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Carbohydrate Signatures of Aquatic Macrophytes and Their Dissolved Degradation Products as Determined by a Sensitive High-Performance Ion Chromatography Method

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The sugar contents of emergent macrophytes from a freshwater lake, a freshwater swamp, and a salt marsh in the southeastern United States were examined together with the dissolved free sugars produced during macrophyte degradation and in natural water samples collected adjacent to macrophyte stands. Simultaneous separation of up to 13 neutral and 2 amino sugars together with 3 uronic acids and muramic acid was achieved by anion-exchange high-performance ion chromatography. As little as 10 pmol or a concentration of 20 nM sugar can be detected by pulsed amperometry, a greater sensitivity for sugar quantification than that of previously reported detection techniques used in conjunction with either gas or liquid chromatographic systems. Optimum conditions for hydrolysis of plant material by using trifluoroacetic acid were determined, and internal standards were used to quantify losses due to matrix effects and solid-phase extraction of samples. Our data demonstrate that ratios of certain indicator sugars in undegraded macrophytes differ significantly from ratios of dissolved free sugars formed during macrophyte degradation, reflecting the complex processes (biological and physical) involved in vascular plant degradation in aquatic ecosystems. Natural water samples collected adjacent to macrophyte beds contained dissolved free sugars at concentrations of 620 nM (lake), 890 nM (freshwater swamp), and 2,300 nM (salt marsh). Sugar signatures of these natural water samples were similar to those of macrophyte degradation products.

Carbohydrates make up a large proportion of the dry weight of vascular plants, accounting, for example, for about 80% of the dry weight of the salt marsh grass *Spartina alterniflora* in eastern U.S. coastal marshes (13). The microbial breakdown of vascular plants in aquatic systems releases carbohydrates in dissolved form. These carbohydrates, either free (mono-, oligo-, and polysaccharides) or bound to other molecules such as humic substances (23), become part of the dissolved organic carbon pool in aquatic environments. From this pool, they are potentially available to bacteria and, via the microbial food web, to higher trophic levels (3).

Measurement of sugars in natural waters not only provides information on bulk-level changes in dissolved carbohydrate concentrations but also may provide an indication of the source material from which the carbohydrates originate. For example, high xylose concentrations in particulate organic material indicate an angiospermic vascular plant source, while arabinose plus galactose can be used to differentiate nonwoody sources (e.g., grasses) from woody sources (e.g., hardwood and softwood trees [6]). Mannuronic acid, a major component of alginic acid, is a potential indicator of algal sources of particulate organic material and is especially indicative of kelp (21, 25). High ribose levels indicate organic matter influenced by plankton (bacteria, phytoplankton, and zooplankton [6]). Finally, sugars are potentially valuable for quantifying microbial biomass on particulate material. Muramic acid can be used to estimate bacterial biomass (8) because its acetylated form is a unique component of the procaryote cell envelope, while glucosamine can be used to estimate fungal biomass (when insects and other arthropods are absent) because of its presence in the cell wall of most fungi (11).

Gas-liquid chromatography or high-performance liquid chromatography (HPLC) has previously been used to determine sugar content in plant material (1, 2, 4, 5, 25, 28, 29) and natural water samples (23). Gas-liquid chromatography requires formation of volatile derivatives, since free sugars are insufficiently volatile for direct analysis. This derivatization increases the time required for the assay and leads to a complex multiplicity of peaks which can be difficult to resolve. HPLC techniques, such as the use of an amino-bonded silica column with refractive index detection, often provide inadequate separation of sugars and poor sensitivity (2, 26) which limit their application to natural water samples. Low-wavelength UV detection (<200 nm) can be more sensitive than refractive index detection but is highly susceptible to interference, requiring significant sample cleanup (26).

We describe here a sensitive, relatively simple technique for the simultaneous determination of amino, neutral, and acidic sugars in plant material and water samples by using high-performance ion chromatography (HPIC). This method is advantageous over techniques used earlier in that a much wider range of sugars can be analyzed (previous techniques were largely restricted to neutral sugars) and better sensitivity can be achieved. To demonstrate the usefulness of this method, we determined carbohydrate signatures of vascular plants from three aquatic environments: a freshwater lake, a freshwater swamp, and a salt marsh in the southeastern United States. Ratios of component monosaccharides of undegraded plant material were compared to ratios of dissolved free sugars formed during microbial degradation of these plants in laboratory microcosms and to ratios in natural waters adjacent to plant stands.

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MATERIALS AND METHODS

Standard preparation. Sugars (Sigma Chemical Co., St. Louis, Mo.) were stored in a desiccator under reduced pressure. As the anion-exchange HPIC column does not differentiate between D- and L-forms of sugars, either enantiomorph was used as a standard. Mannuronic acid lactone was converted to the ammonium salt of mannuronic acid by adjusting a solution of the lactone to a pH of 8 to 9 with an ammonia solution. After 4 h at room temperature, the solution was freeze-dried to remove ammonia. Standard solutions of sugars were filtered through 0.4-μm-pore-size polycarbonate membranes (Nuclepore Corp., Pleasanton, Calif.) and usually used at concentrations of 20 μM, except for fucose and muramic acid which were used at 10 μM and fructose and ribose which were used at 60 μM. All standard solutions were divided into aliquots and stored at −70°C in Nalgene Cryovials (Nalge Co., Rochester, N.Y.) until required. Once thawed, standard solutions were stable for up to a week at 5°C. High-grade deionized water (18 Mohm/cm) passed through an organics removal cartridge (Unipure I system; Solution Consultants Inc., Marietta, Ga.) was employed throughout.

