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Isolation and Quantification of Dissolved Lignin from Natural Waters Using Solid-Phase Extraction and GC/MS

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Solid-phase extraction (SPE) was tested for the isolation of dissolved lignin from diverse natural waters (fresh, estuarine, and marine) in preparation for CuO oxidation. Capillary GC coupled to selected-ion monitoring mass spectrometry (SIM-MS) of CuO oxidation products provides the high sensitivity and precision required for the identification and quantification of trace levels of lignin in seawater. The low blanks and quick cleanup of C_{18} cartridges support SPE for processing such samples. Comparison of SPE with other isolation procedures (direct dry-down and ultrafiltration) has shown that this method quantitatively recovers dissolved lignin and preserves its compositional parameters. The concentration and nature of dissolved organic matter appear to be primary factors that constrain the amount of water that should be processed to obtain quantitative and reproducible recoveries of dissolved lignin using SPE. Highest recoveries of dissolved lignin were obtained at low pH (1.5−4.0) with substantial decreases at pH > 4. Extraction efficiencies were independent of flow rate within a range of five to fifteen bed volumes per minute (50−150 mL min^{-1}), and both refrigeration and freezing were appropriate long-term storage methods for processed cartridges prior to elution of retained dissolved lignin.

The amount of terrigenous dissolved organic matter (DOM) discharged annually by rivers into the oceans accounts for the largest flux of reduced carbon from the continents to the sea.[1−2] Despite large inputs, terrigenous DOM contributes only a small fraction of the total DOM pool in the ocean,[3−4] suggesting high remineralization rates of this highly degraded soil-derived source of DOM. Although photochemical degradation processes may explain parts of this geochemical paradox,[5−7] we still need to better quantify the terrigenous component of marine DOM to understand its fate within the ocean. One way to characterize this fraction is through the identification of molecular tracers unique to land plants and representative of terrigenous organic matter inputs. Lignin is a major and unique structural component of vascular plants[8] and, as such, is an important component of terrigenous DOM exported from land to the sea.[9] The presence of lignin in marine DOM will thus indicate, and in some cases help quantitate, unambiguous inputs of terrigenous DOM to the sea. The unique biochemical signature of lignin provides the added advantage that it helps characterize the source and diagenetic state of vascular plant material[9−11] and thus "fingerprint" riverine DOM. Such "fingerprinting" can provide drainage-basin-specific distinctions of sources of freshwaters to the Coastal Ocean.[12]

Although both XAD extraction and ultrafiltration have been used for the isolation of dissolved lignin from ocean waters,[3−4,7,13−14] it is unclear whether either of these methods result in quantitative recoveries of this terrigenous biomarker. The cleanup procedures for XAD resins are particularly time-consuming (from >5 to 120 h), and following cleaning, these resins still produce substantial contamination that hinders accurate quantification of trace levels of dissolved lignin.[3,13,15] Additionally, extraction efficiencies using XAD resins are strongly limited by operational conditions, with efficiencies dropping markedly at flow rates higher than two bed volumes per minute.[16−17]

Tangential-flow ultrafiltration using 1000 Dalton membranes is noncontaminating and relatively rapid compared to the con-

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ventional XAD procedure.[18] Although the bulk of dissolved lignin (typically 90%) in river DOM is recovered using ultrafiltration, photochemical degradation of lignin in river plumes and coastal environments has been observed to reduce the average molecular size of this terrigenous biomarker.[7] It is thus likely that a substantial fraction of dissolved lignin in the ocean resides as low-molecular-weight (LMW) molecules that escape isolation by ultrafiltration.

In the present study we evaluated solid-phase extraction (SPE) for the isolation of dissolved lignin from dilute matrixes including seawater. Solid-phase extraction is now well recognized as a method for isolating organic compounds from aqueous solutions in preparation for subsequent analysis, and it is used in a wide variety of applications.[17,19−24] We have shown that this method provides extremely low procedural blanks, is rapid and reproducible, and quantitatively recovers dissolved lignin from diverse types of natural waters.

EXPERIMENTAL SECTION

Water samples were collected in the vicinity of the University of Texas at Austin-Marine Science Institute in Port Aransas, Texas. The environments sampled include the Nueces River (NR), the Guadalupe River (GR), the Aransas Pass tidal inlet (AP), and the open waters of the Gulf of Mexico (GOM). All samples (20−200 L) were collected in acid-washed Nalgene carboys from the surface except for the GOM samples which were collected with Niskin bottles at a depth of 50 m. Water samples were filtered through a 0.2-μm pore-size polycarbonate filter (Nucleopore) to remove particulates. Two to four 10-mL subsamples were removed and frozen in muffled glass vials for later determination of dissolved organic carbon (DOC) by the high-temperature catalytic oxidation method using a Shimadzu TOC 5000 analyzer.[25]

