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Improved Analysis of Dissolved Carbohydrates in Stream Water with HPLC-PAD

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This paper describes improvements made to the determination of dissolved carbohydrates in stream water with high-performance liquid chromatography and pulsed amperometric detection (HPLC-PAD). We eliminated interference from dissolved oxygen, separated xylose and mannose along with other molecules, reduced the chromatographic peak shift associated with carbonate accumulation in the column to less than 1% for all samples, and achieved an 8% increase in recovery of hydrolyzed carbohydrates by replacing ion-exchange desalting cartridges with evaporative removal of HCl under N_2. These modifications lowered detection limits to less than or equal to 0.4 nM for 10 different monosaccharides and enhanced our ability to follow the dynamics of these molecules in stream ecosystems. In the determination of dissolved free monosaccharides, average relative precision was 1.3% and recovery ranged from 92 to 109%. For dissolved total saccharides, average relative precision was 3.3%. Concentrations were stable when filtered (0.2 μm) stream water was stored for 1 day at 22 °C, 1 week at 4 °C, or 1.5 months at −20 °C.

Carbohydrates constitute a small, though biologically important component of the dissolved organic carbon (DOC) in streams and rivers,[1,2] and their determination at the molecular level has been used to characterize biogeochemical and ecological processes.[3−7] The HPLC-PAD technique provides enhanced analytical reliability[8] and low detection limits, making it possible to generate an accurate and extensive database for the biogeochemistry of saccharides in aquatic environments.[8−14] The low detection limits of the HPLC-PAD method (2−10 nM) represent improved sensitivity over either colorimetric assays (0.7 μM detection limit) that cannot reveal molecular composition or gas chromatography methods (0.15 μM detection limit) that involve a concentration and derivatization step.[8,9,12,13] However, problems have been noted with HPLC-PAD, including the following: (1) the interference of dissolved oxygen (DO), (2) carbonate accumulation within certain analytical columns accompanied large peak shifts, (3) the coelution of molecules under isocratic conditions, and (4) contamination or loss of analytes during the desalting process following acid hydrolysis for the determination of dissolved total saccharides (DTS).[13] Here we report modifications that resolve each of these deficiencies. Additionally, we report on studies with DFMS handling and include recommendations for sample storage prior to analysis.

EXPERIMENTAL SECTION

Equipment. All analyses were performed with a Dionex 500 chromatography system, consisting of a PEEK version GP40 gradient pump module, an ED40 electrochemical detector, and an EG40 eluent generator. The system also included a Gilson 234 autoinjector with a 250-μL sample loop. An anion-exchange analytical column (4 × 250 mm, PA1; Dionex), packed with nonporous polymeric resin with an anion-exchange capacity of 100 μequiv, and fitted with the corresponding guard column (4 × 50 mm), was used to separate monosaccharide molecules. A carbonate retarder column (ATC-1, Dionex) was used to remove traces of carbonate from the eluent. The system was controlled with PeakNet 5.11 chromatography software (Dionex). Settings of the detector were as follows: E1 = 100 mV, t1 = 400 ms; E2 = −2000 mV, t2 = 10 ms; E3 = 600 mV, t3 = 1 ms, E4 = −100 mV, and t4 = 60 mS. The output range was 10−20 nC. A Thelco Laboratory Oven (Precision) was used for sample hydrolysis and a Thermolyne Dri-Bath (Sybron/Thermolyne) coupled with nitrogen manifold was used for the removal of HCl from hydrolyzed samples.

Chemicals. Carbohydrate standards were obtained as the purest available grades (Fluka, Aldrich). Stock solutions of standards (1 mM) were prepared in 10% aqueous acetonitrile (HPLC grade, Baxter). The solution was stored in 10-mL vials at −20 °C. Working stocks of monosaccharide standards (10 μM) were prepared in deionized (DI) water and stored in a refrigerator (4 °C). Calibration standards (10−500 nM) were prepared from aqueous serial dilutions of the working stocks and were refriger-