Acid hydrolysis. Green culms (leaves) of the sedge *Carex walteriana* were collected from the Okefenokee Swamp, Georgia, in December 1989 and April 1990; *S. alterniflora* (salt marsh cordgrass) and the rush *Juncus effusus* were collected from Sapelo Island, Georgia, and L Lake at the Savannah River Site, South Carolina, respectively, in April 1990. All plant samples were stored at −70°C until processed and then freeze-dried, ground to 425-μm-size particles, and stored in a desiccator at 5°C. The material was hydrolyzed with 2 M trifluoroacetic acid (TFA) (1) in glassware combusted at 550°C for at least 6 h. Triplicate samples of dried plant material (0.010 g), together with 300 μl of TFA (Sigma; 13 M, protein sequencing grade), 200 μl of mannosamine or 6-deoxyglucose (200 μM, internal standard for hydrolysis, rotary evaporation and resin cleanup steps), and 1,450 μl of water, were briefly bubbled with nitrogen and then heated in a nitrogen atmosphere at 121°C in an oil bath for 1 h in 8-ml Pyrex tubes with Teflon-lined screw caps. The tubes were cooled in ice, and the hydrolysate was transferred to heated, round-bottom flasks together with two water rinses of each tube. To each hydrolysate was added 10 μl of 50% (vol/vol) aqueous glycerol to minimize losses of sugars on drying (7, 14). The aqueous acid was removed by rotary evaporation under reduced pressure at a temperature not exceeding 40°C. The sugars were reconstituted in either a solution consisting of 100 μl of 2-deoxyglucose (200 μM) plus 400 μl of water (if losses due only to the resin cleanup step were to be determined) or 500 μl of water only. The 2-deoxyglucose can be added only after the acid is removed, as it does not survive acid treatment.

Before injection onto the HPIC, the samples were cleaned up by solid-phase extraction to prevent contamination of the column. Sample contaminants such as phenolic and hydrophobic compounds were removed with polyvinylpyrrolidone resin (Onguard-P; Dionex Corp., Sunnyvale, Calif.) and divinylbenzene reversed-phase resin (Onguard-RP; Dionex), respectively. Divinylbenzene is preferable to C_{18} reversed-phase resin because it is stable over a wider pH range, and, even after rotary evaporation for acid removal, our samples remained at a low pH. Before use, each Onguard-P cartridge was flushed with 4 ml of water while each Onguard-RP cartridge was flushed with 10 ml of methanol followed by 20 ml of water. The wet resin was transferred to a microcentrifuge tube and centrifuged for 2 min at 10,000 rpm in a swinging-bucket microcentrifuge (model 59A; Fisher Scientific Co., Pittsburgh, Pa.). The supernatant was discarded, and the resin was centrifuged twice more, with the supernatant being removed each time. Approximately 100 mg (wet weight) of each prepared resin was simultaneously added to a reconstituted sample, and the mixture was vortexed and centrifuged for 5 min at 10,000 rpm. The supernatant and top layer of resin were aspirated and filtered through 0.45-μm-pore-size membranes in Microfilterfuge tubes (Rainin Instrument Co., Inc., Woburn, Mass.). After filtration, the samples were stored at 5°C if they were to be analyzed within 1 week; otherwise they were stored at −70°C. The use of mannosamine or 6-deoxyglucose as an internal standard allows the recovery of sugars to be calculated relative to the internal standard recovery so that losses due to processing and matrix effects are quantified. Mannosamine and 6-deoxyglucose were chosen for internal standards because they do not have a record of natural occurrence in plant and microbial material, are easily separated from other sugars, and survive TFA hydrolysis with little loss (see Table 1). Correction factors for destruction of sugars by TFA were calculated by dividing the percent survival of the internal standard by the percent survival of the sugar of interest (see Table 1). The conversion factors 0.88 and 0.90 for pentoses and hexoses, respectively, were used to convert monosaccharides to polysaccharides (i.e., anhydro monosaccharides).

The destructive effects of 4 M TFA hydrolysis compared to those of 2 M TFA hydrolysis on the survival of standard sugars were examined because a concentration of 4 M TFA is required to completely hydrolyze plant polysaccharides containing amino sugars (19). After hydrolysis of triplicate samples in 2 or 4 M TFA, standard sugar solutions were dried by use of a rotary evaporator, reconstituted, and filtered as described above for plant material. Cleanup resins were not used on samples containing standard sugars only.

The potential effect of the plant matrix on sugar recovery after 2 M TFA hydrolysis was examined by spiking triplicate samples of plant material (*C. walteriana*) with standard sugars (see Table 2) and hydrolyzing with acid. Matrix effects were determined by comparing the recoveries from standard solutions with those from standard solutions added to the plant matrix, after subtraction of plant matrix sugars alone. To examine the effect of varying the sugar-to-plant-material ratio on the recovery of sugars after acid hydrolysis, triplicate tubes containing 0.010 g of dried plant material and 2 M TFA were spiked with 110 or 9,000 nmol of fucose and then hydrolyzed. 2-Deoxyglucose was used as an internal standard to correct for losses due to solid-phase extraction of hydrolysates.

Solid-phase extraction. To determine whether the Onguard-P, -RP, or -Ag resins (Dionex) used for sample cleanup selectively removed certain sugars from solution, triplicate 500-μl samples of standard sugar solutions were treated with each resin as described above. Onguard-Ag resin was added to sugar solutions with and without 20‰ sea salts (Sigma) to examine the effect of salt concentration on possible resin-sugar interactions. The Onguard-Ag resin was self-indicating; the color of the wet resin no longer changed from dark gray to white when all the chloride had been removed from solution.

To develop a method for separating inorganic salts from sugars in marine water samples, six resins (amino [Alltech Associates Inc., Deerfield, Ill.]), cyano, diol, aromatic sulfonic, quaternary amine, and polyethyleneimine [PEI; J. T.

Baker Inc., Phillipsburg, N.J.]) were investigated to determine relative binding efficiencies for sugars. To duplicate 8-ml, heated, screw-cap glass tubes were added 200 mg of resin. To prepare the resins, approximately 5 ml of acetonitrile was added to each tube, the suspension was swirled, and, after the resin had settled, the supernatant was aspirated and discarded. Immediately, a solution consisting of 4.00 ml of acetonitrile plus 0.50 ml of 36‰ sea salts (Sigma) plus 0.50 ml of standard sugar solution (concentration of each sugar, 10 to 60 μM; total amount of sugar added, 210 nmol [see Table 3]) was added to each tube. The tubes were sealed and shaken in a horizontal position for 15 min at a rate which kept the resin in suspension. After the tubes were shaken, the resins were allowed to settle and the supernatants were discarded. Approximately 5 ml of acetonitrile was added to each tube, the tubes were shaken briefly, and the supernatant was discarded when the resins had settled. The wet resins were dried by using a stream of nitrogen and then transferred to Microfilterfuge tubes. Sugars were eluted from the resin by adding 180 μl of water three times and centrifuging the tubes after each addition of water to collect the eluted sugars in the filtrate. The total volume of filtered eluant was brought to 500 μl, and aliquots were analyzed by HPIC. To determine the factors affecting the efficiency of sugar binding and recovery, the PEI resin/sugar ratio was reduced by 25 and 75%, and the water eluant/PEI resin ratio was increased threefold in separate experiments. In addition, tetrabutylammonium phosphate was added to the acetonitrile-sugar-sea salt-PEI resin suspension to a final concentration of 2.5 mM. This ion-pairing reagent increases the retention of sugars while reducing the retention of chloride on silica-based, weak-anion-exchange resin (27).