Solid-phase extraction (SPE) of dissolved organic matter was performed on prepacked columns containing 10 g of sorption material composed of octadecyl carbon moieties (C_{18}) chemically bonded to a silica support (C_{18}−SPE Mega-Bond Elut; Varian). Cartridges were pretreated with methanol (100 mL) followed by acidified (pH 2) Milli-Q Plus UV water (50 mL), making sure the sorbent remained wet with water prior to extraction. Filtered water samples were acidified to pH 2 using reagent-grade concentrated HCl, thoroughly mixed, and pumped through the SPE cartridge with a peristaltic pump and silicone tubing (Cole Parmer). The pumping line ran through a silicone stopper that was inserted and fixed in the top of the syringe-like SPE cartridge. By this method, the water (1−50 L) was delivered directly into the headspace of the SPE cartridge and forced by pressure through the sorbent at a flow rate of 50 ± 2 mL min^{-1}. To process four samples simultaneously, we used two peristaltic pumps each mounted with dual pump heads. After the samples were extracted, each C_{18}−SPE cartridge was rinsed with 1 L of acidified (pH 2) Milli-Q Plus UV water to remove residual salts. The cartridges were then stored at 4 °C or in the freezer until further processing. Before elution, we allowed the cartridges to warm to room temperature, and the retained DOM was eluted from the column in one fraction using 50 mL of methanol. The eluent was collected into a muffled glass flask and evaporated to dryness under vacuum using a Savant SVC200 SpeedVac concentrator. During our experiment, we tested the reusability of the C_{18}−SPE cartridges and found that freezing, although not affecting their elution capacity, did substantially decrease their retention capacity in future usage. In addition, although all retained dissolved lignin compounds are eluted with methanol (see later discussion), it is likely that not all adsorbed DOM is removed during elution. Since we do not know how these components may affect the next extraction and because the cartridges are rather inexpensive, we recommend using a new cartridge for each extraction.

The procedures and operating conditions for isolation of different size classes of DOM using tangential-flow ultrafiltration were similar to those described previously by Benner[25] and Benner et al.[18,27] The filtered water samples (35−50l) were processed through two Amicon spiral-wound polysulfone membranes (S10N1) which have a nominal molecular weight cutoff of 1000 Daltons (pore size ≈ 1 nm). The samples were concentrated by a factor of 35−50 to a final volume of ≈1 L (retentate) containing a size fraction (1−200 nm) hereafter referred to as high-molecular-weight (HMW) DOM. After concentration, the estuarine and marine samples were diafiltered with 18 L of Milli-Q water to remove sea salts. Each retentate was first reduced in volume to ∼100 mL by rotary evaporation at 45−50 °C and then dried under vacuum in a Savant SpeedVac concentrator in preweighed muffled flasks. The dried material was scraped from the flask and stored for elemental and lignin analyses.

The solution passing through the membrane (permeate) contained a size fraction (<1 nm or 1000 Daltons) referred to as low-molecular-weight (LMW) DOM. The entire volumes of permeate and diafiltrate were mixed thoroughly, acidified to pH 2, and processed through a C_{18}−SPE cartridge using the same operating conditions used for initial filtered (<0.2 μm) water. Concentrations of DOC (from duplicate 10-mL subsamples) and volumes of water were recorded for each initial filtered water, the ultrafiltered retentate, and the permate/diafiltrate mixture to establish a mass balance and isolation efficiency of DOC as well as the reproducibility of ultrafiltration with different types of water samples.[18,26] For river waters, a direct "dry-down" sample was obtained from the reduction of 1 L of filtered (0.2 μm) water to ∼100 mL by rotary evaporation followed by complete dryness under vacuum in a Savant SpeedVac concentrator according to the above-mentioned procedure. The dried samples were then scraped from the flask and stored for further analyses. The organic carbon (C_{org}) content of dried samples was measured after vapor-phase acidification using a Carlo Erba 1108 CHN analyzer.[28]

The quantification of lignin-derived phenols in dried sediment standards, ultrafiltration samples, and SPE enluents was performed

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Table 1. Retention Times (RT), Target Ions, Relative Response Factors (RRF), Limits of Detection (LOD), and Recoveries (Recov.) for Lignin-Derived Phenols and the Internal Standard Measured under Selected Ion Monitoring (SIM)/MS

compound	symbol	RT[a](min)	target ions[b] (m/z)	RRF[c] (±1 SD)	LOD[d] (pg)	Recov.[e] (%)
ethyl vanillin (std)	EVAL	14.46	*167* 195 238	na	0.74	79.6 (1.2)
vanillin	VAL	12.84	*194* 193 209	0.58 (0.02)	0.32	78.6 (7.3)
acetovanillone	VON	15.07	*193* 208 223	0.76 (0.02)	0.61	91.8 (8.5)
vanillic acid	VAD	18.93	*267* 297 312	1.46 (0.09)	0.96	99.6 (9.4)
syringealdehyde	SAL	17.19	*224* 239 254	0.66 (0.02)	0.21	77.1 (5.8)
acetosyringone	SON	18.92	*238* 253 268	0.98 (0.05)	0.16	92.6 (6.9)
syringic acid	SAD	22.36	*327* 312 342	2.59 (0.15)	0.62	73.8 (8.7)
p-coumaric acid	CAD	23.31	*219* 293 308	0.94 (0.05)	0.29	101.6 (7.1)
ferulic acid	FAD	26.92	*338* 249 323	2.62 (0.17)	0.57	89.9 (10.2)

[a] Ret. Time. On a DB-5MS column (30 m, 0.25-mm i.d.; J&W Scientific) and under a carrier gas (He) flow rate of 1.3 mL min^{-1}. [b] Target ions. Italicized ions represent major ion fragments used for quantitation. [c] RRF is the relative response factor calculated using ethyl vanillin as the internal standard. Values in parentheses represent ±1 SD. [d] LOD calculated using the equation LOD = $(3\sigma)/m$, where σ is the signal variability of the blank and m is the slope of the calibration curve. [e] Recoveries. Calculated from duplicates of spiked UDOM samples. Values in parentheses represent mean % deviation ($100[(n_1 - n_2)/\bar{\chi}]$).

