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Automatic generation of ultra-pure hydroxide eluent for carbohydrate analysis of environmental samples

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Abstract

High-performance anion-exchange chromatography coupled with pulsed amperometric detection under alkaline conditions has significantly improved our understanding of the function of carbohydrates in cycling of terrestrial carbon. The use of NaOH as an eluent has many advantages for anion analysis, but NaOH solutions are also an excellent trap for atmospheric CO_2 resulting in eluent carbonate ion contamination. To minimize the carbonate contamination, eluent generation technology has been marketed that produces ultra-pure hydroxide (OH) eluents on demand. Application of this technology significantly reduced the amounts of OH present in the waste stream and provided faster chromatographic runs with greater sensitivity and precision for determining the monosaccharide composition from environmental samples. Minimizing the salt concentration to $\leq$50 mm from acid hydrolysis extractions injected (25 μl injection volume) on column improved reproducibility of analyte retention times and separations. Eluent generation technology eliminated inconsistent OH eluent preparation that is often due to carbonate contamination. The contamination increases retention time variability for monosaccharides released by hydrolysis of biological samples.

Keywords: Mobile phase composition; Environmental analysis; Monosaccharides

1. Introduction

Liquid chromatography, especially high-performance anion-exchange chromatography (HPAEC), requires careful attention from a trained technician to prepare eluent solutions. Contamination or small variations in preparation techniques results in eluent solutions that may bias the analytical results. This contamination is especially pronounced when OH-based eluents are prepared for AEC analysis of anions or carbohydrates [1]. Use of ultra-pure liquid NaOH has been an acceptable source of OH ions, but once the container is open and in use, adsorption of atmospheric CO_2 is immediate as OH is an excellent trap for CO_2 resulting in carbonate formation. This is a major problem in the use of NaOH-based eluents because carbonate is a stronger eluting ion than OH and can accumulate on the column with low-OH isocratic or gradient chromatography. Sodium hydroxide-based eluents for HPAEC have many advantages over carbonate–hydrogen carbonate eluents that include broader linear response range for analyte detection and higher sensitivity, but the OH-based eluents are not widely adopted because of the preparation problems [1].

Advances in conductivity eluent suppression in anion and cation chromatographic analysis have opened new methods for generation of OH-based eluent for HPAEC analysis of anions and potentially carbohydrates [2]. Generation of high-purity OH is accomplished by electrolysis of water by a platinum electrode within the potassium hydroxide chamber and movement of the K^+ ion across a cation-exchange membrane for every OH generated. This “reagent-free” technology results in the need to prepare only filtered purified water as the eluent and then isocratic or gradient OH eluent is provided on demand by modules within the AEC instrument [3].

The work presented here details the first reported comparison between high-purity KOH eluent generated electrolytically and eluent prepared from purified liquid NaOH for analysis of carbohydrates from plant and soil sources. Rapid and accurate analysis of carbohydrate composition including the hemicellulose and cellulose fractions in environmental samples is extremely important as potential climate change may be mitigated or enhanced by cycling of plant carbohydrates in soil.

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2. Experimental

2.1. Reagents and materials

All monosaccharide standards were obtained from Sigma (St. Louis, MO, USA). The side oat grama (*Bouterloua curtipendula*) and western wheat grass (*Pascopyrum smithii*) plant and soils near the different plants were obtained from US Department of Agriculture (USDA) Forest Service land near Flagstaff, AZ, USA. The plant material was dried and ground to pass a 1 mm sieve and the soil samples were passed through a 2 mm sieve prior to further preparation for monosaccharide composition analysis.

2.2. Monosaccharide extraction

Optimized monosaccharide extraction conditions determined by Martens and Loeffelmann [4] for plant residue carbohydrate analysis required the use of a two-step digestion procedure. First, hemicellulose sugars (arabinose, galactose, glucose, xylose, rhamnose, and mannose) were extracted from 20 mg sample of plant biomass with 800 μl 6 M H_2SO_4, diluted to 1 M after 30 min and autoclaved (121 °C, 104 kPa for 30 min). Second, the residue was washed with two aliquots of 1 ml deionized (DI) water, then dried (60 °C) before addition of 300 μl 18 M (98% reagent) H_2SO_4 (30 min) for cellulose solublization and autoclave hydrolysis (1.5 M H_2SO_4, 30 min). In each digestion, the samples were neutralized to pH 4–5 with 5 M KOH, centrifuged (3000 × *g*) to remove precipitate, the precipitate was washed twice, the supernatants combined, and an aliquot was diluted with Millipore™ purified water for analysis.