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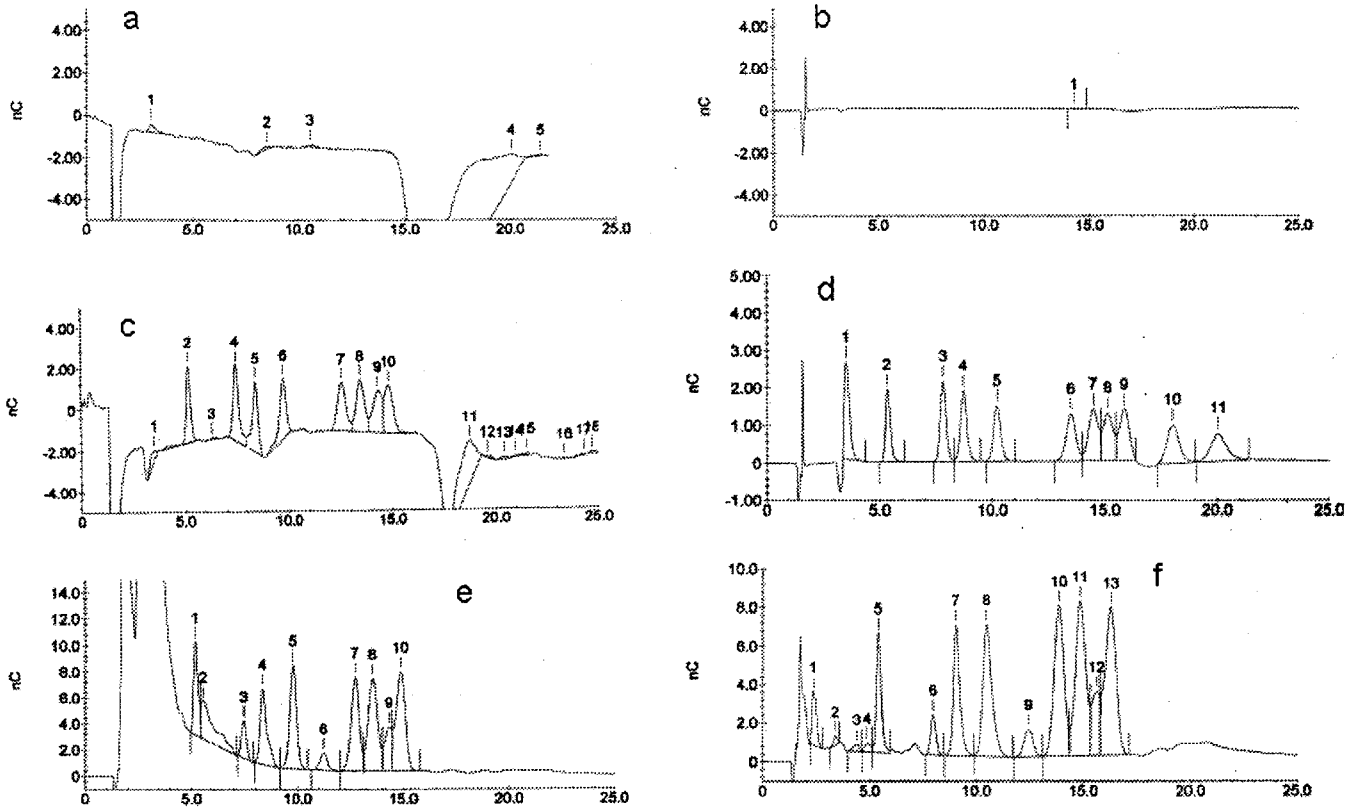

Figure 1. Comparison of chromatograms before and after improvements: (a) and (b) baseline stability with 0.2-μm filtered Nanopure water before and after modification of Dionex 500 system; (c) and (d) from 100 nM monosaccharide standards before and after the modification; and (e) and (f) from hydrolyzed stream samples following conventional desalting and Dri-Bath procedures. Identification in (d): 1, mannitol; 2. fucose; 3. 6-deoxyglucose; 4. rhamnose; 5. arabinose; 6. galactose; 7. glucose; 8. mannose; 9. xylose; 10. fructose; 11. ribose. Identification in (f): 1. mannitol; 5. fucose; 6. 6-deoxyglucose; 7. rhamnose; 8. arabinose; 9. glucosamine; 10. galactose; 11. glucose; 12. mannose; 13. xylose.

ated. Refrigerated standards were replaced weekly. All solutions and HPLC mobile phases were prepared with freshly deionized (DI) water from a Barnstead Nanopure II system (Syborn/Barnstead), and DI water used for the mobile phase was degassed with a water aspirator vacuum. HPLC mobile phases were made by diluting a low-carbonate/sodium hydroxide solution (50:50, w/w; Baker) or were generated as KOH by the EG40 directly from DI water. The mobile phase and degassed DI water used for the EG40 eluent generator were blanketed with purified nitrogen gas under a pressure of 10 psi.

