Analysis of lignin-derived phenols in standard reference materials and ocean dissolved organic matter by gas chromatography/tandem mass spectrometry

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A B S T R A C T

A series of reference materials are proposed for intercomparison and quality control purposes during the quantification of lignin oxidation products (LOP) from diverse environmental matrices. These materials are all easily accessible and certified for diverse organic constituents (NIST and IHSS). They represent a suite of natural environmental matrices (from solids to aqueous) and are characterized by a wide range of organic carbon and lignin concentrations with abundant proportions of all major LOP. The variability of LOP concentrations and signatures for all these materials averages 3–5% and does not exceed 10%. Using these standards, a new quantification method was developed and validated for the determination of low-level CuO oxidation products using capillary gas chromatography–tandem mass spectrometry (GC/MS–MS). Tandem mass spectrometry provides both the high sensitivity and selectivity required for the identification and quantification of trace levels of dissolved lignin. The method is particularly useful for removing interference from co-eluting isotopes generated from the DOM matrix and during glucose amendment procedures of low-carbon samples. Such glucose amendment is not necessary, however, when the CuO to organic carbon weight-to-weight ratio can be kept at a value <200–300.

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1. Introduction

Lignin, the second most abundant naturally occurring polymer after cellulose, is an exclusive and complex structural component of vascular plant tissues (Sarkinen and Ludwig, 1971). Because of its unique structural features, it possesses greater stability to biodegradation than hemicellulose and cellulose and is considered to be among the best-preserved components of vascular plants after deposition (Dignac et al., 2005; Hedges, 1991; Kirk and Farrell, 1987). This recalcitrant nature confers a significant role on lignin in the biogeochemical carbon cycle (Colberg, 1988). In addition, because vascular plants are exclusively terrestrial, lignin is an important contributor to soil organic matter (SOM) (Hedges et al., 1997; Kögel-Knabner et al., 1991), whereas its presence in aquatic environments serves as an unambiguous tracer of terrigenous organic matter (TOM) inputs to such systems (Hedges et al., 1988b, 1982).

Alkaline CuO oxidation is a technique commonly used to analyze lignin in plant tissues and environmental matrices such as soils, sediments, and particulate and dissolved OM (Benner et al., 2005; Gordon and Goñi, 2003; Kuo et al., 2008b; Louchouarn et al., 2000; Otto and Simpson, 2006). Upon oxidation, the lignin macromolecule is hydrolyzed into small methoxylphenyl or phenyl structural units grouped into three separate families: vanillyls (V), syringyls (S), and cinnamyls (C). Each class of lignin oxidation products (LOPs) is in turn comprised of an acid, an aldehyde, and a ketone (for V and S, or only acids (for C). The yields and ratios (S/V, C/V, and acid/aldehyde) of these lignin phenols have been used extensively to identify specific compositional signatures of vascular plant tissues (Goñi and Hedges, 1992a; Hedges and Mann, 1979) and estimate inputs of fresh to highly altered vascular plant carbon to aquatic systems (Benner et al., 2005; Dalzell et al., 2005; Dickens et al., 2007; Gordon and Goñi, 2003; Hedges et al., 2000b; Hernes and Benner, 2003; Houel et al., 2006; Louchouarn et al., 1999; Prabh et al., 1994; Sánchez-García et al., 2008).

The CuO oxidation method has undergone revisions and adaptations since its initial application to aquatic geochemistry some 30 years ago (Hedges and Mann, 1979; Hedges and Ertel, 1982). Improvements have led to a “cleaner” chemistry, a switch to more appropriate surrogates, changes in solvents and their reduction with the use of smaller reaction vessels and solid phase extraction, and recently an increased sample throughput thanks to design improvements in reaction vessels or the use of microwave digestion (Goñi and Hedges, 1992a; Goñi and Montgomery, 2000; Kiern and Kögel-Knabner, 2003; Kuo et al., 2008b; Louchouarn et al., 2000). Gas
chromatography (GC) has been used predominantly over liquid chromatography (LC) for the separation of derivatized LOP during this long analytical history. This choice has been supported by a better separation than that offered by LC systems and the potential for coupling the GC to a mass spectrometer (MS), an option that has become only routinely viable for LC in the last decade. One other major advantage GCMS offers with respect to its LCMS counterpart is its affordability. New GCMS now also permit routine quantification approaches that only the most expensive instruments could perform a few decades ago. Among these, multiple reaction monitoring (MRM) performed during tandem mass spectrometry (MS-MS) brings an added advantage over other monitoring modes such as full scan (FS) or selective ion monitoring (SIM). Although both SIM and MRM provide increased sensitivity over full scan mode during quantification by decreasing overall noise and focusing on few limited ion(s) per scan time, MRM also increases specificity. Because the product ions are unique fragments of specific ion precursors, tandem mass spectrometry avoids the potential artifact generated by co-eluting extraneous analytes yielding similar isotope mass fragments as the target ion(s) (Plomley et al., 2000). Moreover, tandem mass spectrometry yields mass losses that are indicative of functional groups in the precursor ion, strengthening both identification and structure elucidation of the original molecule (Plomley et al., 2000; Zwiener and Frimmel, 2004a).

The advantage of GC/MS-MS for selectivity has been recently documented in the analysis of a suite of analytes including natural saccharidic by-products of incomplete biomass combustion in aerosols (Pashynska et al., 2002), polychlorinated contaminants in standard solutions (Helen et al., 2001; Plomley et al., 2000), pharmaceuticals and emerging contaminants in natural waters (Zwiener and Frimmel, 2004b), and phenolic metabolites in human urine (Bravo et al., 2005). Prior studies have used SIM as a way to improve analytical sensitivity and quantify LOP in dissolved OM extracted from deep ocean waters (Benner et al., 2005; Hernes and Benner, 2002; Opsahl and Benner, 1997; Opsahl et al., 1999). To our knowledge, however, no study has applied MRM for the quantification of these terrigenous biomarkers in environmental media. Our objectives were thus twofold. First we tested a series of standard materials to be proposed as environmental references to assess the consistency of the CuO oxidation procedure across different laboratories and using different quantitative methods. Secondly, we used these standards as well as natural dissolved organic matter (DOM) samples to verify if the MRM mode provided comparable results to the more common FS mode while at the same time resolving interferences observed in difficult, low-level samples.