Soil carbohydrates were extracted by treating 100 mg soil samples with 800 μl 6 M H_2SO_4 for 30 min and then using autoclave digestion (1.5 M H_2SO_4, 30 min) to release monosaccharides. The autoclaved samples were neutralized to pH 4–5 with 5 M KOH, centrifuged (3000 × *g*) to remove precipitate, the precipitate was washed twice, the supernatants combined and an aliquot was diluted for analysis.

Caution: Great care must be exercised to quantitatively retain the small fragments of plant residue that may carry over with the supernatant from the hemicellulose fraction before the cellulose extraction step. In addition, the plant and soil material must be thoroughly washed with water (at least two 1-ml aliquots) to remove residual H_2SO_4 used to extract the hemicellulose fraction before being dried at 60 °C or poor recovery of cellulose-C will result due to charring. Temperatures higher than 60 °C used to dry the material will also result in reduced cellulose recovery as glucose. Great caution must be exercised to avoid any contact with skin when using concentrated H_2SO_4.

2.3. Chromatographic instrumentation

The monosaccharides released were separated on a Dionex DX-500 (Dionex, Sunnyvale, CA, USA) ion chromatograph equipped with an analytical CarboPac PA10 (250 mm × 2 mm i.d.) column and a CarboPac PA10 guard column (50 mm × 2 mm i.d.) at a flow rate of 0.25 ml min^{-1} at ambient temperature. Sample introduction was via a Dionex AS40 autosampler equipped with a 25 μl sample loop. Monosaccharide separation was achieved with a OH gradient as shown in Table 1 for an EG 40 eluent generator equipped with a EGC-KOH cartridge (Dionex Corp).

Table 1
OH gradient, eluent A: 0.22 μm Millipore filtered water (>5 × 10^{-5} dS m^{-1})

Time (min)	Eluent	
	A (%)	EG-40 (mM)
0	100	1
5	100	1
10	100	3
20	100	9
30	100	28
31	100	70
35	100	80
40	100	70

Table 2
NaOH gradient, eluent A: 0.22 μm Millipore filtered water (>5 × 10^{-5} dS m^{-1}); eluent B: 20 mM NaOH; eluent C: 250 mM NaOH)

Time (min)	Eluent		
	A (%)	B (%)	C (%)
0	100	0.0	0.0
10.5	100	0.0	0.0
25.5	75	25	0.0
35.4	0.0	70	30
36.0	0.0	15	85
40.0	0.0	15	85

Monosaccharide separation with prepared NaOH eluents is as shown in Table 2.

The water was thoroughly degassed by heating under vacuum to prevent formation of carbonate from soluble CO_2. Detection was by triple-pulse amperometry with a gold working electrode equipped with a combination pH-Ag/AgCl reference electrode in pH mode. The following working pulse potentials (*E*) and durations (*t*) were used for the detection of the monosaccharides: $E_1 = 0.40$ V ($t_1 = 400$ ms); $E_2 = 1.0$ V ($t_2 = 100$ ms); $E_3 = 0.75$ V ($t_3 = 190$ ms); $E_4 = 0.25$ V ($t_4 = 390$ ms).

3. Results and discussion

3.1. Eluent hydroxide preparation comparison

Table 3 provides the retention times and standard deviations and resolution values for the chromatography of monosaccharide standards for the generated OH eluent ($n = 10$ standards) and prepared OH eluent ($n = 10$ standards). The results show that the chromatography of

Table 3
Chromatographic parameters in detection of monosaccharide composition of carbohydrates by HPAEC–pulsed amperometric detection (PAD) using eluent generation or prepared eluents

Monosaccharide	Parameters					
	Eluent generation OH			Prepared OH eluent		
	t_R[a]	S.D.	R_s[b]	t_R	S.D.	R_s
Fucose	5.25	0.10	–	5.59	0.15	–
Arabinose	9.79	0.23	7.55	11.43	0.39	10.33
Rhamnose	10.53	0.21	1.28	12.51	0.39	1.78
Galactose	11.40	0.31	1.42	13.72	0.54	2.43
Glucose	13.16	0.34	2.92	16.37	0.64	4.66
Xylose	14.82	0.37	2.77	19.07	0.67	4.56
Mannose	15.38	0.35	0.92	20.33	0.49	1.66
Ribose	17.31	0.45	3.37	22.21	0.39	2.76

[a] Retention time (min) and standard deviation (S.D.) were based on 10 (eluent generated) or 10 (prepared eluent) standard injections with monosaccharide concentrations ranging from 1 to 25 mg l^{-1}.