Study Sites and Sample Handling. All samples were collected from four temperate headwater streams located in the Pennsylvania Piedmont and filtered through 0.2-μm nylon filters (Nalgene). Filters were washed with 5 mL of sample. Samples for DTS were acidified to pH 1.1 with 10 M HCl, hydrolyzed at 100 °C for 12 h,[13] and either passed through a Dionex OnGuardA desalting cartridge in the bicarbonate form (anion exchange) or heated and blown down in a nitrogen atmosphere. The desalting cartridge was activated with 5 mL of DI water followed by the same amount of air, at 2 mL/min, and the first 2 mL of sample eluent was discarded. All the glassware was combusted at 500 °C for 6 h.

RESULTS AND DISCUSSION

Elimination of DO Interference. The large negative peak that is associated with DO present in samples can obscure early-eluting molecules such as glucose and fructose, increase baseline noise (Figure 1a), and result in reduced sensitivity of the carbohydrate analysis.[6,8,9,12,13,15] Degassing of sample or eluent does not remove the DO interference.[6,8] Separate degassing of eluent and sample can generate disequilibrium in the system as the mobile phase and sample reach the detector. For example, degassing a sample with He resulted in a positive peak associated with He in a chromatogram.[6] The current design of the Dionex degassing system only degasses the eluent and thus has the same problem. We altered the design by installing an EG40 degassing module between the injector and the column and adjusted the maximum system pressure to less than 3000 psi. This effectively eliminated the DO interference and significantly reduced baseline noise (Figure 1b). With the 0.2-μm filtered DI water sample and a rejection area of 1000 counts, the only peak appearing in the baseline was glucose and it was below the detection limit. Dionex has accomplished the same result by installing a knitted reaction coil after the injector that allows for equilibration of the eluent and sample by extending the time between injection and movement through the analytical column (Jim Thayer, personal communication). Our modification has less dead time, no additional back pressure, and no additional component or cost.

Theoretically, the installation of a degassing unit between the injector and column could introduce peak dispersion, since the unit was composed of a linear tubing assembly. To prevent peak dispersion, we eliminated any unnecessary tubing. A comparison

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Table 1. Precision, Recovery, and Detection Limit for the Determination of DFMS in Stream Water Amended with 100 nM Standards ($\bar{X} \pm$ SD, $n = 3$)

	concentration (nM)			
analyte	stream water	amended stream water	mean recovery (%)	detection limit
mannitol	1.7 ± 0.1	96.8 ± 0.5	95	0.3
fucose	nd[a]	101.0 ± 2.3	101	0.4
rhamnose	nd	105.0 ± 0.8	105	0.4
arabinose	1.1 ± 0.2	103.7 ± 0.9	103	0.4
galactose	nd	103.0 ± 1.1	103	0.3
glucose	11.8 ± 1.9	107.9 ± 0.5	97	0.2
mannose	nd	109.3 ± 1.8	109	0.3
xylose	nd	106.0 ± 2.7	106	0.2
fructose	nd	92.3 ± 1.7	92	0.4
ribose	nd	100.2 ± 1.1	100	0.4

[a] nd, not detectable.

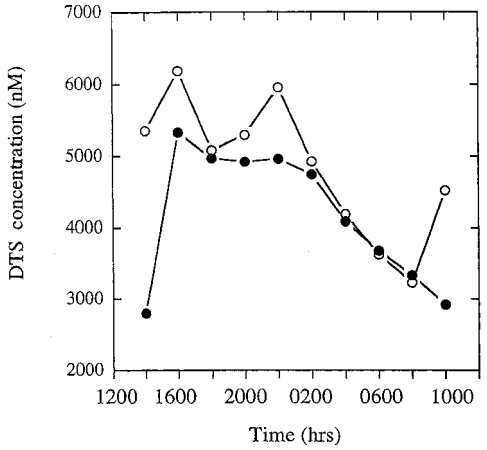

Figure 2. Comparison of analyte concentrations in stream water following the Dri-Bath or anion-exchange procedures: ○, Dri-Bath; ●, anion exchange. To the first and last samples, concentrations were significantly higher for the Dri-Bath procedure (paired *t*-test, $P = 0.05$).

of chromatograms before (Figure 1c) and after (Figure 1d) this modification showed that improved baseline stability was maintained with no observable dispersion, resulting in detection limits less than or equal to 0.4 nM for monosaccharides (Table 1).