using a modified version of the CuO oxidation and extraction scheme of Hedges and Ertel[29] and Goñi and Hedges.[30] The first major modification involves preparation of samples for oxidation. We substituted the overnight N_2 purging of the sample in a glovebox with sparging (30 min) and sonication (1 min) of the NaOH solution (8 wt %/wt) prior to loading the samples and reagents into the reaction minibombs. These were then closed under a N_2 stream to purge the headspace. We introduced this step because SPE enluents needed to be sonicated twice with 4 mL of NaOH to remove the isolated DOM (15−30 mg) and residues adhered to the Savant flasks. We tested this procedure vs the conventional N_2 glovebox purging by oxidizing and analyzing standard sediments prepared with both methods. We also used these sediments to test our second modification, the quantification of lignin-oxidation products (LOP) using selected ion monitoring (SIM) on an HP 5972 mass selective detector vs that achieved by flame ionization detection (FID). Separation of trimethylsilyl derivatives of LOP was performed by gas chromatography on a HP 5890 GC fitted with a DB-5MS capillary column (30 m, 0.25-mm i.d.; J&W Sci., no. 122−5532). The samples were injected into a straight glass liner (J&W Sci., no. 210−4006−5) inserted into the GC injection port and were carried to the capillary column by He (1.3 mL min^{-1}) under a 1/13 split ratio. The GC oven was temperature-programmed from 100 °C, with no initial delay, to 270 °C at 4 °C min^{-1} and held at the upper temperature for 16 min. The GC injector was maintained at 300 °C, whereas the GC/MS interface was maintained at 280 °C. The mass spectrometer was operated in the EI mode (70 eV), and three ion masses were monitored for each lignin-derived phenol (Table 1) during the GC run. Positive identification was performed using retention times and by comparing the relative abundance of the three ions in each sample to those produced by standards. The dynamic ranges and relative response factors for each phenol were determined using a six-point calibration curve. All curves showed strong linearity ($r^2 = 0.98-1.00$) in the concentration ranges (0.9−36 ng μL^{-1}) used. Quantification was then performed using relative response factors and ethyl vanillin (EVAL) as an internal/recovery standard. All samples analyzed were diluted (100−1500 μl pyridine) in order that they would fall within the dynamic range of the calibration curves.

RESULTS AND DISCUSSION

CuO Oxidation/SIM Quantification. The symbols, retention times, target ions, relative response factors, limits of detection, and average recoveries for all eight lignin-derived phenols are presented in Table 1. Under SIM mode, the limit of detection (LOD) for Me_3Si phenol derivatives averages 0.47 ± 0.27 pg (Table 1). These values are about 2−3 orders of magnitude lower than those achieved on GC/FID (0.1 ng).[29] Assuming a minimum sample volume of 100 μL, a maximum injection volume of 2 μL, and a 1/13 split of the injected volume, this limit of detection corresponds to a minimum detectability of about 0.61 ± 0.35 ng of an individual phenol in an oxidation product mixture. This value is again about 2 orders of magnitude lower than that achieved on GC/FID (0.1 μg).[29] The response factors of standard compounds were monitored for every standard curve and showed very limited variability (RSD = 4.7 ± 1.5%) over the 12 months of analyses (Table 1). To evaluate recoveries of individual phenols from the oxidation mixture, we spiked two riverine ultrafiltered DOM (UDOM) samples immediately following CuO oxidation with 50 μL of a standard solution containing all analytes (32−36 ng μL^{-1}). The added spikes (1600−1800 ng of each phenol) were quantitatively similar to the amount of each vanillyl and syringyl phenol generated from the UDOM sample (1500−1800 ng) and about four times higher than the natural cinnamyl phenols (~400 ng). Because we used a small UDOM sample size (~25 mg), the total amount of LOP (natural + spike) present in the spiked solution was within or below the range obtained from several natural river and estuarine UDOM samples analyzed previously. It was a factor of 2−5 higher than total LOP obtained from open ocean UDOM samples. Recoveries of standards averaged 87 ± 10% (Table 1), indicating that lignin-derived phenols were recovered with high efficiency from a complex oxidation matrix.

Within procedural blanks (containing all reagents but no sample), the only phenols positively detected were vanillin (VAL) and vanillic acid (VAD) which averaged 22.0 ± 4.3 and 13.3 ± 7.6 ng/sample, respectively. These levels are 1−2 orders of magnitude

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Table 2. A Comparison of Lignin-Derived Phenol Yields and Parameters Measured from a Reference Coastal Sediment (Sag05) Using GC/FID vs GC/(SIM)MS Methods and Using Preparation in a Glove Box vs Sparging with N_2[a]

lignin parameters	GC/FID[a] ($n = 9$)	GC/(SIM)MS ($n = 11$)	glovebox ($n = 3$)	N_2 sparge ($n = 8$)
λ	3.52 ± 0.17	3.33 ± 0.20	3.44 ± 0.26	3.28 ± 0.16
$\Sigma 8$	8.46 ± 0.41	7.98 ± 0.47	8.26 ± 0.64	7.88 ± 0.40
V	2.73 ± 0.11	2.68 ± 0.17	2.79 ± 0.26	2.63 ± 0.12
S	0.56 ± 0.05	0.45 ± 0.04	0.45 ± 0.03	0.45 ± 0.04
C	0.23 ± 0.04	0.20 ± 0.02	0.20 ± 0.02	0.20 ± 0.03
C/V	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
S/V	0.20 ± 0.02	0.17 ± 0.01	0.16 ± 0.01	0.17 ± 0.01
$(Ac/Al)_V$	0.38 ± 0.05	0.35 ± 0.05	0.36 ± 0.04	0.34 ± 0.05
$(Ac/Al)_S$	0.37 ± 0.05	0.34 ± 0.05	0.34 ± 0.01	0.35 ± 0.06

[a] V, S, C = milligrams of vanillyl, syringyl, and cinnamyl phenols, respectively, normalized to 100 mg of organic carbon. λ = the sum of V, S, and C. $\Sigma 8$ = the sum of vanillyls, syringyls, and cinnamyls expressed in milligrams per 10 g of sediments (dry weight).C/V and S/V = the weight ratios of cinnamyl and syringyl phenols to vanillyl phenols, respectively. $(Ac/Al)_V$ and $(Ad/Al)_S$ = the weight ratios of phenolic acid to phenolic aldehyde for the vanillyl and syringyl phenol families, respectively. ± values represent 1 standard deviation. [b] Values from Louchouarn.[29]

lower than those of procedural blanks (<1 μg of each individual phenol/sample) reported previously by Hedges and Ertel.[29] Extensive testing with all reagents has shown that this contamination is entirely due to trace amounts of phenols present within the NaOH solution. Using HPLC grade reagent (50% w/w; Fisher Scientific) greatly reduces the contamination level.