Water samples. Duplicate 200-ml water samples were collected from L Lake (site 2 of Moran and Hodson [16]) near *J. effusus* beds in August 1990 and from a *C. walteriana*-dominated marsh in the Okefenokee Swamp in February 1991. Water samples were kept cool during transport to the laboratory and then filtered through 0.2-μm-pore-size Nuclepore filters in autoclaved filtration units and stored at −70°C. To each 200 ml of filtered water was added 10 μl of 50% glycerol (to reduce losses of sugars during rotary evaporation) and 25 μl of 6-deoxyglucose (internal standard). After rotary evaporation to dryness, the sugars were reconstituted in 1 ml of water, cleaned with Onguard-P and -RP resins, and filtered (as described above), and 100 μl was injected into the HPIC.

A marine water sample was collected in November 1990 from a salt marsh creek on Sapelo Island, immediately passed through 0.2-μm-pore-size filters, and stored at −70°C. Onguard-Ag resin was used to remove chloride anions from the sample (after flushing the cartridges with 10 ml of water and processing as described above for Onguard-P and -RP resins), before 500 μl was injected into the HPIC.

Dissolved decomposition products from macrophytes. Green culms of *C. walteriana* and *J. effusus* were air dried and ground to 40 mesh (425 μm) in a Wiley Mill. Approximately 3-g amounts of ground macrophyte were added to 1-liter flasks in duplicate, together with 5 ml of microbial inoculum, 850 ml of sterile water, and nutrient supplements reflecting natural concentrations of inorganic nitrogen and phosphorus in each environment. Inocula were prepared from whole-water samples collected adjacent to macrophyte beds in the Okefenokee Swamp (*C. walteriana* microcosms) or L Lake (*J. effusus* microcosms) by concentrating suspended microorganisms over an autoclaved 0.2-μm-pore-size Nuclepore filter. The flasks were stoppered with foam rubber plugs and continuously bubbled with air while stirring (by using magnetic bars) in the dark at approximately 25°C. After 6 weeks, the water in the flasks was filtered sequentially through glass fiber (Whatman GF/F) and 0.2-μm-pore-size Nuclepore filters. To 200 ml of filtered microcosm water was added 10 μl of 50% glycerol and 25 μl of 6-deoxyglucose. After rotary evaporation to dryness, the residue was reconstituted in 1 ml of water, cleaned with Onguard-P and -RP resins, and filtered (as described above) before 100 μl was injected into the HPIC.

Chromatography. Sugars were analyzed by use of a Dionex series 4500i HPIC consisting of a metal-free quaternary gradient pump, an eluant degas module, a microinjection valve, a postcolumn reagent delivery module, and a solvent-compatible pulsed amperometric detector (PAD) equipped with an electrochemical cell containing a gold working electrode and an Ag/AgCl reference electrode. The following pulse potentials and durations were used for sugar analysis: $E_1 = 0.05$ V ($t_1 = 480$ ms); $E_2 = 0.60$ V ($t_2 = 180$ ms); $E_3 = -0.60$ V ($t_3 = 60$ ms). The response time of the PAD was set to 1 s, and the output range was 10,000 nA. Chromatographic data were plotted and integrated by using either an HP 3392A integrator (Hewlett-Packard Co., Avondale, Pa.) set at a full-scale deflection of 16 mV or a model 1020X dual-channel integrator (The Perkin-Elmer Corp., Norwalk, Conn.) which allows storage and manual reintegration of chromatographic data.

Sugars were separated on a Dionex CarboPac PA1 anion-exchange column (4 by 250 mm) fitted with a CarboPac PA guard column at a flow rate of 1.0 ml/min. Eluants which had been filtered through 0.4-μm-pore-size Nuclepore filters were sparged and pressurized with helium. The whole system was maintained at approximately 24°C. Four eluant bottles containing water, 20 mM NaOH, 300 mM NaOH, and 500 mM sodium acetate, respectively, were used. Isocratic elution by using a mobile phase between 0.6 and 16.0 mM NaOH (depending on which sugars were of interest) was used to elute neutral and amino sugars, and a step gradient to 100 mM sodium acetate in 0.6 to 16.0 mM NaOH eluted uronic acids. Eluants were prepared weekly by suitable dilution of low-carbonate 50% (wt/wt) NaOH solution (Fisher Scientific or J. T. Baker). A 10-min column wash with 300 mM NaOH and then a 10-min equilibration with the starting eluant were required to yield highly reproducible retention times for the sugars. If quantification of the three classes of sugars was of interest, an initial eluant of 4.6 mM NaOH for 39 min was used and then a step gradient of 6.0 mM NaOH in 100 mM sodium acetate, a 300 mM NaOH wash, and equilibration in initial eluant were used, resulting in a 70-min run time (see Fig. 1). If some retention time drift was tolerable and uronic acids were not of interest, the column was eluted continuously with 20 mM NaOH to determine most neutral sugars, with injections made at 20-min intervals. As a result of variation in quantification from day to day, because of changes in postcolumn flow rate and buildup of oxidation products on the gold electrode, standard sugar solutions were run about every six injections at the NaOH concentration used to elute sugars in unknown samples. The mean of the standard runs for each day was then calculated for final quantification of unknown samples.

The partial-fill sample loop method was used for sample injection; the loop was flushed with at least four volumes of helium-sparged water between sample injections of 10 to 50% of the loop volume. Samples with volumes greater than 100 μl were also sparged with helium to prevent a dip in the

baseline at 14 min due to dissolved oxygen in the sample. Sample volumes of up to 500 μl in a 1,000-μl loop were injected, but for most samples 100 μl in a 250-μl loop was injected. The exit of the injection valve waste tube was raised to the same level as the injection valve to prevent siphoning of the sample from the injection loop.