Resolution, Precision, and System Reproducibility. To optimize the pH-dependent separation of carbohydrate molecules, we produced carbonate-free eluent directly from DI water with the EG40 eluent generator and compared separations achieved with mobile phases that ranged from 2 to 30 mM KOH. Xylose and mannose could be fully resolved with 2.3 mM KOH, but rhamnose and arabinose coeluted until the KOH concentration was above 11 mM. At a mobile-phase concentration of 21 mM KOH, xylose and mannose separated well enough to quantify, and all other monosaccharides tested could be separated (Figure 1d). Higher concentrations of eluent increased the resolution of xylose and mannose but reduced the resolution of glucose and mannose. The resolution of mannose and xylose peaks with 21 mM mobile phase was an improvement over prior analyses with 16 mM NaOH[13] and compared favorably with the separation of mannose and xylose achieved with a mobile phase of 24 mM NaOH.[6] We observed slight fluctuations in the eluent concentration produced by the EG40 that increased baseline noise. For optimal stability, we prepared 21 mM NaOH from a 50% NaOH solution. Since the difference in retention times between the closely eluting rhamnose and arabinose peaks and the xylose and mannose peaks was as long as 10 min, a gradient eluent program from 2.3 to 12 mM KOH could fully separate all of the saccharides. However, this would result in a loss of sensitivity due to dilution and the increased noise associated with postcolumn base addition.

The accumulation of carbonate in the analytical column, which reduces exchange capacity and deactivates the column, was eliminated by the inclusion of an 8-min cleaning with 300 mM NaOH in the analytical program. Routine maintenance included an initial daily cleaning with 300 mM NaOH at 1 mL/min for 30 min, followed by equilibration with eluent for 35 min, and weekly cleaning with 1 N NaOH for 1 h at 1 mL/min. When the system was maintained in this manner, peak shifts were less than 1% for both standards over 5 injections and hydrolyzed stream water samples over 23 injections. At DFMS concentrations of 100 nM, average relative precision (CV) was 1.3 ± 0.7% ($X_{mean} \pm$ SD) and recovery was between 92 and 109% for individual monosaccharides in stream water amended with standards (Table 1). This represents a substantial improvement over prior analyses reported with the PA1 column where retention times shifted between 8 and 13%, precision was between 3 and 20% for solutions of standards, and an analytical cycle took 10% longer to complete.[13]

Desalting and Neutralization of Hydrolyzed Samples. Samples analyzed for DTS require acid hydrolysis[12,13] followed by neutralization and desalting prior to determination of the constituent monomers. Freshwater samples are typically desalted and neutralized by passing the hydrolysate through an OnGuardA ion-exchange cartridge (Dionex). However, this exposes the sample to potential contamination from the cartridge matrix (Figure 1e), an inevitable introduction of carbonate because the cartridges are saturated with bicarbonate, and removal of charged carbohydrates such as uronic acids and some amino sugars that would interact with the anion-exchange cartridge. Additionally, use of the cartridges is time-consuming and represents an additional cost.

We neutralized and desalted samples with a Dri-Bath method as an alternative to ion exchange. HCl was removed by blowing down the sample (1 mL) in an autosampler vial under a stream of N_2. The sample was first heated to 95 °C for 15 min to facilitate the removal of water, and then the Dri-Bath was turned off and allowed to cool before the sample was completely dry. When dry (~45 min), the gaseous-phase HCl is readily removed. As a precaution, we ensured the complete removal of HCl by adding 100 μL of DI water and repeating the N_2 drying. The dried sample was then redissolved with DI water and sonicated for 5 min. Losses up to 60% of sugars have been reported for some sample drying methods[16,17] but not others.[6,12,18−20] The loss of sugars may be induced through condensation reactions with other organic compounds[16] that can be catalyzed by either basic or acidic conditions. We kept the desalting process in an inert condition by using a nitrogen atmosphere and completing the drying at

