Simultaneous Analyses of Neutral Carbohydrates and Amino Sugars in Freshwaters with HPLC–PAD

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Abstract

**In this study,
we determine concentrations of neutral and amino sugars and a sugar alcohol in freshwaters using high-performance liquid chromatography and pulsed amperometric detection with a single isocratic analysis. Coeluting arabinose, galactosamine, and mannosamine are separated with a mobile phase of 22.8mM NaOH-KOH at a temperature of 17°C. The resolutions are 0.73 and 0.64, respectively. The method separates closely eluting glucose–mannose and mannose–xylose peaks with resolutions of 0.85 and 0.71. Other sugars, fucose, rhamnose, galactose, fructose, ribose, glucosamine, and mannitol are resolved completely. Arabinose and galactosamine are measured in stream, ground, and soil waters that contain dissolved total saccharide (DTS) concentrations of 527 to 1555nM. Failure to distinguish galactosamine from arabinose in those samples results in a 53–82% overestimation of arabinose concentrations and a 1.8–6.5% overestimation of DTS concentrations. The near unity of glucosamine and galactosamine concentrations in stream water samples allows us to suggest a correction factor for historical samples that had been analyzed without resolving galactosamine and arabinose.**

Introduction

Carbohydrates encompass neutral sugars, amino sugars, sugar alcohols, and sugar acids. Of these constituents, neutral sugars are the major components in the dissolved total saccharide (DTS) pool (1) and therefore have received the most attention in analyses of marine (2,3) and terrestrial (4) samples. High-performance liquid chromatography (HPLC) with pulsed amperometric detection (PAD) can provide efficient and accurate determinations of carbohydrate concentrations (5-8). However, previous HPLC–PAD methods did not separate amino sugars from neutral sugars, and thus could overestimate neutral sugar concentrations because of coeluting compounds (9). Amino sugars are widely associated with structural polysaccharides or glycolipids (10), and they have been proposed as biomarkers for organic matter sources and their diagenetic state (11,12). The most widely distributed amino sugars are glucosamine and galactosamine (13). We previously resolved neutral sugars, the sugar alcohol mannitol, and the amino sugar glucosamine (14). However, we were unable to resolve galactosamine and found that it coeluted with arabinose in aquatic environmental samples and, thus, affected the quantitation of neutral sugars. Amino sugars have previously been determined with HPLC–PAD following procedures that first remove neutral sugars through adsorption to ion-exchange resins (9) or destruction with high concentrations (6M) of HCl (Jim Thayer, Dionex personal communication). These approaches allow a reliable quantitation of amino sugars. However, they neither facilitate the simultaneous determination of neutral sugars and amino sugars nor resolve the problem of overestimation of neutral sugars that arises from coelution when neutral sugars are analyzed.

Amino sugars are important structural components of bacterial cell walls, and neutral carbohydrates make up the basic units of plant cells. A full separation of amino sugars and neutral sugars can provide better insight into the origin and processing of these compounds in nature. Here we report, to our knowledge, the first HPLC–PAD method that simultaneously separates neutral carbohydrates, sugar alcohols, and amino sugars (galactosamine, glucosamine, and mannosamine) at the nanomolar level with a single isocratic analysis. Additionally, this work reveals the efficacy of decreasing operating temperatures to accomplish a previously unattainable separation, whereas the use of elevated temperature is more typical in HPLC analysis (15). The new method also was applied to samples representing streams from a broad geographical distribution, as well as soil water, groundwater, and stream water from a single watershed.

Experimental

Equipment

We used a Dionex (Sunnyvale, CA) 500 chromatographic system consisting of an electrochemical detector (ED40), eluent generator (EG40), polyetheretherketone version gradient pump (GP40), and gold electrode. We removed carbonate from the