2. Materials and procedures

2.1. Materials

Standard reference materials used in this study were purchased either from the U.S. National Institute of Standards and Technology (NIST; Gaithersburg, MD) or the International Humic Substances Society (IHSS; St. Paul, MN). These include two estuarine sediments (NIST SRM 1944 and SRM 1941b), atmospheric particles (NIST SRM 1649a), vascular plant soft tissues (NIST SRM 1547), and dried fulvic acid (IHSS 15101F). A summary of the general characteristics (sample matrix and origin) is provided in Table 1. Additional samples used in this study include a sediment sample (SAG05B) collected from the Saguenay Fjord, Canada, as well as DOM extracted from a suite of Arctic Ocean water masses by solid phase extraction (SPE). Lignin constituents of DOM were isolated from these latter samples according to the method described in Louchouarn et al. (2000). Ocean water samples were collected below the surface (55 m), at intermediate depth (800 m), and in deep waters (2000 and 2800 m) during an Arctic Ocean cruise in 2005. All samples were filtered on a 0.2 μm Nuclepore™ filter and were then acidified to pH 2.5 using concentrated HCl (reagent grade). The acidified waters were run through C-18 solid phase extraction (SPE) columns (60 cm² 10 g⁻¹; Bond Elute, Varian Inc.), which were preconditioned prior to sample extraction by running ~50 mL HPLC grade methanol, followed by 100 mL acidified (pH 2.5) MilliQ water. The dissolved lignin extracted on the column was eluted in one fraction using 35 mL HPLC grade methanol into a 250 mL muffled glass flask, and then dried in a Savant SpeedVac (SC210A) for 12–24 h.

2.2. Elemental analyses

Organic carbon (OC) concentrations were determined on dried samples by combustion using a Perkin-Elmer 2400 elemental analyzer. Carbonates were removed prior to analysis by vapor-phase acidification with HCl for 24 h followed by drying at 60 °C for 24 h (Harris et al., 2001; Hedges and Stern, 1984; Hernes and Benner, 2002). The instrument was calibrated with acetalнике, while a series of NIST SRMs (1944, 1941b) was used regularly as verification standards. The mean analytical variability determined from replicate analyses of selected samples was <2%.

2.3. Lignin analysis

Lignin-derived CuO oxidation products (LOP) were determined using the method developed by Hedges and Ertel (1982) and Göñi and Hedges (1992a,b), with modifications (Kuo et al., 2008b; Louchouarn et al., 2000). For particular materials, a homogenized ground sample, providing 2–5 mg OC (Louchouarn et al., 2000) was added to a stainless steel reaction mini-vessel (3 mL; Prime Focus Inc.), pre-loaded with CuO (~330 mg), Fe(NH4)2(SO4)2·6H2O (~100 mg), and a stainless steel ball bearing used to ensure sample stirring during oxidation. Each reaction vessel was then filled with ~3 mL of 2 N Ar-sparged NaOH solution. The DOM samples were treated slightly differently. Each dried SPE eluent was sonicated twice with 1.5 mL of 2 N NaOH (pre-sparged with Ar) to remove the isolated DOM and residues adhered to the Savant flasks. The two 1.5 mL NaOH aliquots were then transferred to a reaction mini-vessel pre-loaded with CuO, Fe(NH4)2(SO4)2·6H2O, and a ball bearing. The intermediate and deep ocean water samples were all amended with an addition of 10 mg of glucose that was also pre-added to the reaction vessel. The headspace of each reaction mini-vessel was purged for 30 min with Ar using a customized purging block (Prime Focus Inc.) and then closed. The vessels (n = 12) were heated at 155 °C for 3 h in a customized Hewlett-Packard 5890 gas chromatograph fitted with a revolving carousel to facilitate sample stirring during oxidation. Trans-cinnamic acid (3-phenyl-2-propenoic acid) and ethyl vanillin (3-ethoxy-4-hydroxy-benzaldehyde) were used as surrogate standards and were added directly (~3–12 μg) to each mini-vessel after cooling. After centrifugation of solids and rinsing (×2) with 3 mL of 1 N NaOH, the aqueous solution was acidified with 6 N HCl (pH ≤ 1) and extracted (×3) with ethyl acetate. Extracts were treated with Na2SO4 to remove residual water and evaporated to dryness using a LabConco™ solvent concentrator. The CuO reaction products were re-dissolved in a small volume of pyridine (~200–500 μL). Following further dilution with pyridine (1:10 to 1:20), an aliquot (75 μL) was transferred to a 1.5 mL glass vial to which 75 μL of N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS; Supelco, PA, USA) was added. The samples were then derivatized under normal atmospheric pressure by heating at 75 °C for 1 h in a 20-wells block heater. After derivatization, each sample was transferred to a 250 μL glass autosampler vial insert. At this point, some samples were spiked with a known amount of trisopropylbenzene (Aldrich, MO, USA; Simpson et al., 2004) to serve as a GC-internal standard and help calculate surrogate recovery.
2.4. GC/MS analysis

