLC grade water (85°); detection: refractive index.
‡The values in parentheses indicate R_s values for HPLC–RI detection.

Table 4. Composition and quantity (*mg/kg*) of aminosaccharides extracted from chitin, organic amendments, organic-amended soil and microbial polymers

Aminosaccharide	Chitin	Poultry manure (PM)	PM-treated soil	Sewage sludge (SS)	SS-treated soil	Straw (ST)	ST-treated soil	Alfalfa (AL)	AL-treated soil	Check soil	*Chromo-bacterium violaceum*	*Hansenula holstii*
Galactosamine	ND*	49	4	188	5	1.41×10^3	5	1.83×10^3	13	1	2.06×10^4	ND*
Mannosamine	ND	39	3	90	3	230	3	685	2	<1	7.02×10^3	ND
Glucosamine	7.15×10^4	38	4	115	5	905	5	1.21×10^3	34	2	8.63×10^3	ND
N,N'-Diacetylchitobiose	ND	187	6	84	3	1.31×10^4	4	6.04×10^3	5	1	1.27×10^4	1.09×10^3
Acetylgalactosamine	ND	18	<1	35	1	149	13	245	1	1	2.38×10^3	ND
Acetylmannosamine	ND	67	2	73	3	390	3	1.57×10^3	0	1	1.96×10^4	ND
Acetylglucosamine	1.21×10^5	60	3	59	2	968	3	1.95×10^3	1	<1	1.23×10^4	ND
Total	1.93×10^5	458	23	644	22	1.72×10^4	36	1.35×10^4	56	7	8.33×10^4	1.09×10^3

*ND, not detected.

detection of glucosamine, galactosamine and mannosamine. The concentration of glucosamine detected in the organic-amended soils was comparable with the values obtained in GC analysis of soils by Benzing-Purdie,[1] although the GC analyses did not detect acetylaminosaccharides present in the samples. The concentration of the total aminosaccharides directly extracted from the animal wastes and plant residues ranged from 0.46 g/kg (poultry manure) through 0.64 g/kg (sewage sludge) and 13.5 g/kg (alfalfa), to 17.2 g/kg for straw (Table 4). Glucosamine was the predominant aminosaccharide in the plant materials. Previous work suggested that aminosaccharides in soil were of microbial origin and not from plant additions.[1,18] However, this study indicates that animal and plant residues may make an important contribution to the aminosaccharide pool in soil. The results also indicate that about 3.7% of the total nitrogen in alfalfa and about 20% of that in straw was present as aminosaccharides. The *Chromobacterium violaceum* polymer contained about 8% aminosaccharides, mainly galactosamine, acetylmannosamine, *N,N'*-diacetylchitobiose and acetylglucosamine whereas, the *Hansenula holstii* polymer contained only a low amount of *N,N'*-diacetylchitobiose (Table 4). Upon decomposition of plant residues in soil, aminosugars released may provide energy for the soil organisms or be complexed in the organic matter fraction.[7,19]

CONCLUSIONS

The HPAC–PAD work described for quantification of aminosacharides allows separation and analysis of a complex mixture of aminosaccharides in plant materials, chitin and soil. This study shows that minimal sample preparation is needed for HPAC–PAD. The combination of an anion-exchange column with triple-pulse amperometric detection and SCX purification results in high selectivity for aminosaccharides. HPAC–PAD is much more rapid, precise, sensitive and selective than HPLC–RI for the determination of aminosaccharides.

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