Sugar amounts of between 200 pmol and 30 nmol were injected to check for linearity in the relationship between peak area and injected amounts of sugar standards. Unknown sugars were identified by their retention times by using at least two different eluant concentrations; two peaks produced by different components may coelute at one NaOH concentration but are unlikely to shift retention time to an identical position at another NaOH concentration. This ability to increase separation of closely eluting sugars by adjustment of the NaOH concentration was useful for quantifying minor sugars which eluted close to a major peak. To optimize the PAD sensitivity and minimize baseline drift, 300 mM NaOH was added to the postcolumn effluent via a mixing tee at a flow rate of approximately 1 ml/min.

Occasionally, loss of sugar resolution and peak area due to sample contaminants required regeneration of the column. Usually, elution with 270 mM NaOH in 50 mM sodium acetate at 0.5 ml/min for 15 h restored column performance. For ionic contaminants, the PAD cell was disconnected and the column was washed with 30-min consecutive elutions of 1 M hydrochloric acid, 100 mM oxalic acid (to chelate metal ions), and 1 M NaOH at 1.0 ml/min, with water rinses between the washes. For hydrophobic contaminants, it was necessary to elute with 4% acetonitrile in 100 mM NaOH at 0.5 ml/min for 15 h, with the PAD cell disconnected from the eluant flow.

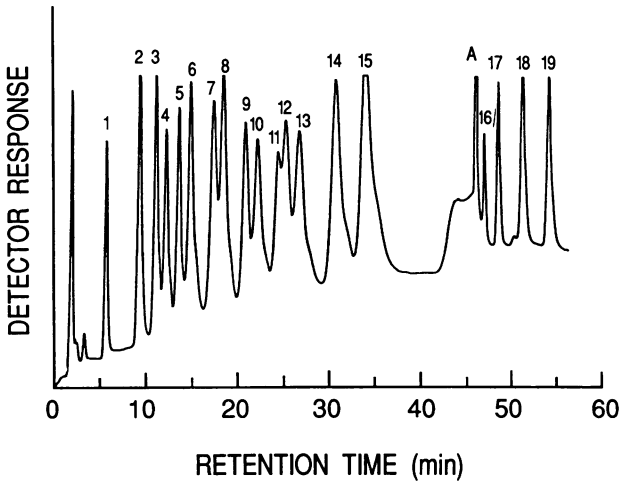

FIG. 1. HPLC chromatogram showing separation of a standard solution of 13 neutral and 2 amino sugars together with 3 uronic acids and muramic acid. Isocratic elution with 4.6 mM NaOH at 1.0 ml/min for 39 min eluted the neutral and amino sugars and then a step gradient to 6.0 mM NaOH–100 mM sodium acetate eluted acidic sugars. Injected amounts of each sugar were 1.5 nmol, except Fuc and MurA (0.75 nmol), and Fru and Rib (4.5 nmol). The column and detector settings used are described in the text. 1, fucose (Fuc); 2, 6-deoxyglucose (6-dGlc; internal standard); 3, 2-deoxyglucose (2-dGlc; internal standard); 4, rhamnose (Rha); 5, arabinose (Ara); 6, mannosamine (ManN; internal standard); 7, glucosamine (GlcN); 8, galactose (Gal); 9, glucose (Glc); 10, lyxose (Lyx); 11, xylose (Xyl); 12, mannose (Man); 13, sucrose (Suc); 14, fructose (Fru); 15, ribose (Rib); 16, muramic acid (MurA); 17, galacturonic acid (GalA); 18, glucuronic acid (GlcA); 19, mannuronic acid (ManA); A, peak due to the acetate step gradient. Note baseline drift for 39 min despite isocratic elution.

RESULTS AND DISCUSSION

Chromatography. The HPIC method successfully separated in a single chromatographic run all components in a standard aqueous solution containing 13 neutral and 2 amino sugars, together with three uronic acids and muramic acid (Fig. 1). Without prior sample concentration, as little as 10 pmol or a concentration of 20 nM (standard error = 23%; n = 4) of sugar can be quantified by pulsed amperometric detection with a signal-to-noise ratio of 3 on a regular basis. Sugar concentrations of 5 to 10 nM can be quantified for at least five injections after cleaning the gold electrode in the PAD cell; cleaning takes only a few minutes and can be done daily. Mopper et al. (15a) report twice this sensitivity when an AS6 column (Dionex) with an eluant of >20 mM NaOH and no postcolumn addition of 300 mM NaOH are used. Without postcolumn addition, samples are not diluted before detection but baseline drift can increase.

Injection of more concentrated (10 to 60 μM) sugar solutions (Fig. 1) resulted in a reduction of the average detection standard error to 1.4% (n = 4), varying between 0.4% for sucrose and 2.8% for muramic acid. There was no significant difference in quantification of the same amount of sugars injected at concentrations which differed 10-fold, i.e., triplicate 50-μl injections of a standard sugar solution compared to 500-μl injections of a standard sugar solution diluted 10-fold ($P > 0.12$, t test). A linear relationship ($r^2 \geq 0.999$) was found between peak area and amount injected for each of the 19 standard sugars in the range of 200 pmol to 30 nmol (n = 4). The separation and limit of quantification of sugars described in the present study are a considerable improvement over earlier high-performance liquid chromatographic techniques where, for example, galactose was poorly separated from glucose (2) and refractive index detection was several orders of magnitude less sensitive than pulsed amperometric detection (26). In addition, sugar detection by pulsed amperometry is approximately 2 orders of magnitude more sensitive than by gas-liquid chromatography with flame ionization detection, where the practical detection limit is approximately 1 nmol (25). Also, there is no need for derivatization of sugars before analysis by HPIC, and problems of peak multiplicity in gas chromatography analysis (5) do not occur in HPIC because each sugar produces only one peak.

A change of eluant concentration does not affect each sugar retention time to the same extent, e.g., the elution order of galactose and glucosamine in *C. walteriana* hydrolysate was reversed when the initial eluant was changed from 0.6 to 11.0 mM NaOH (Fig. 2). By running the same sample with at least two different NaOH concentrations, the possibility that unknown components will coelute with a sugar of interest or that two sugars will coelute is minimized. Moreover, the number of unknown peaks is relatively small, illustrating the selectivity of this technique for sugars among the wide range of TFA hydrolysis products produced from plant material and the diversity of compounds present in natural water samples. Because of the variability in the slope of the baseline from run to run, the data storage and manual reintegration facility of the model 1020X integrator was necessary for precise quantitation of chromatographic peaks, especially for natural water samples with low concentrations of sugars.