Replicate analyses (n = 2−11) of a wide range of sample matrixes (sediments, ultrafiltered DOM, SPE-isolated DOM, and dried down freshwater DOM), produced relative standard deviations for phenol yields and lignin parameters that did not exceed 15% and averaged 5.7 ± 4.5% overall. Analytical mean deviation due to repeated injections (n = 3 and 4) of two samples averaged 1.9 ± 1.5% accounting for ∼33% of the total analytical variability. The remaining variability is due to chemical procedures (oxidation and extraction) and sample heterogeneity.

We have used an estuarine sediment[31] to test the sparging/sonication procedure vs the N_2 glovebox step and the SIM vs FID modes of quantification (Table 2). Under SIM analysis, no significant difference (t-test; $P > 0.01$) was observed in lignin yields and parameters between the two preparation procedures (Table 2). In contrast, the comparison of FID vs SIM quantification did show some differences (t-test; $P < 0.01$), but these were restricted to the yields of syringyl phenols and the S/V ratio (Table 2). If this resulted from slightly different temperature programs and higher oxidative conditions, cinnamyl phenols, the most temperature-sensitive lignin-derived phenol family,[15,31] should also show significant differences. Because this is not the case, we believe that the difference in syringyl yields represents, instead, a reduction in the integration of coeluting peaks under the SIM mode vs FID. In any case, the difference is slight and total lignin yields ($\Sigma 8$) do not differ significantly (t-test; $P > 0.01$) between the two modes of quantification (Table 2). Therefore, quantification of lignin-derived phenols with SIM yields results and a precision that are comparable to those obtained with FID.[29−33] The added advantages of a higher sensitivity and positive identification allow for an accurate quantification of samples containing only trace levels of lignin.[4,34]

A final consideration needed to be addressed, however, before this method could be applied to samples containing low amounts of both lignin and total organic carbon. Although the absolute yields of the eight major lignin-derived phenols produced are linearly correlated to the mass of sediments and associated C_{org} oxidized (Figure 1a), the intensive ratios used to characterize lignin composition, source, and diagenetic state deviate at low organic matter contents (C_{org} < 2 mg; Figure 1b−d). The destruction of syringyl compounds, as well as the transformation of vanillin (VAL) and ferulic acid (FAD) into vanillic acid (VAD) and of *p*-coumaric acid (CAD) into *p*-hydroxybenzoic acid, have previously been reported as side reactions associated with the lignin oxidation processes.[7,15,29,31] During the analytical procedure, "superoxidation" of the sample is responsible for conversion of aldehydes to corresponding carboxylic acids,[15,29,31] for preferential losses of syringyl phenols, and for overall decreases in phenol yields in samples containing little organic matter.[29] In our experiment, superoxidation appears to be responsible for some signature changes observed in samples with low organic matter content, because the observed decreases in C/V and S/V ratios (Figure 1b−c) are paralleled by strong increases in $(Ad/Al)_v$ ratios (Figure 1d). In low organic matter samples, $(Ad/Al)_s$ ratios decreased substantially (Figure 1d), suggesting that no transformation of aldehyde to acid moieties occurs in the syringyl family. However, syringyl phenols appeared to be sensitive to oxidative conditions as they decreased by 20 and 40% in the 1-mg and 0.5-mg C_{org} samples, respectively. This behavior is consistent with experimental data[7] that show strong preferential losses of syringyl phenols, particularly syringic acid, upon photooxidation of dissolved lignin. The vanillyl phenols remained constant in the 1-mg sample and increased slightly in the 0.5-mg sample, suggesting formation of vanillic acid from cinnamyl phenols. The same trends were also observed at low organic matter loadings in small DOM samples (see discussion below).

Further testing with addition of lignin-free carbon sources has shown that this effect can be abated by adding 5−15 mg of glucose (2−6 mg of C_{org}) to prevent superoxidation of the lignin polymer (Figure 1b−d). Procedural blanks for glucose added to sample-free reagents (24.5 and 22.2 ng of VAL and VAD per sample, respectively) were similar to reagent blanks and demonstrated that addition of glucose does not increase phenol yields in small samples. Reduction in the amount of CuO (from 1 g to 250 mg) also reduces adverse oxidation effects but to a much lower extent than glucose amendment (data not shown). Hence, to ensure a stable response in the oxidation reaction, a minimum sample mass amounting to ≥2 mg of natural C_{org} should be used. Amendment with lignin-free glucose can be considered in cases of samples with masses of <2 mg of C_{org}.