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eluent with an anion trap (ATC-1, Dionex) and amino acids with an amino trap (P/N 46122, Dionex). PeakNet 5.11 chromatography software (Dionex) was used to control the system, and analyte separation was performed with either a Dionex PA1 or PA10 analytical column. Both columns use 10-µm diameter polystyrene–divinyl benzene substrate with ion-exchange capacity of 100 µeq. However, substrate agglomeration differs, with 580 nm microbead quaternary ammonium functionalized latex used with the PA1 and 460nM difunctional ammonium ion used with the PA10. These differences result in lower back pressure with the PA1 (1100 psi) compared with the PA10 (> 2300 psi). Effects of these differences on the application of anion and amino trap columns for carbohydrate analysis will be discussed later. Settings for the detector and instrument modifications to remove dissolved oxygen have been previously described (14). Nitrogen was used to pressurize the eluent reservoirs at 10 psi. Samples were introduced with a Gilson (Middleton, WI) 234 autoinjector and 250-µL sample loop. We hydrolyzed samples (100°C, 12 h, pH ~ 1.0) in a laboratory oven (Precision, Winchester, VA). Following hydrolysis, residual hydrochloric acid was removed either by evaporation with a Thermolyne Dri-Bath (Sybron/Thermolyne, Dubuque, IA) coupled with a nitrogen manifold or by passage through a Dionex OnGuardA anion exchange desalting cartridge in the bicarbonate form (14).

Chemicals

Sugar standards were purchased from Fluka/Aldrich (Buchs, Switzerland) in the purest available grades and low carbonate NaOH (w/w, 50% solution) was obtained from J.T. Baker (Philipsburg, NJ). Deionized (DI) water was prepared with a Barnstead Nanopure II system (Sybron/Barnstead, Boston, MA) and used to make monosaccharide standard working solutions (10µM) diluted from standard stocks (1mM) that were prepared in 10% aqueous acetonitrile (HPLC grade, Fisher, Fair Lawn, NJ) and stored in the freezer (–20°C) for up to 6 months. Our calibration standards (10–500nM) were made in 10-mL vials via aqueous serial dilution and adjusted to a pH of approximately 1.0 with 10N HCl. The calibration standards were analyzed with a procedural standard calibration method, that was the aqueous calibration standards were prepared and processed in exactly the same manner as a sample. The HPLC mobile phases were either generated directly from DI water with the EG40 as KOH or made by diluting the low-carbonate NaOH solution.

Method optimization

To determine the optimum condition for a simultaneous separation of neutral and amino sugars, we varied the eluent concentration between 10 and 30mM and the operating temperature by adjusting the ambient room temperature with an air conditioner. Details of the analytical procedure, sample handling, and analysis were described previously (14). The effect of ionic strength was studied by adding KCl in a range of I = 0 to 6mM. Resolution was also assessed with carbohydrate-free stream water that was prepared by UV-irradiation and then spiked with calibration standards. We calculated precision from replicate analyses of stream water and investigated recovery by spiking stream water with known amounts of monosaccharides after filtration, but before hydrolysis and removal of HCl. Recovery of amino sugars was further investigated by comparing concentrations following either the evaporative removal of HCl under N_2 or chemical removal of HCl with ion-exchange resin in two samples each of soil water, groundwater, and stream water. This information was also used to evaluate the possible loss of carbohydrates through the cartridge.

Study sites and sampling

Samples were collected from 12 streams located in watersheds within the Pennsylvania Piedmont, New Jersey Pinelands, and Evergreen Tropics of northwestern Costa Rica. In addition to stream water, samples of spring water, groundwater, and soil water were collected from the White Clay Creek watershed in southeastern Pennsylvania. We used precombusted glass bottles to collect grab samples of surface waters, a peristaltic pump to collect groundwater from shallow (~ 2 m) polyvinyl chloride wells, and soil water from vacuum lysimeters installed at a depth of 1 m. Wells and lysimeters were purged 24 h prior to sampling. We immediately filtered [0.2-µm nylon filters (Nalgene, Rochester, NY)] and acidified (pH ~1.0 with HCl) samples and kept them refrigerated (5°C) until analysis. All glassware was precombusted at 500°C for 6 h.

Results and Discussion

Method optimization

We previously resolved neutral monosaccharides with an isocratic elution and a mobile phase concentration of 21mM KOH-NaOH (14). However, our previous method only focused on neutral carbohydrates, and arabinose and galactosamine coeluted as one large peak. Separation of arabinose and galactosamine on the PA1 column began at a mobile phase concentration above 21.0mM and temperatures of 19–20ºC and was lost above 23.5mM. The best separation of arabinose and galactosamine occurred at an eluent concentration of 22.8mM (pH = 12.6) at a temperature of approximately 17°C. Even though the increase in pH shifted the peaks of glucose, mannose, and xylose, we success-