Recovery of sugars after TFA hydrolysis. Standard sugar

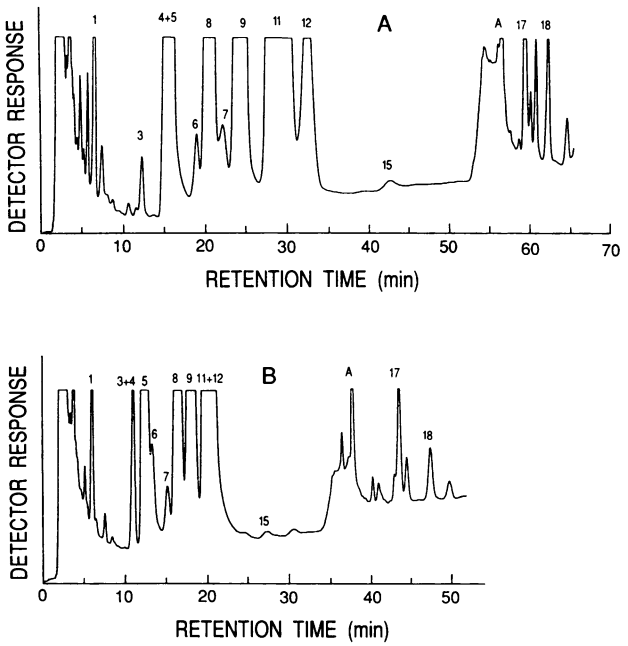

FIG. 2. Typical HPLC chromatograms of *C. walteriana* TFA hydrolysis products. (A) Isocratic elution with 0.6 mM NaOH for 50 min and then a step gradient to 8.0 mM NaOH–100 mM sodium acetate. (B) Isocratic elution with 11.0 mM NaOH for 31 min and then a step gradient to 16.0 mM NaOH–100 mM sodium acetate. HPLC conditions are described in the text. Peak identification is described in the legend to Fig. 1.

recovery after 2 M TFA hydrolysis for 1 h varied between 33 and 92%, excluding 2-deoxyglucose and fructose (which are completely destroyed), and sucrose (which forms fructose degradation products and glucose) (Table 1). Walters and Hedges (25) used 0.5 M TFA at 135°C for 2 h and also found that recoveries of standard solutions of galacturonic acid and ribose were low. Our recoveries of neutral and amino monosaccharides after 4 M TFA hydrolysis were similar to values reported previously (19). Hydrolysis with 4 M TFA, which completely hydrolyzes polysaccharides containing amino sugars (19), produced greater degradation than 2 M TFA of every free sugar examined; in the worst case, only 19% of the galacturonic acid standard was recovered (Table 1). Glucosamine content of *C. walteriana*, after hydrolysis with 4 M TFA, was found to be 19% greater than that after 2 M TFA treatment (after correcting for the 9% greater loss of free glucosamine with 4 M TFA compared to 2 M TFA; Table 1). This 19% increase in recovery of glucosamine from plant material is identical to the greater recovery reported when 4 M hydrochloric acid (which releases all of the glucosamine in polysaccharides) was used rather than 2 M TFA to hydrolyze glycopeptides (10), indicating that all of the glucosamine-containing polysaccharide in *C. walteriana* was hydrolyzed by 4 M TFA. Because of the greater degradation of free sugars with 4 M TFA, 2 M TFA was routinely used for acid hydrolysis of plant material and the glucosamine content measured was increased by 19% to correct for incomplete hydrolysis of glucosamine-containing polysaccharides by 2 M TFA.

TABLE 1. Survival of standard monosaccharides after TFA hydrolysis and rotary evaporation

Monosaccharide	% Recovery (mean ± SE)[a] with a TFA concn of:	
	2 M	4 M
Fucose	86 ± 0.9	72 ± 3.5
6-Deoxyglucose	89 ± 1.0	ND[b]
Rhamnose	82 ± 1.0	69 ± 2.4
Arabinose	81 ± 1.5	74 ± 2.0
Mannosamine	91 ± 0.9	83 ± 1.0
Glucosamine	92 ± 2.0	83 ± 0.9
Galactose	81 ± 1.4	73 ± 1.2
Glucose	87 ± 1.5	84 ± 2.7
Lyxose	57 ± 1.2	42 ± 0.9
Xylose	71 ± 1.8	52 ± 0.9
Mannose	83 ± 1.2	70 ± 2.4
Ribose	57 ± 1.0	ND
Muramic acid	81 ± 2.3	ND
Galacturonic acid	33 ± 1.8	19 ± 2.3
Glucuronic acid	67 ± 0.7	61 ± 1.3
Mannuronic acid	78 ± 2.0	ND

[a] $n = 3$.
[b] ND, not determined.

TFA does not hydrolyze α-cellulose to glucose effectively (25), and concentrated sulfuric acid pretreatment is required to completely hydrolyze the polymer (6). The disadvantage of sulfuric acid, however, is that it is not volatile and, after hydrolysis, neutralization steps are required. These steps involve precipitate formation that complicates isolation and may provide a substrate for sugar adsorption and subsequent loss. Since TFA was used in the present study (because of its high volatility and high yields of monosaccharides from noncellulosic polysaccharides), our reported yields of glucose underestimate the total glucose content by the fraction of unhydrolyzed α-cellulose. TFA is usually the hydrolysis medium of choice to obtain neutral and amino monosaccharides and uronic acids from plant material, sediments, plankton, and glycoproteins (1, 2, 19, 25).

The plant matrix had a significant effect on monosaccharide recovery after 2 M TFA hydrolysis; on average, spiked sugar recovery (i.e., recovery of standard sugars hydrolyzed in the presence of plant material) was only 76% ± 2.0% of standard sugar recovery after hydrolysis (Table 2), which is similar to the previously reported spiked sugar recovery of 85% in a variety of matrices (5, 6). A fucose spike of 9,000 nmol was recovered with an efficiency of 63% ± 4.7%, which is not statistically different from the 57% ± 2.6% recovery of a 110-nmol fucose spike (Table 2), indicating that the amount of the sugar spike does not affect its recovery after acid hydrolysis. The lower monosaccharide recovery after TFA hydrolysis of sugars in the presence of plant material may be due to Maillard compounds (deoxyketosyl-amino acids and melanoidins), formed when mixtures of carbohydrates and amino acids are heated (12). The Maillard compounds would be expected to elute at times different from that of the sugar component alone and may not be detected by pulsed amperometry. Therefore, the amount of sugar involved in Maillard compound formation would not be quantified along with free sugars.