Isolation of Dissolved Lignin (SPE vs Ultrafiltration). Examination of DOC mass balances was used as a quality control

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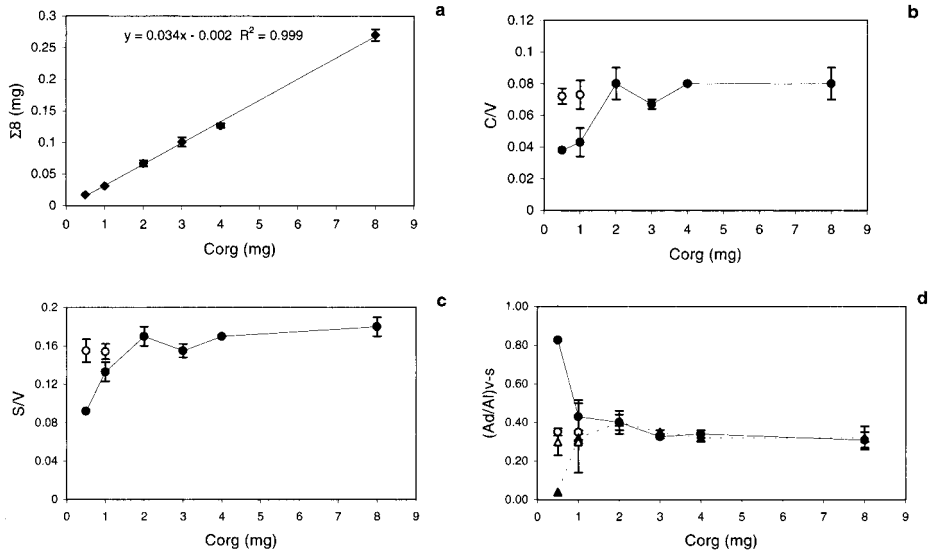

Figure 1. Total lignin-derived phenols and parameters vs organic carbon content (C_{org} in mg) during oxidation of various amounts of Sag05 sediments. (a) Sum of lignin-derived phenols (Σ8 in milligrams). (b) The ratio of cinnamyl to vanillyl phenols (C/V). (c) The ratio of syringyl to vanillyl phenols (S/V). (d) The ratio of acids to aldehydes in the vanillyl and syringyl families ((Ad/Al)*v* and (Ad/Al)*s*, respectively). (± values represent 1 standard deviation). Graphs a, b, and c: (●) represents untreated sediments and (○) represents sediments amended with glucose. In graph d: (●) and (○) represent (Ad/Al)*v* values for untreated and treated sediments, respectively, whereas (▲) and (△) represent (Ad/Al)*s* values for untreated and treated sediments, respectively.

Table 3. A Comparison of Measured Lignin-Derived Phenol Concentrations (Nanograms per Liter for GOM Samples and Micrograms per Liter for AP and NR Samples) and Parameters Obtained Using the C_{18}-SPE Method and the Ultrafiltration/C_{18}-SPE Mass Balance (sum of HMW and LMW Fractions) in Three Types of Natural Waters

	GOM[a]		AP[a]		NR[a]	
parameter	C_{18}	HMW + LMW	C_{18}	HMW + LMW	C_{18}	HMW + LMW
(ng L^{-1} or μg L^{-1})	90.2 ± 3.6	91.0 ± 14.2	3.2 ± 0.4	3.1 ± 0.1	10.6 ± 0.7	9.9 ± 0.6
S/V	0.65 ± 0.07	0.54 ± 0.17	0.87 ± 0.02	0.82 ± 0.05	0.90 ± 0.02	0.90 ± 0.11
C/V	0.20 ± 0.03	0.17 ± 0.06	0.20 ± 0.02	0.16 ± 0.01	0.22 ± 0.0	0.18 ± 0.03
$(Ad/Al)_V$	1.77 ± 0.30	1.12 ± 0.31	1.28 ± 0.07	1.13 ± 0.09	0.72 ± 0.0	0.83 ± 0.09
$(Ad/Al)_S$	1.00 ± 0.23	0.73 ± 0.21	0.87 ± 0.04	0.76 ± 0.07	0.61 ± 0.06	0.64 ± 0.13

[a] GOM, Gulf of Mexico; AP, Aransas Pass; NR, Nueces River

for the carbon isolation efficiency and reproducibility of the ultrafiltration procedure using the 1000 Dalton MW cutoff membrane. Replicate mass balance calculations indicate that an average of 98 ± 6% of the DOC in the initial water samples was accounted for in the combined retentate and permeate/diafiltrate fractions (HMW + LMW; Table 4). The average recoveries of DOC for river, estuarine, and marine waters were 42, 32 ± 0.3, and 22 ± 1.1%, respectively. These values are consistent with previous tests with ultrafiltration[18,26−27] which showed that carbon contamination and adsorption during sample handling are minimal and that the recovery of DOM by ultrafiltration decreases considerably from freshwater to open-ocean environments.

Blanks for the SPE method were determined by extracting two preconditioned C_{18} cartridges (250 mL of methanol followed by 50 mL of acidified Milli-Q water) with 50 mL of methanol and analyzing the eluates for lignin content. These replicate blanks yielded results that were not significantly different (*t*-test; $P > 0.01$) from procedural CuO blanks (all reagents without sample). In contrast, the methanol eluate of a C_{18} cartridge prior to cleaning yielded levels of individual lignin-derived phenols that were 2−10 fold higher than those obtained from procedural blanks. These results demonstrate that the cartridges carry some trace contamination and that the cleaning step is required for removing any lignin phenols present in the sorbent material. The low blanks and quick cleanup step (5−10 min/C_{18} cartridge) are advantageous relative to XAD resins that require lengthy cleaning procedures and still produce some contamination with a variety

Table 4. Concentrations of Dissolved Organic Carbon (DOC), Percentages of DOC Recovered Using Tangential-Flow Ultrafiltration, and Weight Percentages of Organic Carbon (Wt% OC) in the Recovered Isolate as Determined by Flash Combustion of Dried Powders in a CHN Analyzer

sample	salinity	DOC (μM)	volume (L)	%DOC recovered	% initial DOC[a]	wt% OC (CHN)
GOM#1	36.2	78	50	23.4	105	3.96
GOM#2	36.2	87	50	21.1	n.d	3.38
AP#1	22.0	219	35	31.7	97.3	9.32
AP#2	22.0	218	35	32.3	99.6	10.0
NR		336	50	41.6	91.0	7.98

[a] % Initial DOC = $((DOC_{concentrate} + DOC_{permeate})/DOC_{initial\ water}) \times 100$.