[b] Resolution between neighboring peaks was calculated by $[2(t_{R,y} - t_{R,x})/(W_x + W_y)]$ where t_R is retention time of the two analytes and W is the width at baseline. Since fucose was the first eluting peak, no resolution was given for fucose and the solvent front (1.83 min).

monosaccharides found in biological samples [4] had a greater precision with use of generated OH eluents compared to the prepared eluents when evaluated over a 3-day time period. Liu et al. [5] reported major improvements in retention time reproducibility when eluents were generated versus prepared OH-based eluents for early eluting inorganic anions and organic acids. Chromatographic traces of the monosaccharide standards are shown for the generated OH eluent (Fig. 1) and for the prepared eluents (Fig. 2) given for each day of chromatographic analysis during a 3-day sample analysis schedule. The traces show that acceptable separation or resolution between eluting peaks was obtained for each method of eluent preparation and that resolution values were near or exceeded 1.0 for each monosaccharide with the different eluent preparation methods and are given in Table 3. The results suggest that the use of eluent generated on demand resulted in better precision and lower waste stream OH concentrations than use of prepared eluents from a low-carbonate NaOH source.

Monosaccharide analysis in our laboratory is conducted on acid-hydrolyzed plant and soil samples. The use of sulfuric acid to release monosaccharides and NaOH to neutralize the acidity before chromatography generates a high salt matrix (≥2.0 M salts). Development of methodology for monosaccharide analysis from biological samples [6] found that a strong NaOH wash (minimum of 200 mm OH) following the monosaccharide separation was necessary to remove potential interferences and regenerate the OH ion on the column exchange sites for stable retention times. A low OH concentration gradient is required to separate the early eluting monosaccharides with these conditions, but the low OH concentration can result in exchange of counter ions such as Cl^- and carbonate for OH groups on the exchange sites. The loss of OH from the exchange sites will result

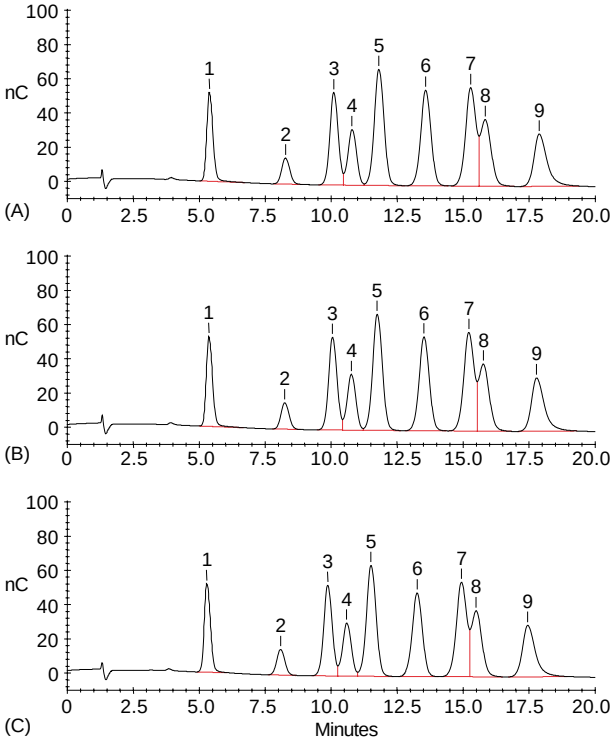

Fig. 1. Chromatograms of monosaccharide standards (4 μg ml^{-1}) in a ≤50 mM salt matrix separated by an eluent generated gradient detected by HPAEC–PAD on (A) day 1; (B) day 2; and (C) day 3. Peaks: 1, fucose; 2, 2-deoxy-glucose (internal standard); 3, arabinose; 4, rhamnose; 5, galactose; 6, glucose; 7, xylose; 8, mannose; 9, ribose.