We compared sugar recoveries after hydrolysis in solutions with the same final concentrations of TFA (2 M) but differing volumes. A threefold reduction in the ratio of 2 M TFA to a standard sugar solution had no effect on the degradation of the standard sugars, while the same reduction in the ratio of 2 M TFA to plant material (*C. walteriana*) resulted in approximately a 20% decrease in monosaccharide recovery. Therefore, it is important to hydrolyze samples

TABLE 2. Recovery of monosaccharides from spiked *C. walteriana* after 2 M TFA hydrolysis

Monosaccharide	Amt (nmol) of monosaccharide[a]				% of spike recovered	% Recovery of spike/standard value[b]
	Recovered in unspiked *C. walteriana*[c]	Added (spike)	Recovered in spiked *C. walteriana*[c]	Recovered from spike		
Fuc	110	110	173	63	57	66
Rha	125	220	273	148	67	82
Ara	3,740	5,720	6,820	3,080	54	67
ManN	0	220	141	141	64	70
GlcN	36	220	184	148	67	73
Gal	1,010	5,720	4,460	3,450	60	74
Glc	1,180	5,720	5,200	4,020	70	80
Xyl	6,440	5,720	9,900	3,460	60	85
Man	180	220	317	137	62	75
Rib	18	660	320	302	46	81
GalA	222	220	273	51	23	70
GlcA	54	220	182	128	58	87

[a] $n = 3$.
[b] Standard monosaccharide recovery values are presented in Table 1.
[c] Amount of *C. walteriana*, 0.010 g. Average percent standard errors of the mean for unspiked and spiked monosaccharides were 3.6 and 7.0%, respectively.

with the same volume as well as the same concentration of TFA when making quantitative comparisons of sugars in plant material. A possible explanation for this effect is that plant material may contain components which partially neutralize the added acid, resulting in poorer recoveries of monosaccharides due to incomplete hydrolysis of polysaccharides.

Mannosamine or 6-deoxyglucose was used as an internal standard because these sugars survived TFA hydrolysis well (Table 1) and could easily be separated from other sugars (Fig. 1 and 2A and B). Neither sugar was found in *C. walteriana* tissue that was hydrolyzed in the absence of these internal standards nor in natural water samples. Under the HPIC conditions used in this study, inositol and sorbitol were found to elute between the void volume and fucose among a number of unidentified peaks in hydrolysates of plant material (Fig. 2A and B). Sugar alcohols, therefore, cannot be easily determined under these conditions and the internal standards used in gas-liquid chromatography (e.g., adonitol [6]) are not suitable for this technique. Glycerol, which was used to reduce losses of sugars during concentration and drying steps (7, 14), eluted in the void volume and did not affect sugar determinations.

Rotary evaporation of a standard sugar solution after TFA hydrolysis gave approximately 70% better recovery than freeze-drying in a Speed Vac Concentrator (Savant Instruments Inc., Farmingdale, N.Y.), even though glycerol was added to hydrolysates immediately before both rotary evaporation and freeze-drying. Rotary evaporation has been reported previously to be milder than freeze-drying when concentrating monosaccharides and amino acids (7, 14) and is therefore recommended.

Effect of sample cleanup on sugar recovery. Onguard-Ag resin, used to remove chloride from marine water samples, completely removed monosaccharides containing an amino group (mannosamine, glucosamine, and muramic acid) both in the absence and presence of 20‰ artificial seawater. There was no statistical difference in the recovery of 10 neutral monosaccharides and 3 uronic acids in the absence and in the presence of artificial seawater ($P > 0.29$; t test), indicating that the presence of sea salts does not affect Onguard-Ag resin-monosaccharide interactions. For Onguard-Ag resin, recoveries of 10 neutral monosaccharides (mean, 88.5% ± 1.6%) and 3 uronic acids (mean, 88.7% ± 1.9%) were not significantly different ($P > 0.96$).

The effect on sugar recovery of a mixture of equal amounts of wet Onguard-P and -RP resins (used to remove humic and hydrophobic compounds, respectively) was also investigated. Recoveries of 10 neutral monosaccharides (mean, 88.2% ± 0.9%), 2 amino monosaccharides (mean, 86.5% ± 0.5%), and 3 uronic acids together with muramic acid (mean, 85.8% ± 3.4%) were not statistically different ($P > 0.35$). Sucrose recoveries after Onguard-Ag or Onguard-P and -RP treatment were similar to those of the neutral monosaccharides and uronic acids. Therefore, sample cleanup can be accomplished effectively with little loss of sugars in most cases. Any one of the three different types of sugars investigated can be used as an internal standard for all three types during the resin treatment step, except for amino sugars (such as mannosamine) treated with Onguard-Ag resin.

Extraction and concentration of sugars in seawater. None of the six resins used to extract sugars from 20‰ artificial seawater was efficient for all of the neutral, amino, and acidic sugars tested (Table 3). All six resins gave relatively good recovery (23 to 52%) of a disaccharide (sucrose), although whether this technique is generally efficient for purification and concentration of oligosaccharides in inorganic salt solution requires further investigation. The diol resin was best for uronic acids and muramic acid. The relatively low recoveries of mannuronic acid for all of the resins might be due to reversion to the lactone form, which probably affected binding efficiency to the resin. PEI resin gave the best recoveries for xylose and ribose, which are indicators of particulate material input from angiospermic vascular plants and planktonic microorganisms, respectively (6). Leachate from the amino resin produced a rapidly increasing baseline on the chromatogram, which made quantification of later eluting sugars difficult.