Separation and quantification of trimethylsilyl (TMS) derivatives of CuO oxidation by-products were performed using gas chromatography–mass spectrometry (GC/MS) with a Varian Ion Trap 3800/4000 system fitted with a fused silica column (VF 5MS; 30 m × 0.25 mm i.d., 0.25 μm film thickness; Varian Inc.). Each sample was injected, under splitless mode, into a deactivated glass liner inserted into the GC injection port and using He as the carrier gas (~1.0 mL min⁻¹). The GC oven was programmed from 65 °C (with a 2 min initial delay) to 300 °C (held 10 min) using a 4 °C min⁻¹ temperature ramp. The GC injector and GC/MS interface were maintained at 280 °C and 270 °C, respectively. The mass spectrometer was operated in the electron ionization mode (EI, 70 eV) using either full scan (FS) or multiple reaction monitoring (MRM). Compound identification was performed using GC retention times and by comparison full mass spectra (FS mode) or precursor/product spectra (MRM mode) with those of commercially available standards. Under FS mode, the instrument was operated in the mass range m/z 50–500 and 1–3 target ions (including the base ion) were selected for quantification (Table 2). For operation in the MRM mode, a specific precursor ion for each compound of interest was selected and its product ions monitored (Table 3). The scan time in MRM mode was 0.5 s. The condition of collision-induced dissociation (CID) was optimized for each compound by selecting the amplitude of dissociation (0.6–1.2 V) that maximized the signal to noise ratio (S/N), which was calculated using the Varian MS Workstation software (version 6.6). In all cases, the amplitude selected yielded an S/N for each daughter product that was >10.

Quantification was performed using relative response factors (RRF) adjusted to the surrogate standard and determined using multiple injections (n = 6) of a one-point calibration solution. The standard deviation of the RRFs was less than 5%, whereas its value corresponded to that determined with a 5-point calibration curve (r² = 0.996–0.999) with concentrations of each lignin-derived phenol ranging from 0.1 to 3.8 ng μL⁻¹. The method detection limit (MDL) for each LOP was determined as kS, where k is 3 and S is the standard deviation of repeated analyses (n = 7) of low-level standard spikes (~0.8 pg) (Louchouarn et al., 2009). This index of sensitivity is slightly different than the common limit of detection (LOD) since it is based on the variability of the analytical signal of a series of standards that went through the entire extraction procedure. The MDL averaged 3–15 pg/μL and 0.5–1.5 pg/μL for FS and MRM modes, respectively (Tables 2 and 3). This is an equivalent of 10−30 and 50–300 pg/L in ocean water samples for each monitoring mode, respectively. In solid samples, the MDL is equivalent to 15–90 and 3–8 ng/g for each monitoring mode, respectively.

### Table 1

Reference materials used for lignin quantification in different environmental matrices.

<table>
<thead>
<tr>
<th>Material</th>
<th>Name</th>
<th>ID</th>
<th>Production/Collection method</th>
<th>Organic carbon (wt.%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estuarine sediment</td>
<td>Baltimore Harbor</td>
<td>SRM 1941b</td>
<td>Sampled from the Baltimore Harbor in the Chesapeake Bay area (USA)</td>
<td>3.0 ± 0.2¹</td>
</tr>
<tr>
<td>Estuarine sediment</td>
<td>NY/NJ Waterway</td>
<td>SRM 1944</td>
<td>Sampled from the NY/NJ Harbor in the Hudson Estuary (USA)</td>
<td>4.4 ± 0.3²</td>
</tr>
<tr>
<td>Aerosol</td>
<td>Urban dust</td>
<td>SRM 1649a</td>
<td>Atmospheric particulate matter collected in 1976–77 in the Washington D.C. area (USA), over a period in excess of 12 months. The material was then sieved through a fine-mesh sieve (~125 μm) and issued as SRM 1649 in 1982. Re-certified in 1990 and issued as SRM 1649a. Dried leaves (coronet variety) were initially ground milled to pass a 1 mm screen. At NIST, the ground leaves were further separated to a particle size of approximately 75 μm.</td>
<td>17.7 ± 0.2²</td>
</tr>
</tbody>
</table>
| Plant tissue            | Peach leaves          | SRM 1547     | Dried leaves, 20 years ago, the 14C-labeled recovery standard used initially during LOP quantitation ([Hedges and Ertel, 1982]) was replaced with chemical analogs, or surrogate standards, such as ethyl vanillin (EVAL) and Trans-cinnamic acid (CIAD; [Goff and Hedges, 1992a]). Since then, studies have used these in isolation or combination sometimes without giving specific information as to which was used for quantitation. Recent publications still show this dual option with some citing a preference for EVAL as a surrogate (Dalzel et al., 2005, 2007; Louchouarn et al., 2000; Tareq et al., 2006; Tareq et al., 2004), others for CIAD (Benner et al., 2005; Hernes and Benner, 2002; Hernes et al., 2007; Houel et al., 2006; Kuo et al., 2008b; Sánchez-García et al., 2008), and yet a third group using both simultaneously ([Goff and Thomas, 2000; Goff et al., 2000; Gordon and Goff, 2003; Opsahl et al., 1999; Tesi et al., 2007]). Despite this diversity, however, no study has shown that results are comparable using either of these surrogates. One particular issue that may lead to differences in quantification is the higher volatility of ethyl vanillin and its increased susceptibility to evaporation during solvent drying and exchange. Monitoring the EVAL to CIAD ratio has been a way to determine if ethyl vanillin has suffered excessive losses and thus may lead to a potential overestimation of less volatile analytes of interest. In the present study, we compared total lignin yield quantitation using both surrogates and making sure that the EVAL/CIAD ratio remained within ±10% of the average surrogate solution. As shown in Fig. 1, all values are comparable for both surrogates across a range of LOP yields that are relevant to most environmental studies. Additionally, the recovery of each surrogate (calculated using trisopropylbenzene) did not differ significantly from each other (68 ± 13% and 65 ± 13% for CIAD and EVAL, respectively, in SRM 1941b). These recoveries were also relatively similar to those reported (73 ± 3% and 71 ± 6% for CIAD and EVAL, respectively) from the extraction of pure standard solutions using ethyl acetate under liquid–liquid extraction ([Goff and Montgomery, 2000]). These results suggest that, assuming no selective evaporative losses of EVAL occurred during solvent dry-down in prior studies, all published data produced under both quantification methods are comparable within the average analytical error of the CuO method itself (±5–10%). In the following section, all data discussed were quantified using CIAD as the surrogate standard. 3.2. Standard reference materials