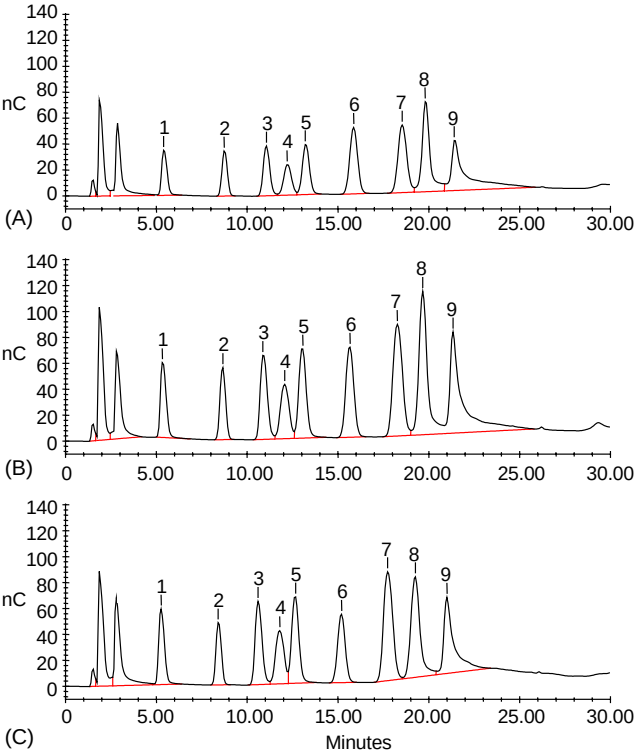

Fig. 2. Chromatograms of monosaccharide standards separated by a prepared eluent gradient detected by HPAEC–PAD on (A) day 1 (4 μg ml^{-1}); (B) day 2 (8 μg ml^{-1}); and (C) day 3 (8 μg ml^{-1}). Peaks as in Fig. 1.

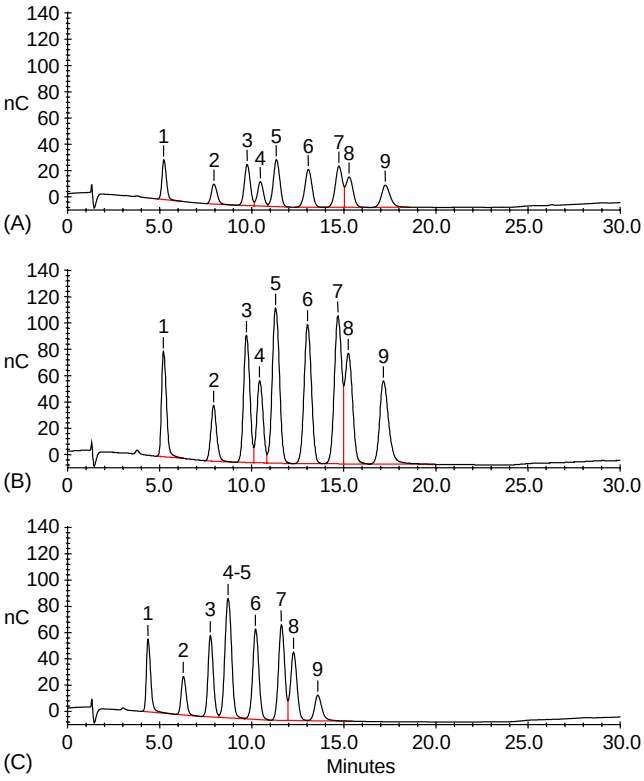

Fig. 3. Chromatograms of monosaccharide standards in a ≥100 mM salt matrix separated by an eluent generated gradient detected by HPAEC–PAD on (A) day 1 (2 μg ml^{-1}); (B) day 2 (8 μg ml^{-1}); and (C) day 3 (4 μg ml^{-1}). Peaks as in Fig. 1.

in retention time instability for the subsequent monosaccharide analyses. Use of the eluent generation module (EG40) limits the production of OH ions to 100 mm, half the minimum 200 mm OH concentration required to produce stable monosaccharide elution times for the prepared OH eluent (Table 3 and Fig. 2). The high concentration of OH required for prepared eluents is the result of carbonate contamination during the preparation and storage of eluents and the 200 mm OH concentration is required to counter higher carbonate concentration also present in the 200 mm OH column rinse. Initial eluent generated chromatographic runs with hydrolyzed soils samples containing ≥100 mm salts resulted in unstable retention times and merging of the rhamnose and galactose peaks, as shown in Fig. 3C. The effect of salt concentration was investigated as a source of the retention time instability because eluent generation produces carbonate-free eluent. By increasing the dilution of the stock solutions with water, the matrix salt concentration were reduced to ≤50 mm in standards and samples, and resulted in stable retention times as shown for monosaccharide standards in a 50 mm salt matrix (Fig. 1).