The ion-pairing reagent, tetrabutylammonium phosphate, did not affect recovery of sugars from PEI resin. However, a threefold increase in the volume of water used to elute sugars from this resin produced a 60% increase in sugar recovery. Approximately 9 ml of water per g (dry weight) of PEI resin appeared to produce optimum elution of sugars from PEI resin. Reductions in the PEI resin-to-sugar ratio of

TABLE 3. Recovery of sugars from 20‰ artificial seawater after solid-phase extraction and elution of sugars with various resins[a]

Resin	% Recovery of[b]:																		Avg SE (%)
	Fuc	2-dGlc	Rha	Ara	ManN	GlcN	Gal	Glc	Lyx	Xyl	Man	Suc	Fru	Rib	MurA	GalA	GlcA	ManA	
Amino	14	17	13	19	27	19	24	21	17	24	21	46	24	12	23	7	9	<4	19.4
Cyano	4	6	4	7	12	18	12	17	5	7	12	30	10	4	16	25	23	9	10.5
Diol	7	9	5	9	24	31	17	21	6	32	17	39	15	5	39	48	50	10	9.4
Sulfonic	11	12	8	15	14	20	25	39	14	16	16	23	23	11	11	17	12	7	11.3
Quat-amine	9	14	6	14	28	42	25	26	14	38	24	52	19	6	33	35	35	6	12.0
PEI	12	24	12	22	28	14	22	10	22	18	25	50	30	18	35	9	12	5	6.5

[a] Details of the procedure are given in the text.
[b] $n = 2$.

25 and 75% resulted in 56 and 70% losses in sugar recovery, respectively. The optimum amount of resin to use for each sample is an important consideration and should be determined for each type of sample. The recovery of 6-deoxyglucose by using PEI resin was much lower than that of 2-deoxyglucose, making 2-deoxyglucose the preferable internal standard for PEI solid-phase extraction without acid hydrolysis.

Unfortunately, we found PEI resin bound salts as well as sugars, and the salts apparently blocked binding sites for sugars. For natural seawater samples (>30‰), with free sugars in low concentration, it was necessary to wash the resin with tetrabutylammonium phosphate in 80% acetonitrile to remove most of the salts after incubation with seawater-acetonitrile. Furthermore, when the sugars were eluted from the resin, Onguard-Ag solid-phase extraction was required to remove chloride ions before injection into the HPIC. The recovery of 2-deoxyglucose (internal standard) was only 14% after the above processing was completed. Because the recovery of most sugars was less than that for 2-deoxyglucose (Table 3), we do not recommend the use of PEI resin to concentrate sugars in >30‰ seawater. However, the technique may work well for brackish water where the concentration of interfering salts is lower, although this possibility requires further investigation.

Sugar content of natural samples. Concentration of water samples (from microcosms, L Lake, or the Okefenokee marsh) by rotary evaporation from 200 ml to 1 ml produced only an approximately sevenfold improvement in the detection limit of 20 nM (corrected to original sugar concentrations). This was due to the extensive resin treatment of concentrated samples required to remove humic and other interfering substances by using Onguard-P and -RP. The limit of detection for seawater samples without concentration was 30 nM because of losses resulting from solid-phase extraction of chloride.

The major component sugars of angiospermic cellulose and hemicellulose (i.e., glucose, arabinose, galactose, and xylose) were the dominant free sugars in the macrophyte hydrolysates, dissolved macrophyte degradation products, and natural water samples collected in macrophyte-dominated areas (Table 4). Vascular plant nonstructural sugars representing the initial photosynthate (glucose, fructose, and sucrose) and possibly derived from storage polymers of glucose and fructose were also major dissolved free sugars in the macrophyte degradation products and natural water samples in most cases. Glucose, at a concentration of up to 890 nM, was the major dissolved free sugar in the three natural water samples. Glucose and fructose were reported to be the dominant dissolved free sugars in a variety of seawater samples from estuarine to oceanic (15, 15a), and we found this to be the case for salt marsh water also (Table 4), although we found higher sugar concentrations than reported previously. The uronic acids were not a major component of the dissolved-free-sugar pool, although in systems where algae such as kelp are present, mannuronic acid could be an important indicator of algal source input (25). Glucosamine was not detectable in water and microcosm samples. Some, and possibly all, of the glucosamine in the macrophytes (Table 4) may result from the chitin constituent of fungal cell walls. Fungi have been found growing on the surfaces of *S. alterniflora* and other emergent macrophytes, particularly in the standing dead stage of decomposition (20); insects and other nonfungal sources of chitin were not present on the plant samples.

Less than 20% of α-cellulose is hydrolyzed to glucose by TFA (25), resulting in a glucose value much lower than expected for macrophyte tissue. Correcting for the low recovery of glucose, carbohydrates account for 50% or more of the dry weight of the three macrophytes. Previously reported monosaccharide constituents of wheat straw (9) and the neutral detergent fiber fraction of six forage grasses (29), determined by 2 M TFA hydrolysis, are, in decreasing order, xylose > arabinose = glucose > galactose ≥ rhamnose. The order of the same constituents in the three macrophytes analyzed in the present study is identical. The glucose contents of *S. alterniflora* (28) and a *Carex* species (6), after concentrated sulfuric acid pretreatment and then hot sulfuric acid hydrolysis, were approximately double the xylose contents; otherwise, the relative contents of neutral sugars after sulfuric acid treatment were similar to those found in the present study. This confirms that TFA hydrolyzes all plant polysaccharides well except for α-cellulose.

Dissolved-free-sugar concentrations in lake and swamp waters analyzed in the present study were generally higher than those reported for marsh and river samples from Oregon (23), for example, 60 nM dissolved monosaccharides for Klamath Marsh samples (23) compared to 527 nM for L Lake, 748 nM for the Okefenokee marsh, and 1,265 nM for the Sapelo salt marsh (arabinose, xylose, mannose, galactose, and glucose concentrations only). However, concentrations in our samples were well within the ranges reported for various freshwater and marine environments (24). Total dissolved organic carbon concentrations average 5 mg of C $liter^{-1}$ for L Lake and the Sapelo marsh (18, 22) and 35 mg of C $liter^{-1}$ for the Okefenokee marsh (18). Dissolved free monosaccharides thus account for only a few percent (<5%) of the total dissolved organic carbon in these samples.