The analytical precision and “accuracy” of the major CuO oxidation products and related parameters was tested in the past with a series of
reference materials that were shared across different groups. One such material, developed in John Hedges’ lab in the 1980s, was the Lake Washington Standard Mud (1WSM) (Goñi and Montgomery, 2000; Louchouarn et al., 1997). Because of the dwindling stock of this standard, P. Louchouarn developed another sediment standard (SAG05B) and cross-referenced its analyses with respect to those of the LWSM (Houel et al., 2006; Louchouarn et al., 2000). Recent analyses of this standard (Table 4) show no significant differences with values reported in the literature since the mid-1990s (Houel et al., 2006; Louchouarn et al., 1997, 2006, 2000). However, because of the need for materials that are widely accessible to the entire geochemical community, we sought a series of environmental mixtures that could be used as references for LOP analyses in diverse applications. The mixtures selected include estuarine sediments (NIST SRM 1649a), plant soft tissues (NIST SRM 1547), and dried fulvic acid (IHSS SRM 1941B), atmospheric particles (NIST SRM 1649a), and pesticides). These high concentrations are consistent with long-term watershed disturbances and historically high industrial and urban point source inputs to these coastal regions (Dickhut et al., 2000; Yan et al., 2005, 2006). The NY/NJ waterway sediments, however, are particularly enriched in such hydrocarbons suggesting larger inputs of anthropogenic organics in this harbor system. The four fold differences in lignin concentrations (28, mg 100 mg OC−1 dry weight) and three fold differences in lignin yields (λ8, mg 100 mg OC−1) also point to higher inputs of terrigenous OM to the NY/NJ sediments (Table 4), which result from natural processes and/or higher anthropogenic impact to the watersheds draining into the NY/NJ coastal system (pulp and paper mills, agriculture, deforestation; Houel et al., 2006; Louchouarn et al., submitted for publication, 1999)). The internal lignin ratios suggest that both sediments are comprised of high proportions of angiosperm tissues (high S/V ratios), although the Baltimore Harbor sediments are slightly more enriched in vascular plant soft tissues (C/V ratios). Acid to aldehyde ratios (AD/Al) vary between 0.3 and 0.3 mg 100 mg OC−1, and do not exceed 10% (Table 4).

The two coastal sediments were chosen because of their differences in grain size (NIST certificates of analysis) and terrigenous OM content (Table 4). The sediments of the NY/NJ waterway system are characterized by coarser sediment fractions and slightly higher OC content than those of the Baltimore Harbor (4.4 vs. 3.0%, respectively; Table 1). Both sediments serve as certified materials for high-level hydrocarbon content in sediments (i.e. PAHs, PCBs, chlorinated pesticides). These high concentrations are consistent with long-term watershed disturbances and historically high industrial and urban point source inputs to these coastal regions (Dickhut et al., 2000; Yan et al., 2005, 2006). The NY/NJ waterway sediments, however, are particularly enriched in such hydrocarbons suggesting larger inputs of anthropogenic organics in this harbor system. The four fold differences in lignin concentrations (28, mg 100 mg OC−1 dry weight) and three fold differences in lignin yields (λ8, mg 100 mg OC−1) also point to higher inputs of terrigenous OM to the NY/NJ sediments (Table 4), which result from natural processes and/or higher anthropogenic impact to the watersheds draining into the NY/NJ coastal system (pulp and paper mills, agriculture, deforestation; Houel et al., 2006; Louchouarn et al., submitted for publication, 1999)). The internal lignin ratios suggest that both sediments are comprised of high proportions of angiosperm tissues (high S/V ratios), although the Baltimore Harbor sediments are slightly more enriched in vascular plant soft tissues (C/V ratios). Acid to aldehyde ratios (AD/Al) vary between 0.3 and 0.3 mg 100 mg OC−1, and do not exceed 10% (Table 4).
these two standard materials thus makes them ideal candidates for QA/QC controls on lignin quantification from sedimentary materials that contain varying amounts of terrigenous OM.

Similarly, we sought a readily available standard material that could represent fresh plant OM. Because woody materials are virtually devoid of cinnamyl phenols (C, (Goñi and Hedges, 1992a; Hedges and Goñi, 1992b)), we selected an angiosperm soft tissue, which contains all major lignin-derived phenols (Goñi and Hedges, 1992a). No OC content is provided in the certificate of analysis from the dried peach leaf standard reference material (NIST 1547), so the one presented in Table 1 (45.8±0.4 wt.% was produced in our lab (Goñi et al., 2004)). In addition, the large proportion of syringyl phenols in angiosperm tissues (S/V=1.0±0.0) is characteristic of angiosperm tissues, whereas the low (Ad/Al)−s ratios (0.22–0.27) confirm the fresh nature of this standard material (Hedges et al., 1988a). Hence, because of its diverse composition, the dried peach leaf SRM (NIST 1547) could be useful as a potential reference material to test recoveries of all lignin constituents in samples receiving large inputs of relatively unaltered vascular plant OM (wetlands, soil litter, etc.).