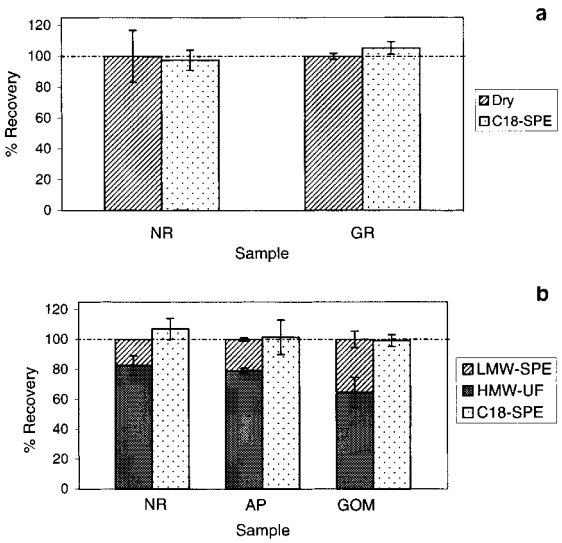

Figure 2. Percent recoveries of dissolved lignin-derived phenols (± 1 SD) isolated using the C_{18}−SPE method: (a) C_{18}−SPE values vs those obtained from the oxidation of dried river DOM; (b) C_{18}−SPE values vs those obtained from the sum of HMW and LMW fractions isolated by ultrafiltration and SPE, respectively. NR, Nueces River; GR, Guadalupe River; AP, Aransas Pass Inlet; GOM, Gulf of Mexico.

of aromatic compounds that "leach" off the resin during extraction.[3,13,15,36]

To test for quantitative recoveries of dissolved lignin from natural water samples, we compared lignin yields using C_{18}−SPE with those obtained from (1) direct dry down of freshwater samples and (2) the sum of the retentate and the permeate/diafiltrate fractions (HMW + LMW) isolated by ultrafiltration and C_{18}−SPE, respectively, from freshwater and marine samples. Using the first approach, the C_{18}−SPE method recovered 101 ± 4% of the lignin-derived phenols isolated from the direct dry-down of water samples from two different rivers (Figure 2a). The range in lignin concentrations within these two rivers spanned 1 order of magnitude (7.9 ± 0.5 and 70.8 ± 2.7 μg L^{-1} for the Nueces and Guadalupe Rivers, respectively), showing quantitative recoveries of the SPE method at different lignin and DOM loadings. In some instances, however, we observed a large variability (~17%) in lignin yields obtained from dried down solids (i.e., NR; Figure 2a). This may have resulted from large amounts of inorganic salts ($C_{org} \approx 0.3\%$) present in the isolated solids creating interferences and/or heterogeneities in the samples.

The second approach was designed to test recoveries of dissolved lignin in dilute matrixes with high salt content (i.e. estuarine and open ocean waters), but it was also tested on river water. The total concentrations of lignin-derived phenols (HMW + LMW) within the three sample types studied ranged 2 orders of magnitude with values of 91.0 ± 14.2 ng L^{-1}, 3.1 ± 0.1 μg L^{-1}, and 9.9 ± 0.6 μg L^{-1} for the GOM, AP, and NR samples, respectively (Table 3). In this data set, the C_{18}−SPE method recovered 103 ± 3% of lignin-derived phenols obtained from the combined size fractions (HMW+LMW; Figure 2b). These results indicate that solid-phase extraction provides quantitative recoveries of dissolved lignin in natural waters with an average precision (~6.5 ± 3.0%) comparable to that of sediment analyses.

Finally, we compared the lignin signatures obtained with the C18−SPE method vs those obtained from the mass balance of HMW and LMW fractions. A good match in signatures between these two methods is crucial since lignin compositional parameters are particularly useful in characterizing the source and diagenetic state of terrigenous organic matter in natural samples.[9−12,37−39] No significant difference (*t*-test, $p < 0.01$) was observed in most parameters generated using the C_{18} SPE method and the HMW+LMW mass balance (Table 3). Two values (the (Ad/Al)*v* and C/V ratios from the GOM and AP samples, respectively) did show differences between the HMW+ LMW and C_{18} methods, but the overall results suggest that, in addition to its efficient recoveries, the C_{18}−SPE method preserves the signatures of the lignin-derived compounds dissolved in freshwater and saltwater.

Special Considerations. To use the C_{18}−SPE isolation to investigate the cycle of dissolved lignin in natural waters, the C_{18} Mega Bond Elut cartridges must have well-defined and reproducible retention characteristics. A number of parameters were tested to identify key variables that affect extraction efficiencies of dissolved lignin with C_{18}−SPE. These included the volume and pH of water samples processed, the flow rate of extraction, and the storage of SPE cartridges prior to elution.

The rationale behind testing the extraction efficiency of the C_{18}−SPE cartridges as a function of volume of water was twofold: (1) to determine the maximum volume of water that could be processed through the cartridge before the retention efficiency was significantly reduced and (2) to observe the retention characteristics of the C_{18} phase for dissolved lignin as a function of DOM type (i.e., freshwater vs marine). Previous work with a smaller type of C_{18}−SPE cartridge (500 mg) and unacidified estuarine waters indicated a DOC isolation efficiency of 38% and a linear response in DOC retention within a range of 0.10−0.60 μg of C_{org}, after which the efficiency was reduced.[40] If the retention capacity of C_{18}−SPE for DOC was exclusively related to the initial concentration of DOM in the water sample, then a very simple relationship could be derived from the above-mentioned experiment and saturation curves would be directly dependent on the mass of sorbent material within the cartridge. However, earlier studies[17,19] demonstrated that both the quantity and the quality of DOM influence the retention efficiency and saturation of the C_{18} material.