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Table 2. Influence of Sample Storage Conditions on Concentrations of DFMS (nM)

time (days)	T (°C)	monosaccharides[a]									
		mannitol	fucose	rham	arab	galac	gluc	mann	xylose	fruc	ribose
0	22	96.8	101.0	105.0	103.7	103.0	107.9	109.3	106.0	92.3	100.2
		[0.5]	[2.3]	[0.8]	[0.9]	[1.1]	[0.5]	[1.8]	[2.6]	[1.7]	[1.1]
1	22	95.9	89.8	91.6	88.3	101.1	119.2	97.7	103.7	103.9	87.8
2.5	22	28.8	5.2	nd[b]	nd	9.6	1.4	11.6	nd	nd	nd
7	5	102.2	93.3	94.3	90.0	96.6	93.0	107.0	92.9	195.2	100.3
		[7.5]	[6.9]	[0.5]	[1.6]	[1.9]	[3.4]	[0.8]	[1.9]	[2.2]	[3.3]
15	5	2.5	10.6	14.2	nd	nd	nd	nd	nd	nd	nd
30	5	ND	4.5	nd	nd	nd	nd	nd	nd	nd	nd
15	−20	102.4	97.7	103.0	102.9	98.9	110.5	105.5	98.0	100.7	100.8
		[2.2]	[3.5]	[7.2]	[2.8]	[2.2]	[2.0]	[6.4]	[3.0]	[9.1]	[3.2]
45	−20	106.5	105.3	117.9	106.5	97.7	111.7	105.9	98.0	101.7	106.2
		[3.1]	[5.1]	[5.2]	[4.6]	[4.4]	[3.5]	[4.5]	[2.8]	[0.6]	[3.6]

[a] Data presented as mean and [standard deviation], $n = 3$; others were single determinations. [b] nd, nondetectable with our method.

lower temperatures. A comparison of ion-exchange and Dri-Bath procedures with replicate subsamples of stream water collected during a storm from April 17 to 18, 2000, showed that concentrations of DTS in samples processed with our Dri-Bath procedure were equal or higher than samples processed with ion exchange (Figure 2). When DTS concentrations were averaged over all 10 stream samples, the Dri-Bath procedure resulted in a value of 8% higher than ion exchange. However, the accuracy of the DTS analysis cannot be verified without authentic reference materials. Furthermore, a comparison of chromatograms from the Dri-Bath and ion-exchange methods showed that the Dri-Bath resulted in a more stable baseline, which should ensure better detection limits and enable the identification of more components within the carbohydrate pool (Figure 1e and f). The Dri-Bath method is also conducive to a high through-put of samples, as the number of samples that can be processed simultaneously within a period of 1.5 h is only limited by the number of heating blocks and manifold lines available. Last, a series of five samples from three streams, with DOC concentrations ranging from 1.21 to 1.54 mg of C/L and DTS concentrations ranging from 69.2 to 94.1 μg of C/L, were analyzed in triplicate for DTS. The coefficient of variation (CV) associated with a total of seven to eight individual aldoses per sample ranged from 0.5 to 4.5%, with an average of 3.3 ± 1.5% (X_{mean} ± SD). This CV includes all the errors in sample collection, filtration, hydrolysis, neutralization, desalting, and analysis.

Sample Storage Prior to Analysis. Carbohydrates are susceptible to microbial decomposition, so the handling and storage of samples prior to analysis is important. Previous research found that samples for the analysis of DTS could be stored at room temperature for at least 21 days without losing saccharides after they were filtered (0.2 μm) and acidified to pH 1.1.[13] Typically samples collected for the determination of DFMS are not acidified, and when an autosampler is used, they may be held at room temperature for several hours prior to analysis. Concentrations of DFMS in filtered samples held at room temperature were stable during the first 24 h, but over 97% of DFMS was consumed after 54 h (Table 2). Cold storage at 4 °C showed reasonable stability for 1 week, and samples stored frozen (−20 °C) were unaltered for at least 45 days (Table 2). Rapid freezing of samples is an effective means of long-term storage.

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