Because of widespread interest and rapid development in black carbon (BC) research in the last few years (Hedges et al., 2000a; Masiello, 2004), several reference materials have been developed for intercomparison and quality control purposes during the quantification of the broad spectrum of BC constituents (Hammes et al., 2007, 2008; Kuo et al., 2008a; Reddy et al., 2002). One of these is the Urban Dust standard reference material (NIST 1649a), which is comprised of atmospheric particulate matter collected in the Washington DC Navy Yard during a year’s time in 1976–1977 (Currie et al., 2002). Although this SRM was collected in an urban setting and was thought initially to contain predominantly fossil fuel-derived combustion by-products, it was shown that close to 40% of the OC in this sample is modern carbon (Currie et al., 2002; Reddy et al., 2002), derived either from biomass combustion or from plant OM (microbe debris, pollen). The high concentration of levoglucosan, an exclusive biomarker of biomass...
content of pollen grains (Hu et al., 1999; Keil et al., 1998) show that in debris and/or pollens. The few studies that have evaluated the LOP volumes per minute (Amador et al., 1990). Later methods seeking to isolate dissolved LOP in atmospheric particulate matter could also include fine plant micro-debris and/or pollens. The few studies that have evaluated the LOP content of pollen grains (Hu et al., 1999; Keil et al., 1998) show that in general, these are characterized by unusual lignin phenol composition showing a particular enrichment in cinnamyls and virtually no syringyls irrespective of plant species. For example, their C/V ratios vary from 1−3, in angiosperm pollens, to 13−35, in some gymnosperm pollens. Most significantly, their p-coumaric to ferulic acid ratio (Cd/Fd) can reach extremely high values (>100) though a four order of magnitude range has been reported across angiosperm and gymnosperm species (Hu et al., 1999; Keil et al., 1998). In the Urban Dust SRM, the high Cd/Fd ratio (∼9.0) points to the significant presence of pollen, though the high S/V ratio (∼1.0) also suggest that other angiosperm plant micro-debris are present in substantial amounts. Although several studies have used solvent-extractible lignin methoxyphenols as markers of biomass combustion in atmospheric aerosols (bin Abas et al., 2004; McConnell et al., 2007; Simoenet, 2002), we are not aware of any that have quantified LOP in atmospheric particulate matter. With the increased analytical sensitivity afforded by GC/MS and LC/MS systems, this could now be considered in filter samples containing as little as a few mg of material. This is possible since mineral dilution, of the sort present in soils and sediments, is much less important in atmospheric particulate matter. Hence, because of its relatively large LOP concentration and yield, and the substantial presence of all major lignin phenols, SRM 1649a could be used in studies seeking to assess Aeolian transport of vascular plant micro-debris.

Previous work on lignin has shown that LOP can also be significant molecular components in dissolved OM from soil and wetland porewaters (Benner and Opsahl, 2001; Ertel et al., 1986; Hernes and Benner, 2003; Louchouarn et al., 2000), and coastal to open ocean seawater (Benner et al., 2005; Hernes and Benner, 2002; Louchouarn et al., 2000; Opsahl and Benner, 1997; Opsahl et al., 1999). Despite the growing number of such studies, there still exist no uniform methodology of dissolved OM isolation for LOP quantification leading to an intrinsic heterogeneity in the operational definition of what might be called dissolved LOP. Initial isolation approaches used XAD resins (Ertel et al., 1986, 1984; Meyers-Schulte and Hedges, 1986), though this method is particularly time-consuming (from 5 to 120 h), and efficiencies are strongly limited by operational conditions, with efficiencies dropping markedly at flow rates higher than two bed volumes per minute (Amador et al., 1990). Later methods seeking to isolate dissolved lignin have used C-18 SPE columns (Hernes and Benner, 2002, 2003; Louchouarn et al., 2000), tangential-flow ultrafiltration (Benner et al., 2005; Hernes and Benner, 2003; Louchouarn et al., 2000), and even direct freeze-drying (Louchouarn et al., 2000). Although the latter method ensures the complete isolation of DOM from fresh waters, it is not feasible for seawater due to the dilution and potential interference generated by solid salts in the large volumes needed to isolate enough dissolved OM required for the CuO oxidation. Similarly, although the bulk of dissolved lignin (typically 90%) in river dissolved OM is recovered using ultrafiltration, a substantial fraction of dissolved lignin in the ocean occurs as low molecular weight molecules that escape isolation by ultrafiltration (Hernes and Benner, 2002; Louchouarn et al., 2000). A combination of ultrafiltration followed by SPE on the permeate has now made it possible to determine lignin concentrations and compositions in both high and low molecular weight components of dissolved OM (Benner et al., 2005; Hernes and Benner, 2002, 2003; Louchouarn et al., 2000), whereas C-18 SPE column also quantitatively recovers the total dissolved lignin from most water samples (Louchouarn et al., 2000). Because of the large heterogeneity of size fractions and material sources in colloidal and dissolved OM, no standard can be expected to represent the full range of materials found in rivers to the open ocean. However, solid phase samples comprised almost exclusively of fresh materials (plant debris) or mineral matrices (soils and sediments) are even less representative of the OM found dissolved in aqueous matrices. Although the fulvic acid sample chosen here is strongly terrigenous in its character and enriched in lignin-derived constituents (∼38 = 1.6 ± 0.10 mg g−1), it may still be used as a better reference material than sediment and soil materials. Its composition suggests that the fulvic acid is derived predominantly from angiosperm sources (S/V = 0.79 ± 0.02) with moderate leachate inputs from soft tissues (C/V = 0.35 ± 0.00). The high (Ad/Al) − s ratios (0.99 ± 0.05 and 0.85 ± 0.02, respectively) confirm that this material contains a high proportion of oxidatively altered materials and/or acid moieties selectively leached from the parent plant tissues (Hernes et al., 2007; Sánchez-García, 2007). The very high 3.5 Bd/V ratio is consistent with the reported increased concentration of 3.5 Bd in terrigenous OM sorbed to fine soil and sediment particles (Dickens et al., 2007; Houel et al., 2006; Sánchez-García et al., 2008) further supporting the interpretation that the isolated dissolved OM has been selectively leached and/or is comprised of material that has been extensively altered during its transfer from the plant tissues to the aqueous phase.