The results from our volume experiments with river and estuarine waters (Figure 3a) indicate that differences in extraction efficiency (here quantified as LOP isolation) do occur among diverse water samples. However, lignin retention for all samples was linear up to a similar upper limit of processed LOP (52.4 ± 4.7 μg) regardless of water type. When LOP isolation is expressed as a function of total DOC passed through the cartridge (Figure 3b), extraction efficiency seems to be controlled by the relative amount of dissolved lignin present in the waters. Although the

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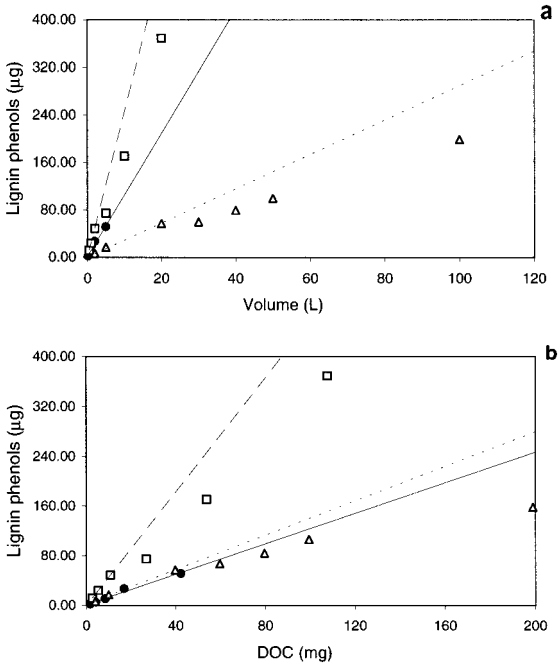

Figure 3. Measured concentrations of lignin-derived phenols (μg) vs (a) volume (L) and (b) DOC (mg) of two river samples (NR) collected in summer (●) and winter (□) and estuarine waters (AP: △) isolated using C_{18}−SPE. Linear extraction for: (—) NR summer samples (0.2−5 L); (-----) NR winter samples (0.5−2 L); and (·····) AP samples (2−20 L).

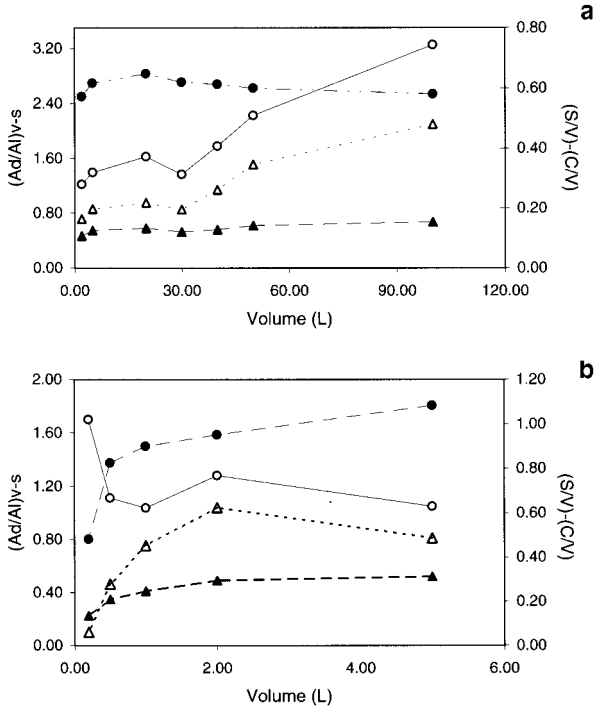

Figure 4. Compositional parameters obtained from measured lignin-derived compounds isolated using C_{18}−SPE from (a) estuarine waters (AP) and (b) river waters (NR summer). (Ad/Al)*v*, ○; (Ad/Al)*s*, △; S/V, ●; and C/V, ▲.

riverine and estuarine waters (NR summer and AP samples, respectively) showed over a four-fold difference in DOC concentrations (8.5 vs. 2.0 mg L^{-1}, respectively), they were characterized by similar carbon-normalized lignin yields (123 and 139 μg/100 mg C_{org}, respectively) and showed a similar behavior during extraction. The efficiency of extraction from these two waters was linear up to a processed DOC value of ∼40 mg, whereas in the winter river samples it decreased markedly at a processed DOC value >11 mg (Figure 3b). These results suggest that it is the proportion of extractable components, not the total quantity of DOM, that controls the upper limit of terrigenous DOM extracted by the C_{18}−SPE cartridges.

Potential losses of components during SPE extraction, although typical of chromatographic columns and adsorption processes,[40−41] raise some concerns about the utilization of C_{18}−SPE cartridges for the isolation of dissolved lignin. Major signature changes could occur during these losses due to selective "fractionation" of lignin moieties during sorption. Indeed, the decrease in retention observed in our experiments is accompanied by a selective loss of certain lignin components, resulting in substantial changes in specific, but not all, lignin compositional parameters (Figure 4). In the estuarine samples (AP), the acid/aldehyde ratios of lignin-derived phenols increase by a factor of 2 after ∼30 L, whereas S/V and C/V ratios remained stable throughout the experiment (Figure 4a). In constrast, in low-volume extractions (and thus low C_{org} retained) we observed signature changes that were similar to those presented in Figure 1b−d and are related to the superoxidation of lignin in samples with low organic matter content.