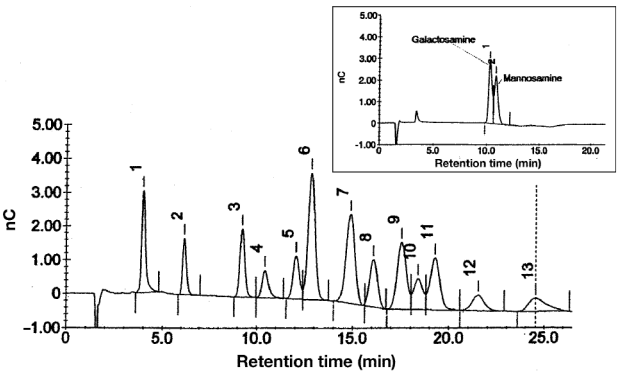

Figure 1. Chromatogram from the optimized method (200nM). Peak identification: (1) mannitol, (2) fucose, (3) 6-deoxyglucose (internal standard), (4) rhamnose, (5) arabinose, (6) galactosamine, (7) glucosamine, (8) galactose, (9) glucose, (10) mannose, (11) xylose, (12) fructose, and (13) ribose. Note: the retention time of galactosamine decreased as indicated in the insert because of column aging. The inserted chromatogram was run 1.5 years after the main chromatogram shown in this figure.

fully resolved all components under investigation (Figure 1). Complete separation was obtained for mannitol, fucose, rhamnose, glucosamine, galactose, fructose, and ribose, and the resolutions for the coeluting peaks of arabinose-galactosamine, glucose-mannose, and mannose-xylose were 0.73, 0.85, and 0.71 respectively. The detector response to galactosamine was approximately threefold greater than to arabinose with our experimental conditions (Figure 1).

Temperature can influence the resolution of neutral sugars and widely different column temperature optima have been suggested (5,16). With our eluent concentrations, there was no need to control the temperature for the determination of neutral sugars at normal room temperature (~ 20–25º). However, temperature exerted a crucial influence on the separation of arabinose and galactosamine. We observed that separation of arabinose and galactosamine diminished significantly above 20.5°C. Optimal temperatures and concentrations ranged from 19.5°C and 23.5mM to 16°C and 21mM. Within these settings, ionic strength of the samples did not need to be controlled to obtain the desired resolution of amino sugars and neutral carbohydrates. Currently, a commercial temperature controller is not available for our purpose (Pers. Comm. Dionex), and it is possible that precise temperature control would enhance resolution and thus broaden the optimal eluent concentration range beyond that reported here. Mannosamine, which can be abundant in glycoproteins and glycolipids, tends to coelute with galactosamine. Our method separated mannosamine from galactosamine with a resolution of 0.64 (Figure 1), however we have not detected mannosamine in any watershed samples.

The PA10 column has been reported to partially resolve arabinose and galactosamine at the micromolar level, although the resolution of mannose and xylose was limited (17). That investigation did not report on the influence of temperature, a parameter central to resolving arabinose, galactosamine, and mannosamine. It is therefore difficult to repeat those results. Additionally, the use of a relatively low eluent concentration may preclude the sensitivity required to determine carbohydrate concentrations at the nanomolar level that is typical of natural waters. The PA10 column was designed to eliminate the interference of dissolved oxygen (DO), which severely affected the quantitation of carbohydrates at the nanomolar level with the PA1, by pushing the DO dip to the end of the chromatogram of neutral sugars. However, the design of PA10 results in a large default back-pressure (2800 psi detected from our experiment and 2300 psi reported by the manufacturer) with a lower maximum allowable back-pressure (3500 psi compared with 4000 psi for the PA1). This caused difficulties when anion-trap and amino-trap columns, essential for the analysis of carbohydrates at the nanomolar level, were installed online to remove carbonate and amino acids, respectively. Additionally, the high-default back-pressure exhibited by the PA10 prevented our use of an efficient online column cleaning program between injections. That cleaning, which we used with the PA1, significantly reduced peak shifts, a common problem for the carbohydrate analyses with HPLC–PAD (14). Our previous modification of the Dionex 500 system eliminated the DO interference in carbohydrate analyses (14). Without DO interference, the lower default back-pressure of the PA1 provided greater flexibility for sensitive analyses. The present study demonstrates that separation on a PA1 column is superior to that reported with a PA10 column, and the method with PA1 is more applicable to environmental samples in which individual constituents of DTS pool are present in the nanomolar concentration range.