3.3. Lignin oxidation products measured by tandem MS: particulate and dissolved OM

Tandem mass spectrometry brings an added advantage over selective ion monitoring (SIM). Although both provide increased sensitivity over full scan mode during quantification by focusing on few limited ion(s) per scan time, and thus decreasing overall noise, MRM mode also provides increased selectivity. Because the monitored ions are unique products from specific ion precursors, tandem mass spectrometry avoids the potential artifact of co-eluting isotope mass fragments that is possible during SIM. Moreover, MRM yields mass losses that are indicative of functional groups in the precursor ion strengthening both identification and structure elucidation of the original molecule. For example, upon MRM all acid moieties in the CuO oxidation by-products are characterized by the loss of the carboxylic functional group (m/z 44; Fig. 2) confirming their initial acid functionality. Similarly, other common losses in the LOP series include aldehyde functional groups (m/z 29) from vanillin and syringaldehyde, as well as the losses of one to three methyl groups (m/z 15) released from the attached trimethylsilyl derivatizing agent (Figs. 2 and 3).

The advantage of added selectivity may be required during oxidation of DOM because of the need to protect the low-organic samples from super-oxidation during the CuO oxidation procedure. It was shown previously that an OC loading of less than 2 mg in each reaction vessel may lead to significant alterations of the lignin by-products and indicator ratios (particularly S/V, C/V, and (Ad/Al); Louchouarn et al., 2000). This issue can be resolved for most samples through the addition of a small amount of lignin-free glucose (Louchouarn et al., 2000). Glucose amendment was also shown to increase LOP recoveries from mineral soil horizons (Amelung et al., 1999) suggesting that it stabilizes LOP yields in OM-poor, mineral-rich matrices. In some DOM samples with low-level OC content, however,
EI mass spectra obtained from the TMS derivative of vanillic acid under a) full scan mode, and b) from ion m/z 297 using MRM mode employing a 1.0 V CID amplitude. In figure b), fragment ions m/z 282 and 267 correspond to a loss of one and two methyl ions (m/z 15), respectively, from the TMS group, whereas fragment ion m/z 253 corresponds to a loss of the carboxylic functional group (m/z 44).
Fig. 3. EI mass spectra obtained from the TMS derivative of syringaldehyde under a) full scan mode, and b) from ion m/z 224 using MRM mode employing a 1.0 V CID amplitude. In b), fragment ion m/z 195 corresponds to the loss of the aldehyde functional group (m/z 29).
this procedure seems to produce complex by-products that co-elute with several of the LOPs as well as the surrogate standard yielding isotopic fragments that interfere with the SIM mode (Fig. 4). For example, extraneous fragments observed during the surrogate’s peak elution time (m/z 333, 305, 231, 221, 217, 204 and 147) are all known cleavage by-products of TMS-glucose (Shimasaki et al., 1995). Because of the 1 m/z unit separating some of these fragments and the surrogate’s target ions (205 and 220; Table 2), isotopes from glucose fragments can generate large quantitative interferences leading to an underestimation of LOPs. Additional interferences were also observed during segments from major vanillyl products (vanillin and vanillic acid). One solution to this issue is to select target precursor/product pairs to increase the selectivity of the quantification method and substantially improve the chromatography (Fig. 5).

We compared the MRM results of two of the standards discussed earlier (SRM 1941b and IHSS-FA) with those obtained from conventional full scan mode to assess the applicability of the former monitoring mode to LOP quantification (Table 5). With one exception (see below), no significant difference (t-test; P>0.01) was observed in lignin yields and parameters between the two monitoring procedures (Table 5). The only significant difference (t-test; P<0.01) was observed for the (Ad/Al)s of the fulvic acid sample (but not for the standard sediment). This result seems to be driven by a close to significant difference in syringic acid quantitation using the two monitoring modes (P=0.02). However, the average values of these ratios were still within ~10% of each other suggesting that the small differences observed between the two quantitation modes are within the analytical precision reported for the method (see Section 3.1). The general statistical similarities between the two modes of quantification confirm that the MRM characterization of LOP in environmental media is comparable to that produced using full scan monitoring methods.

In addition, we reproduced a previous test, using glucose amendment (10 mg), to verify that lignin signatures could be preserved during CuO oxidation even under low sample-OC loading (Louchouarn et al., 2000). Approximately 10–15 mg of SRM 1941b (~0.3–0.4 mg OC) were oxidized with and without glucose amendment and analyzed both under full scan and MRM modes. Despite the low OC loadings, the unamended LOP signatures only showed minor shifts from the average values (Table 5). In particular, although the (Ad/Al)v signature of the unamended sample did show a significant increase with respect to standard conditions (~2 mg OC) and glucose amendment (Table 5), it was not as large as that reported earlier by Louchouarn et al. (2000). This moderate shift is consistent with earlier findings that a reduction in the amount of CuO (from 1 g to 250 mg) helped reduce adverse oxidation

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![Fig. 4. Spectrum of an Arctic DOM sample showing a large co-elution during the CiAD peak elution time yielding fragments typical of derivatized glucose (m/z 147, 204, 217, 221, 231, 305, and 333; identified with an asterisks on spectrum) and of derivatized Trans-cinnamic acid (m/z 145, 161, 205 and 220; identified with a star on spectrum).](image-url)
effects in low-level samples (Louchouarn et al., 2000). The new design of the mini-reaction vessels brings the total amount of CuO used down from 1 g to ~300 mg thus supporting the lower oxidation observed in unamended samples.

Super-oxidation of the lignin macromonomer thus seems to be avoided above a specific weight-to-weight ratio of CuO to OC loaded in the reaction vessels. Although it seems that a 400–500:1 ratio is an upper limit above which super-oxidation becomes substantial.

Table 5
Concentrations and relative ratios of CuO oxidation products in standard estuarine sediments (NIST SRM 1941b) and dried fulvic acid sample (IHSS 1S101F). FS: full scan mode; MRM: multiple reaction mode. SRM1941b-low represents loadings of 0.3–0.4 mg OC in the reaction vessel (10–15 mg sediment), whereas SRM1941b+glucose corresponds to the same loading amended with 10 mg glucose. All data were produced using CIAD as a surrogate standard.