These findings have implications for the isolation of dissolved lignin by SPE, because changes in acid/aldehyde ratios from fresh to altered particulate and dissolved materials ((Ad/Al)*v*, 0.1−0.7 fresh; 0.8−4.0 altered)[7,9−11,32,39,42−43] are used as indicators of diagenetic changes. We can see from Figure 4 that both (Ad/Al)*v* and (Ad/Al)*s* ratios are variable due to column loading. Moreover, there is also variability of these ratios under "optimal" column-loading conditions (i.e., (Ad/Al)*v* for 2−30 L in AP samples; Figure 4a). These results suggest (Ad/Al) data should be interpreted cautiously as diagenetic indicators of terrigenous DOM. On the basis of these results, we recommend an extraction of 2−4 L of river water (4−10 mg DOC L^{-1}), 10−20 L of estuarine water (1−5 mg DOC L^{-1}), and 30−40 L of ocean water (0.5−1.5 mg DOC L^{-1}) using the C_{18}−SPE cartridges.

Because organic acids are important components of DOM that exist predominantly as anions at the pH of natural waters (pH 6−8), water samples should be acidified (pH 2) to protonate these ions before solid-phase extraction of DOM is performed. Solid-phase extraction of natural DOM[17,44] and model acidic analyte[23] has shown substantial decreases in retention at pH > 4. We tested the isolation efficiency of dissolved lignin from estuarine waters over a broad range of pH (1.5−8.0). Although no significant difference (*t*-test; $P > 0.01$) was observed in isolated lignin concentrations at pH ≤ 4, the retention of dissolved lignin-derived phenols decreased by as much as ∼40% at higher pH (Figure 5). Two additional tests performed at a later time on acidified and

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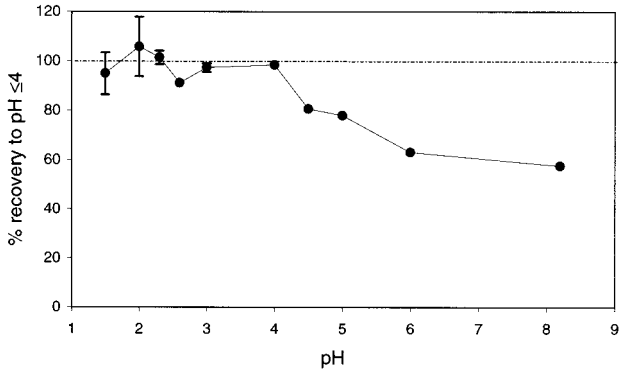

Figure 5. Percent recoveries of dissolved lignin-derived phenols (±1 SD) relative to average values obtained at pH 2−4. Estuarine waters (AP) isolated using C_{18}−SPE.

unacidified estuarine waters showed a similar decrease in extraction efficiency with unacidified waters accounting for 50 ± 1% of the value obtained at pH 2. During one of these additional tests, we collected the SPE permeate of the unacidified fraction and, after acidification to pH 2, extracted it with an additional C_{18}−SPE cartridge. The mass balance of the unacidified fraction plus acidified permeate amounted to 107% of the value obtained from the whole filtered water (pH 2), suggesting quantitative recoveries of sequential extractions of both acidic and apolar fractions of dissolved lignin. When we tested freshwater samples (Nueces River; $n = 2$), only 22 ± 4% of the lignin isolated at pH 2 was retained from unacidified waters. These results suggest that dissolved lignin exists as a heterogeneous mixture of components with varying chemical characteristics.[17,45]

The flow rate of extraction is an important parameter because it ultimately determines sample process time. This is particularly critical during fieldwork where sampling and processing times are limited. Previous experiments with XAD resins have shown that DOM extraction efficiencies drop markedly at flow rates higher than two bed volumes per minute.[16−17] In contrast, DOM extraction efficiency for C_{18}−SPE was shown to be independent of flow rate over a range of more than 10 bed volumes per minute.[17,20] In the present study, we extracted water samples on the C_{18} cartridges at a flow rate equivalent to five bed volumes per minute (50 mL min^{-1}). With large seawater samples (>30 L), this flow rate is equivalent to >10 h of processing time. We tested the extraction at 10 and 15 bed volumes per minute (100 and 150 mL min^{-1}, respectively) in order to see if we could reduce sample processing times. No significant difference (*t*-test; $P > 0.01$) could be observed between the three flow rates (Table 5), so processing time can be reduced by a factor of 2−3. However, we recommend using a flow rate of 50−100 mL min^{-1} because at higher flow rates the increased pressure in the pumping line creates a strong potential for leaks and sample loss.

Table 5. Measured Lignin-Derived Phenol Concentrations (μg L^{-1}) in Estuarine Waters (AP) Isolated by C_{18}−SPE under Three Different Flow Rates and Storage Conditions[a]

	Flow Rate *n*	(μg L^{-1})
50 mL min^{-1}	2	2.94 ± 0.20
100 mL min^{-1}	2	2.76 ± 0.16
150 mL min^{-1}	2	2.80 ± 0.26
	storage *n*	(μg L^{-1})
fresh	2	1.90 ± 0.15
fridge	2	1.76 ± 0.05
freezer	1	1.82

[a] Samples for the two experiments were collected at different times. Absolute variations about the mean are given.

Finally, this method was specifically designed for isolating trace amounts of lignin from seawater and was intended to be used in the field. Therefore, we tested two methods for the storage and preservation of extracted samples. We extracted a series of estuarine water samples ($n = 6$) and split the cartridges into three groups. The first group was eluted and analyzed immediately, whereas the second and third were stored at 4 and −20 °C, respectively, for one month prior to elution and analysis. No significant difference (*t*-test; $P > 0.01$) was observed among the three treatments (Table 5), indicating that storage under cold or frozen and dark conditions was efficient and did not lead to any significant loss of dissolved lignin.

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