Table I. Precision and Recovery of DTS in Stream Water Amended with 99nM Standards

Analyte	Concentration (nM) (x ± SD) Stream water (n = 4)	stream water (n = 2)	Amended Precision (%RSD)*	Recovery† (%)
Mannitol	11 ± 1	107 ± 1	0.9	96
Fucose	184 ± 4	285 ± 4	1.4	100
Rhamnose	195 ± 8	294 ± 5	1.7	100
Arabinose	272 ± 24	418 ± 3	0.7	112
Galactosamine	37 ± 2	120 ± 3	2.5	88
Glucosamine	39 ± 1	128 ± 2	1.6	92
Galactose	248 ± 7	335 ± 5	1.5	96
Glucose	252 ± 7	333 ± 8	2.4	95
Mannose	101 ± 4	209 ± 6	2.9	104
Xylose	231 ± 8	315 ± 8	2.4	95
Fructose	14 ± 2	92 ± 0	0.0	81
Ribose	nd‡	105 ± 1	1.0	105

* RSD, relative standard deviation (x/SD).
† Recovery, measured concentration/theoretical concentration.
‡ nd, nondetectable.

Recovery, precision, and linear range

Recovery of carbohydrate monomers added to stream water ranged from 81% to 112% (Table I). This recovery range is wider than what we previously published (14). In the present study, we added the standard before hydrolysis, whereas previously the standard was added after hydrolysis, but before the removal of HCl. In contrast to hydrolysis procedures that have resulted in the destruction of fructose and ribose by high concentrations of sulfuric acid (6), our more mild acidic hydrolysis conditions, acid evaporation, and analytical procedures resulted in a partial loss of fructose (19%) and no analytically significant loss of ribose. Amino sugars were not lost during the evaporative removal of HCl under N_2, whereas the use of ion exchange following hydrolysis, as described in the Experimental section, resulted in amino sugar losses of 10% and 17% from two soil waters, 28% and 40% from groundwaters, and 31–44% from stream waters. The partial removal of amino sugars that we observed with ionic exchange differs from the complete removal suggested for both amino sugars and uronic acids in marine waters (18). The complete removal of uronic acids by ion exchange is facilitated by their negatively charged carboxylic functional groups. Amine groups are positively charged (protonated) under acidic conditions. They are neutral above a pH of approximately 6. Because the Dionex OnGuardA Desalting Cartridge gives a pH value of approximately 5.5, amine functional groups are weakly basic in the solution. Thus, a partial loss of amino sugars could be expected in the filtered samples. We made no attempt to correct our data for recovery, as the monosaccharides released during sample hydrol-

ysis would have been derived from many types of polymers and absolute recoveries cannot be measured without first knowing the structure of polysaccharides present.

Precision of this method, assessed as the percent relative standard deviation, ranged from 0% to 2.9% (Table I). We obtained a linear response ($R^2 \geq 0.99$) from 10 to 500nM, values that bracket the natural range of monosaccharide concentrations in our samples, except for ribose that had a value of $R^2 = 0.98$, perhaps as the result of losses during hydrolysis. However, our procedural calibration analysis may compensate the loss and thus minimize the analytical error. A quadratic model increased the R^2 value for each standard curve by ≤ 0.01.

Application to watershed samples

Analyses of stream water, spring water, soil water, and groundwater samples from a Pennsylvania Piedmont watershed showed good resolution with the new method (Figure 2A–D). In these samples, molar concentrations of arabinose ranged from 4.5% to 13.9% of the DTS pool, and galactosamine ranged from 1.0% to 3.8%. Without the present method modification, we would have not only overestimated arabinose concentrations by 53–82% and underestimated galactosamine, but also overestimated DTS by 1.8–6.5% (Table II) because of differences in detector response to the individual molecules.