3.2. Plant and soil carbohydrate composition

Carbohydrates play a major role as structural (e.g. cellulose and hemicellulose) components of plants and provide a major source of energy for microbial processes in soils. Martens [7] reported that different plant residues have different decomposition rates in soil and the decomposition rates were related to the chemical composition of the plant residue. Since carbohydrates are the greatest source of C cycling in the terrestrial environment [7], the quantitative composition of different plant residues and the impact of plant biochemistry on carbon mineralization and cycling must be known for soil C sequestration to be managed as a means to mitigate atmospheric CO_2 concentrations.

Table 4
Properties of side oat grama and western wheat grass and the soils (0–5 cm depth) in proximity with the different plants[a]

Material	Concentration (mg g^{-1})			
	Carbon	Nitrogen	Total carbohydrates	Glucose[b]
Side oat grama plant	489	8.40	498	315 (63)
Soil bulk	6.15	0.70	2.15	0.44 (20)
Soil inside	13.7	1.27	5.10	1.66 (33)
Soil outside	8.87	0.77	2.65	0.58 (22)
Western wheat grass plant	492	26.8	338	158 (47)
Soil bulk	6.20	0.61	2.40	0.42 (18)
Soil near	7.84	0.94	2.69	0.66 (25)

[a] Soils sampled included bulk soils in interspace areas between plant communities, soil inside of the growth ring of side oat grama and outside the growth ring (10 cm) and soils near groups of western wheat grass plants and 10 cm away from plant group.
[b] The value in parentheses indicate the percentage glucose recovered of total carbohydrates recovered.

The total carbon and nitrogen content, and total carbohydrate and total glucose concentrations for the two species of rangeland vegetation, a side oat grama and a western wheat grass and the related soils are given in Table 4. The

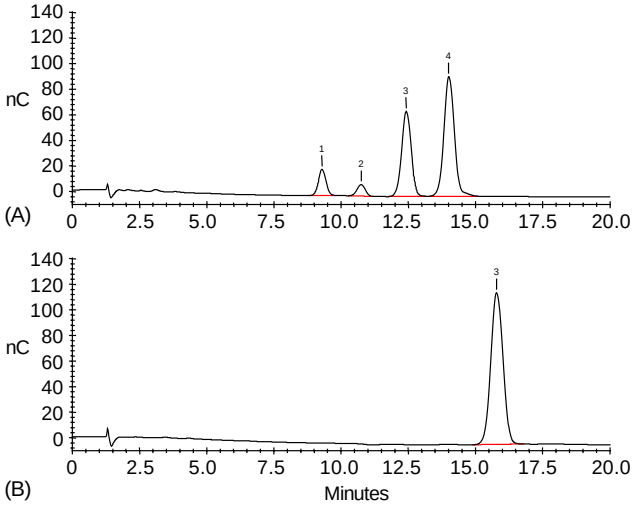

Fig. 4. Chromatograms of side oat grama plant tissue in a ≤50 mM salt matrix separated by an eluent generated gradient detected by HPAEC–PAD for (A) hemicellulose fraction and (B) cellulose fraction. Peaks: 1, arabinose; 2, galactose; 3, glucose; 4, xylose.