Carbohydrate signatures of natural samples determined by HPIC have the potential to provide information on the origin of detrital material in freshwater and marine environments,

TABLE 4. Monosaccharide concentrations in undegraded macrophytes compared to dissolved free sugars formed during their degradation and dissolved free sugars in water samples collected adjacent to the macrophyte beds in natural environments

Site	Sample	Concn[a] of sugars														Avg SE (%)
		Fuc	Rha	Ara	GlcN[b]	Gal	Glc	Xyl	Man	Suc	Fru	Rib	GalA	GlcA	ManA	Total	
J. effusus beds (L Lake)	*J. effusus* undegraded plant	1.7	3.2	83.2	0.90	24.1	32.5[c]	180	5.6	ND[d]	ND	0.48	23.6	6.8	0	362	6
	J. effusus degradation products	<3	19	160	<3	18	56	13	<3	67	77	42	<3	<3	<3	450	63
	Natural water	<3	13	99	<3	43	350	23	12	7	59	14	<3	<3	<3	620	9
C. walteriana prairie (Okefenokee Swamp)	*C. walteriana* undegraded plant	1.7	4.0	97.4	0.60	37.4	89.0[c]	220	0.55	ND	ND	0.36	23.8	2.4	0	477	4
	C. walteriana degradation products	<3	<3	210	<3	15	52	30	15	40	65	26	33	33	<3	520	20
	Natural water	<3	<3	20	<3	16	690	18	4	<3	98	3	26	12	6	890	14
S. alterniflora marsh (Sapelo Island)[e]	*S. alterniflora* undegraded plant	5.9	2.4	59.9	2.5	24.8	81.7[c]	260	13.6	ND	ND	0.63	8.8	4.2	0	464	11
	Natural water	<30	<30	160	<30	140	890	45	<30	40	840	150	ND	ND	ND	2,300	32

[a] Sugars in natural water samples and macrophyte degradation products are expressed in nanomolar concentrations while constituent macrophyte plant sugars are expressed in milligrams per gram (dry weight). $n = 2$, water and degradation products; $n = 3$, macrophyte compositions.
[b] Macrophyte compositions are corrected for 19% greater recovery of GlcN with 4 M TFA hydrolysis.
[c] Glucose values for undegraded plant material are underestimates because of incomplete hydrolysis.
[d] ND, not determined.
[e] *S. alterniflora* degradation products were not analyzed.

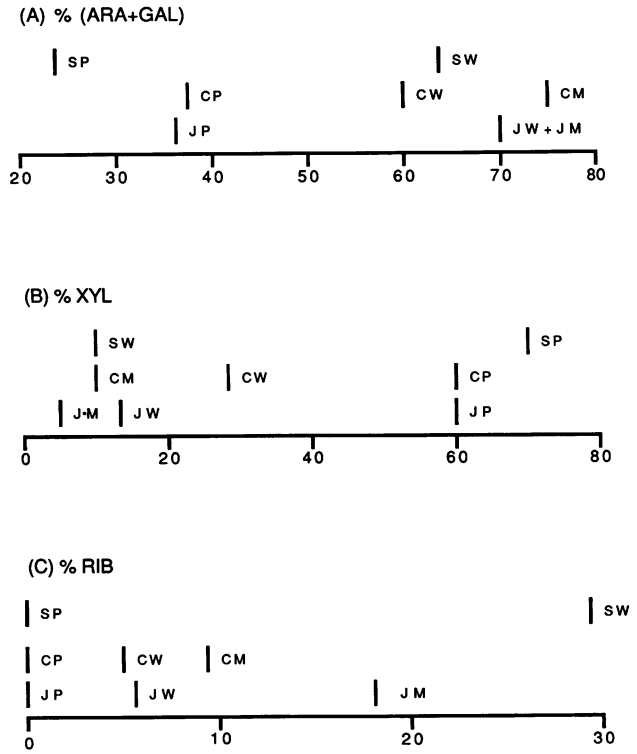

FIG. 3. Weight percent arabinose plus galactose (A), xylose (B), and ribose (C) in emergent macrophytes, their dissolved degradation products, and natural water samples from the macrophyte beds. Only Fuc, Rha, Ara, Gal, Xyl, Man, and Rib were considered for weight percent calculations (6). Abbreviations: J, *J. effusus*; C, *C. walteriana*; S, *S. alterniflora*; P, plant; M, microcosm samples of dissolved degradation products; W, water. Weight percent values were calculated from the data shown in Table 4.

provided that biochemically consistent compositional differences exist in the carbohydrate signatures of potential sources. Although glucose is the predominant sugar in natural water samples, the source of the glucose, whether from structural or nonstructural plant material or from nonplant sources, cannot be distinguished; therefore, glucose is of little value as an indicator sugar for source material. However, Cowie and Hedges (6) found that weight percent parameters of certain neutral sugars on a glucose-free basis allowed different biological sources of particulate material from a coastal environment to be distinguished; high-percent ribose indicated a planktonic (bacteria, phytoplankton, and zooplankton) influence on the sugar content of particulate organic material, while high-percent xylose indicated an angiospermic influence and high-percent arabinose plus galactose indicated contributions from nonwoody vascular plant tissues. In the present study, we applied the weight percent parameters to dissolved free sugars as well as the sugar content of particulate source material for the two freshwater systems and the salt marsh. The weight percent sugar parameters calculated from *J. effusus*, *C. walteriana*, and *S. alterniflora* plant compositions (Fig. 3) are within the previously published ranges for grasses (6), although *S. alterniflora* has a higher-percent xylose than for the grasses studied previously.

The sugar signature of macrophyte source material and that of dissolved free sugars formed during plant degradation were substantially different (Fig. 3). The percent arabinose

plus galactose was about twofold lower in the undegraded plants than in their dissolved degradation products, while the percent xylose was at least sixfold higher in the undegraded plants than in their degradation products. Differences in ratios of sugars between intact plant material and plant degradation products may be due to differential solubilization of sugars from plant detritus (via physical leaching [4] and/or enzyme-mediated breakdown), to differential microbial utilization of particulate and dissolved plant-derived sugars, to precipitation and complexation of certain components of the dissolved sugar pool, or to a combination of these processes. In this study, we found evidence for preferential release of arabinose plus galactose from degrading plant material and preferential retention of the structural sugar xylose. A similar divergence of chemical signatures of undegraded aquatic vascular plant tissue and the dissolved products formed from the plant during degradation has been described for the lignin component of *S. alterniflora* (17).

Ribose content also differed between plant material and microcosm samples, with the percent dissolved free ribose in macrophyte degradation products at least 50-fold higher than the percent ribose in intact macrophyte tissue. This difference may reflect extreme biological recalcitrance of free ribose released from degrading plant material relative to other free sugars or, more likely, nonplant (e.g., microbial) sources of free ribose. Natural water samples collected near macrophyte beds (Fig. 3) reflected the sugar signatures of the dissolved degradation products of the macrophytes.

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