Corrections of historic data

Historic data that did not consider the coelution effect of galactosamine and arabinose could be corrected to more accurately describe the concentrations of arabinose and DTS, if glucosamine could be quantitated. As an example, we present data in which arabinose, galactosamine, and glucosamine were quantitated in 67 analyses of waters from 12 different streams located in the Pennsylvania Piedmont, New Jersey Pinelands, and Evergreen Tropics in Costa Rica. The co-occurrence of galactosamine and glucosamine in each of the samples, and the relative constancy of the molar ratios of the two amino sugars (galactosamine: glucosamine = 0.81 ± 0.03, range 0.42 to 1.00) allowed us to estimate galactosamine concentrations based on the regression of galactosamine versus glucosamine (Figure 3) for samples analyzed prior to the detection of galactosamine. The consistency of this relationship over a broad geographical range suggests that the two amino sugars were derived from a common and widespread source. Glucosamine is a major component of chitin and galactosamine is found in glycolipids, but speculation about the common source or the meaning of this relationship in freshwater is beyond the scope of this study. With an average detector

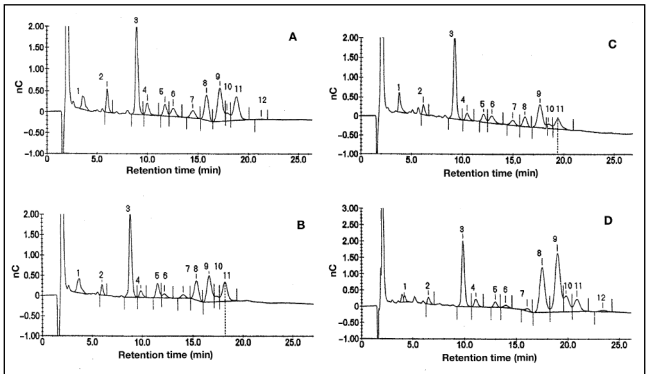

Figure 2. Chromatograms in White Clay Creek watershed samples: (A) stream water, (B) spring water, (C) groundwater, and (D) soil water. Peak identification: (1) mannitol, (2) fucose, (3) 6-deoxyglucose (internal standard), (4) rhamnose, (5) arabinose, (6) galactosamine, (7) glucosamine, (8) galactose, (9) glucose, (10) mannose, (11) xylose, and (12) fructose.

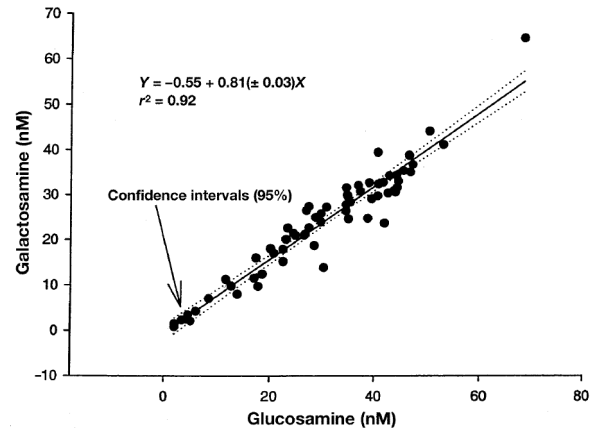

Figure 3. Relation between galactosamine and glucosamine concentrations in waters derived from 12 streams. The SD of the slope was 3.

Table II. Influence of Method Optimization on Estimation of Arabinose, Galactosamine, and DTS Concentrations (nM)

Site	Date	Arabinose before optimization*	Arabinose after optimization†	Arabinose over estimation (%)	Galactosamine*	DTS*	DTS over estimation (%)†
Well 11	2/17/02	180 ± 2	99 ± 7	82	36 ± 1	1022 ± 35	4.4
Well 34	2/17/02	127 ± 23	73 ± 24	74	20 ± 1	527 ± 60	6.5
Lysimeter 001	2/17/02	78 ± 5	51 ± 5	53	10 ± 0	863 ± 59	2.0
Lysimeter 004	2/17/02	85 ± 3	52 ± 3	64	12 ± 0	1149 ± 48	1.8
Stream water	11/16/01	190 ± 4	117 ± 3	62	45 ± 0	1555 ± 2	1.8
Stream water	12/07/01	157 ± 2	89 ± 1	76	36 ± 1	1215 ± 9	2.6

* Data expressed as $x \pm$ SD ($n = 2$).
† [Concentration of arabinose before optimization – (arabinose concentration + galactosamine concentration)]/DTS.