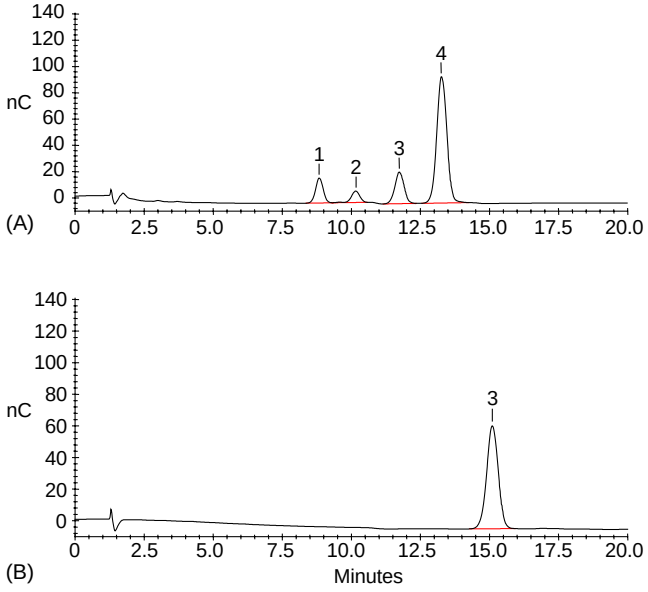

Fig. 5. Chromatograms of western wheat grass plant tissue in a ≤50 mM salt matrix separated by an eluent generated gradient detected by HPAEC–PAD for (A) hemicellulose fraction and (B) cellulose fraction. Peaks as in Fig. 4.

chromatographic results for the hemicellulose and cellulose components for the side oat grama and western wheat grass are shown in Figs. 4 and 5, respectively. Although the carbon content of most semi-arid range soils is lower than temperate soils, the large expanse of the semi-arid soils in the USA (>25% of total land) requires a better understanding of carbon cycling in these soils. The side oat grama had lower nitrogen content and higher carbohydrate, glucose concentration and percentage of total carbohydrate concentration as glucose compared with the western wheat grass plant tissue (Table 4). It is of interest that the soils in the proximity of the different plants also had different amounts of total carbohydrates and glucose concentration and percentage of total carbohydrates as glucose (Table 4). Soils near the side oat grama had a higher carbon content than soils near the western wheat grass plants that may be the result of the growth pattern of the two grasses. The side oat grama is a bunch-type grass that will grow in concentric circles from the initial plant, while the western wheat grass vegetative growth is less dense and emerges from rhizomes each spring. It appears that the western wheat grass has a smaller impact on the organic carbon content of the rangelands compared with the side oat grama that may be related to the nitrogen content and carbohydrate concentration of the vegetation.

A carbon/nitrogen ratio has been used as a measure of availability of the plant tissue to soil microorganisms. The side oat grama had a carbon/nitrogen ratio of 58 versus the carbon/nitrogen ratio of 18 for the western wheat grass tissue or a three-fold difference. To determine the rate of organic matter mineralization, the two plant materials were added to the same soil and the carbon evolved from the soil as CO_2, measured by an infrared gas analyzer (Qubit Systems, Kingston, Canada) was determined for a period of 92 days (Fig. 6). The results showed that the difference between total carbon evolved from the side oat grama and the western wheat grass during the 92 days mineralization experiment approximated the difference in the carbon/nitrogen ratio. Thus, the increase in the soil carbon near the side oat grama plants may be due to the slower mineralization of the plant tissue and over time would result in greater soil carbon and carbohydrates as reported in Table 4.

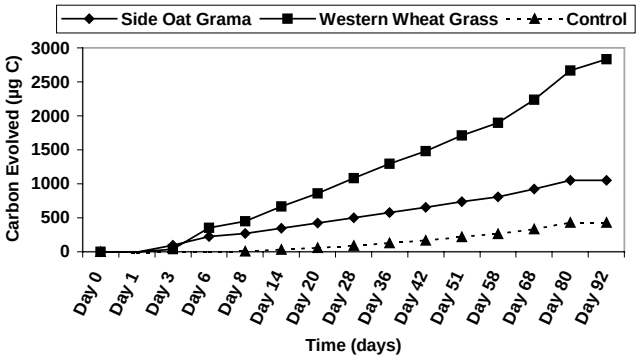

Fig. 6. Evolution of carbon from plant mineralization studies over a 92-day incubation as measured by infrared gas analysis for the side oat grama and western wheat grass plants.

The use of module-generated OH ions results in a greater retention time precision for the analysis of monosaccharides compared with the preparation of low-carbonate NaOH. The eluent generation technology limits the OH concentration in laboratory waste streams and has potential to shorten chromatographic run times while maintaining resolution between eluting peaks. Application of the eluent generation technology to digestions of environmental samples determined that the matrix salt concentration resulting from acid digestions and subsequent neutralizations can impact chromatographic resolution and that matrix salt concentrations need to be maintained at ≤50 mm for stable retention time analysis.

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