<table>
<thead>
<tr>
<th></th>
<th>SRM1941b</th>
<th>IHSS-1S101F</th>
<th>SRM1941b-Low</th>
<th>SRM1941b+glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FS</td>
<td>MRM</td>
<td>FS</td>
<td>MRM</td>
</tr>
<tr>
<td>V</td>
<td>0.12 ± 0.01</td>
<td>0.12 ± 0.00</td>
<td>0.73 ± 0.06</td>
<td>0.79 ± 0.07</td>
</tr>
<tr>
<td>S</td>
<td>0.10 ± 0.01</td>
<td>0.10 ± 0.00</td>
<td>0.58 ± 0.04</td>
<td>0.61 ± 0.05</td>
</tr>
<tr>
<td>C</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.00</td>
<td>0.26 ± 0.01</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>( \lambda )(^{8} )</td>
<td>0.24 ± 0.01</td>
<td>0.25 ± 0.00</td>
<td>1.57 ± 0.11</td>
<td>1.68 ± 0.14</td>
</tr>
<tr>
<td>( \lambda )(^{8} )</td>
<td>0.80 ± 0.04</td>
<td>0.83 ± 0.00</td>
<td>0.30 ± 0.02</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>S/V</td>
<td>0.84 ± 0.03</td>
<td>0.87 ± 0.01</td>
<td>0.80 ± 0.03</td>
<td>0.77 ± 0.02</td>
</tr>
<tr>
<td>C/V</td>
<td>0.24 ± 0.02</td>
<td>0.28 ± 0.00</td>
<td>0.36 ± 0.02</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>(( \text{Ad}/\text{Al} ))(^{v} )</td>
<td>0.41 ± 0.03</td>
<td>0.45 ± 0.00</td>
<td>1.02 ± 0.07</td>
<td>1.07 ± 0.13</td>
</tr>
<tr>
<td>(( \text{Ad}/\text{Al} ))(^{s} )</td>
<td>0.33 ± 0.03</td>
<td>0.39 ± 0.02</td>
<td>0.87 ± 0.04</td>
<td>0.99 ± 0.04</td>
</tr>
<tr>
<td>3.5 ( \text{Bd}/V )</td>
<td>0.14 ± 0.01</td>
<td>0.16 ± 0.00</td>
<td>3.39 ± 0.39</td>
<td>2.21 ± 0.25</td>
</tr>
<tr>
<td>C/\text{Fad}</td>
<td>3.08 ± 0.29</td>
<td>2.71 ± 0.00</td>
<td>5.46 ± 0.44</td>
<td>4.66 ± 0.05</td>
</tr>
</tbody>
</table>

\(^{a}\) \( \Sigma \) 8: concentrations of 8 major lignin oxidation products (V, S, and C) in mg g\(^{-1} \) dry weight.

\(^{b}\) \( \lambda \) 8: carbon-normalized yields of 8 major lignin oxidation products (V, S, and C) in mg 100 mg\(^{-1} \) OC.
(assuming no molecular O$_2$ is present in the vessel), further work is required to demonstrate what the exact stoichiometric threshold is for the production of stable LOP yields and signatures, and if indeed such threshold is constant across different matrices. To be conservative, however, one should probably try to maintain an OC loading below a 200–300:1 CuO:OC weight-to-weight ratio. In cases where this is not possible (low OC content in deep ocean waters or mineral soil horizons), amendment with lignin-free glucose can be considered to prevent substantial alteration of LOP signatures.

Finally, we tested the MRM quantitative mode on four Arctic Ocean DOM samples characterized by wide ranging LOP concentrations (Table 6). Although we present the data for all three phenol families in Table 6, we only compare the sum of vanillyl and syringyl phenols ($\Sigma$$_6$, Table 6) between both modes of quantitation. The conversion of the amino acid tyrosine to the cinammyl phenol p-coumaric acid has indeed been shown to interfere with the accurate quantification of lignin-derived cinamyl phenols in open ocean DOM samples, which are characterized by a substantially higher proportion of proteins than lignin moieties (Hernes and Benner, 2002). To avoid the potential interference of protein-derived constituents in such types of samples, the lignin totals thus only include the six vanillyl and syringyl phenols (Benner et al., 2005; Hernes and Benner, 2002; Opsahl et al., 1999).

Intermediate to deep-water samples show depleted lignin levels with values one order of magnitude below those from surface waters (55 m). These values are consistent with the few dissolved lignin data available from Arctic Ocean showing the influence of terrigenous OM inputs in Arctic Ocean surface waters (Benner et al., 2005; Opsahl et al., 1999; Walker et al., in press). Because of the nature of these samples (extracted on SPE column on the field), no replicates were available for analysis, which prevents any statistical comparison between values as in the previous discussion. However, we can suggest that parameters are comparable between the two monitoring modes if they lie within the analytical reproducibility of the method (~10%). Although the yields for syringyl phenols are comparable (within 10% of each other) across all DOM samples, some differences are observed between the two quantification modes for vanillyl phenols and all ratios involving these compounds. A large interfering peak in the vanillic acid region of the chromatogram was observed in all subsurface water samples and led to a lack of detection of this phenol under FS mode. This generated lower LOP yields and (Ad/Al)$_v$ ratios of zero for the three intermediate to deep-water samples. The analysis of these samples under MRM removed the impact of the interference and allowed the characterization of vanillic acid in all samples. The absence of interference in the surface samples was probably due to the lack of glucose amendment and/or to the high lignin yield in this sample. Under such conditions, both FS and MRM results are similar (Table 6).

### Table 6

Concentrations and relative ratios of CuO oxidation products in DOM samples extracted from surface, intermediate, and deep waters of the Arctic Ocean. All intermediate and deep ocean DOM samples were amended with 10 mg glucose: FS: full scan mode; MRM: multiple reaction mode. All data were produced using C1AD as a surrogate standard.