response of 3 for galactosamine versus arabinose, we can correct the arabinose concentrations for stream water samples by subtracting the value of 2.3 × the moles of glucosamine. For instance, we analyzed a stream sample collected from West Creek within the Pennsylvania Piedmont prior to our optimization. Concentrations of DTS, arabinose, and glucosamine analyzed were 1528, 224, and 50nM, respectively. The corrected concentration of arabinose would be calculated as: $[C_{Ara}] = 224 - 2.4 \times 50 = 109$nM. Concentration of galactosamine (GlN) was estimated as: $50 \times 0.81 - 0.55 = 40$ (nM). Then the concentration of DTS could be adjusted as: $[DTS]_{adj} = [DTS]_{orig} - [C_{Ara}]_{orig} + [C_{Ara}]_{adj} + [C_{GlN}] = 1528 - 224 - 109 + 40 = 1453$ (nM), where adj and orig represent adjusted and originally analyzed concentrations, respectively.

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References

1. G.O. Aspinall. *Polysaccharides*. Pergamon Press, New York, NY, 1970.
2. T. Hama and K. Yanagi. Production and neutral aldose composition of dissolved carbohydrates excreted by natural marine phytoplankton populations. *Limnol. Oceanogr.* **46:** 1945–55 (2001).
3. P. Hernes, G.I. Hedges, M.L Peterson, S.G. Wakeham, and C. Lee. Neutral carbohydrate geochemistry of particulate material in the central equatorial Pacific. *Deep-Sea Research* **43:** 1181–1204 (1996).
4. S. Opsahl and R. Benner. Characterization of carbohydrates during early diagenesis of five vascular plant tissues. *Org. Geochem.* **30:** 83–94 (1999).
5. R.D. Rocklin and C.A. Pohl. Determination of carbohydrates by anion exchange chromatography with pulsed amperometric detection. *J. Liq. Chromatogr.* **6:** 1577–90 (1983).
6. N.H. Borch and D.L. Kirchman. Concentration and composition of dissolved combined neutral sugars (polysaccharides) in seawater determined by HPLC–PAD. *Mar. Chem.* **57:** 85–95 (1997).
7. T.J. Gremm and L.A. Kaplan. Dissolved carbohydrates in streamwater determined by HPLC and pulsed amperometric detection. *Limnol. Oceangr.* **42:** 385–93 (1997).
8. P. Kerhervé, B. Charriére, and F. Gadel. Determination of marine monosaccharides by high-pH anion-exchange chromatography with pulsed amperometric detection. *J. Chromatogr.* **718:** 283–89 (1995).
9. K. Kaiser and R. Benner. Determination of amino sugars in environmental samples with high salt content by high-performance anion-exchange chromatography and pulsed amperiometric detection. *Anal Chem.* **72:** 2566–72 (2000).
10. A.L. Lehninger. *Biochemistry*. Worth Publishing, New York, NY, 1975.
11. B. Dauwe and J.J. Middelburg. Amino acids and hexosamines as indicatore of organic matter degradation state in North Sea sediments *Limnol. Oceanogr.* **43:** 782–89 (1998).
12. V. Ittekkot, W.G. Deuser, and E.T. Degens. Seasonality in the flux of sugars, amino acids, and amino sugars to the deep ocean: Panama Basin. *Deep-Sea Res.* **31:** 1057–69 (1984).
13. G. Liebezeit. Amino sugars in Bransfield Strait and Weddel Sea sediments. *Senckenbergiana maritime.* **23:** 29–35 (1993).
14. X.-H. Cheng and L.A. Kaplan. Improved analysis of carbohydrates in stream water with HPLC–PAD. *Anal. Chem.* **73:** 458–61 (2001).
15. B.A. Bidlingmeyer. Trends in reversed-phase HPLC column practice: 1997. *J. Chromatogr. Sci.* **35:** 392–400 (1997).
16. C. Panagiotopoulos, R. Sempéré, R. Lafont, and P. Kerhervé. Subambient temperature effects on the separation of monosaccharides by high-performance anion-exchange chromatography with pulse amperometric detection Application to marine chemistry. *J. Chromatogr.* **920:** 12–22 (2001).
17. J.B. Jahnel, P. Ilieva, and F. H. Frimmel. Detection of glucosamine in the acid hydrolysis solution of humic substances. *Fresenius' J. Anal. Chem.* **360:** 827–29 (1998).
18. A. Skoog and R. Benner. Aldoses in various size fractions of marine organic matter: implications for carbon cycling. *Limnol. Oceangr.* **42:** 1803–13 (1997).

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