<table>
<thead>
<tr>
<th></th>
<th>AOSOS 44 (2800 m)</th>
<th>AOSOS 33 (2000 m)</th>
<th>AOSOS 30 (800 m)</th>
<th>AOSOS 33 (55 m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOC$^a$</td>
<td>0.59</td>
<td>0.60</td>
<td>0.77</td>
<td>1.04</td>
</tr>
<tr>
<td>V</td>
<td>49.0</td>
<td>71.1</td>
<td>80.7</td>
<td>99.2</td>
</tr>
<tr>
<td>S</td>
<td>43.2</td>
<td>41.9</td>
<td>60.9</td>
<td>56.7</td>
</tr>
<tr>
<td>L</td>
<td>39.1</td>
<td>38.0</td>
<td>10.0</td>
<td>8.0</td>
</tr>
<tr>
<td>$\Sigma$$_6$</td>
<td>92.2</td>
<td>113.1</td>
<td>141.5</td>
<td>155.9</td>
</tr>
<tr>
<td>$\Sigma$$_3$</td>
<td>15.57</td>
<td>19.23</td>
<td>23.59</td>
<td>25.89</td>
</tr>
<tr>
<td>S/V</td>
<td>0.88</td>
<td>0.59</td>
<td>0.75</td>
<td>0.57</td>
</tr>
<tr>
<td>$\Sigma$/V</td>
<td>0.80</td>
<td>0.53</td>
<td>0.12</td>
<td>0.08</td>
</tr>
<tr>
<td>(Ad/Al)$_v$</td>
<td>0.00</td>
<td>0.63</td>
<td>0.33</td>
<td>0.00</td>
</tr>
<tr>
<td>(Ad/Al)$_s$</td>
<td>0.39</td>
<td>0.41</td>
<td>0.32</td>
<td>0.33</td>
</tr>
<tr>
<td>3.5 Bd/V</td>
<td>1.59</td>
<td>1.08</td>
<td>0.91</td>
<td>0.72</td>
</tr>
<tr>
<td>C/Fl/Fd</td>
<td>0.83</td>
<td>0.89</td>
<td>1.28</td>
<td>1.42</td>
</tr>
</tbody>
</table>

$^a$ DOC: dissolved OC concentrations in mg L$^{-1}$.

$^b$ $\Sigma$ of six major lignin oxidation products (V and S) in mg L$^{-1}$.

$^c$ Ad/Al$_s$: carbon-normalized yields of 6 major lignin oxidation products (V and S) in µg 100 mg$^{-1}$ DOC.

### 3.4. Implications

About three decades ago, the late John Hedges adapted the CuO oxidation method, originally developed by the wood chemistry industry, to the quantification of phenolic by-products of lignin oxidation in environmental matrices (Hedges and Ertel, 1982; Hedges and Mann, 1979). He then applied this method to characterize inputs of terrestrial organic matter to aquatic systems (Hedges et al., 1988b, 1982), and in doing so opened up a very broad field of research which now includes studies on sources and reactivity of plant-derived OM in soils, the impact of hydrology fluctuations and land use on carbon cycling and fluvial transport, terrigenous OM export to the coastal and open ocean, mercury cycling and green house gas emissions in natural and man-made lakes, and impacts of climate change on terrestrial and aquatic systems. Despite its widespread applicability and unambiguous identification of vascular plant-derived OM, the CuO oxidation method suffers from some limitations, one of which being a relatively narrow analytical window for the characterization of terrigenous OM in complex environmental matrices (Gordon and Goñi, 2003; Klap et al., 1999; Kuo et al., 2008b). Despite the large number of compounds that have been characterized by this method (Goñi and Hedges, 1990, 1992b, 1995; Goñi et al., 2000), alterations of lignin phenylproplyl units during biological to thermal degradation (Hatcher, 1990; Hedges et al., 1988a; Klap et al., 1999; Kuo et al., 2008b; Opsahl and Benner, 1995) have the potential to remove the common lignin oxidation products from the analytical window of the CuO oxidation method suggesting a “loss” of terrigenous organic matter from the sample (Kuo et al., 2008b). This issue can be somewhat controlled through the choice of appropriate terrigenous OM end-members (i.e. soil OM instead of fresh plant materials) and some characterization of the degradation state of the terrigenous OM (Farella et al., 2001; Gordon and Goñi, 2003; Houel et al., 2006; Sánchez-García et al., 2008). In addition, this method does not provide a quantitative indication of absolute lignin content in any sample. Instead, lignin yields and signature ratios are operationally defined by the analytical conditions adopted during oxidation with temperature and the presence of oxygen bearing the most influence on the yield and proportion of individual monomers released from the complex lignin macromonomer (Hedges and Ertel, 1982; Louchouarn, 1997). Because the oxidation systems have evolved over the years to include systems with markedly different temperatures and time conditions (i.e. microwave ovens (Goñi and Montgomery, 2000)), there is a need for “standardized” materials that can be used as references to test both the variability and relative consistency of the method (in this particular case, “accuracy” should be replaced by inter-laboratory consistency). The criteria for selecting the few reference materials presented here included easy accessibility, already recognized and certified parameters (i.e. OC, N, metals, organics), as well as a range of matrices and LOP concentrations that represent the typical sources or sinks of terrigenous OM in aquatic environments (Table 1). We recognize that these materials are by no means an exhaustive representation of the potential materials reaching aquatic environments. Obvious additions would include surface and deep soil samples with wide ranging OM content as well as dissolved OM samples extracted from different aquatic systems (coastal waters, tropical vs. boreal lakes and rivers). The suite of standards presented, however, provide a starting point for the verification of LOP quantification in matrices that range from high mineral to mostly OM content (sediments vs. DOM and leaves, respectively). A similar precision
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