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HPLC Purification of Higher Plant-Dervied Lignin Phenols for Compound Specific Radiocarbon Analysis

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The ability to measure the radiocarbon content of compounds isolated from complex mixtures has begun to revolutionize our understanding of carbon transformations on earth. Because samples are often small, each new compound isolation method must be tested for background carbon contamination (C_{ex}). Here, we present a new method for compound-specific radiocarbon analysis (CSRA) of higher plant-derived lignin phenols. To test for C_{ex}, we compared the $\Delta^{14}C$ values of unprocessed lignin phenol containing standard materials (woods, leaves, natural vanillin, and synthetic vanillin) with those of lignin phenols liberated by CuO oxidation and purified by two-dimensional high-pressure liquid chromatography (HPLC) coupled to mass spectrometry (MS) and UV detection. We assessed C_{ex} associated with (1) microwave assisted CuO oxidation of bulk samples to lignin phenol monomers, (2) HPLC purification, and (3) accelerator mass spectrometry (AMS) sample preparation. The $\Delta^{14}C$ of purified compounds (corrected for C_{ex}) agreed, within error, with those of bulk materials for samples that were $>10\ \mu g$ C. This method will allow routine analysis of the $\Delta^{14}C$ of lignin phenols isolated from terrestrial, aquatic, and marine settings, revealing the time scale for the processing of one of the single largest components of active organic carbon reservoirs on earth.

Higher-plant derived organic carbon represents a significant fraction of the earth's annual primary productivity and is also an important reservoir of fixed carbon, both living and dead. Organic matter (OM) derived from higher plants in terrestrial ecosystems can be processed to soil debris or converted to carbon dioxide through respiration. Once in aquatic ecosystems, such as rivers, more biological processing occurs as plant-derived OM makes its way to the ocean. Once in the ocean, it can be further transformed in the marine dissolved and particulate organic carbon pools, buried in coastal marine sediments, or converted to carbon dioxide. Traditionally, the radiocarbon age of bulk carbon in terrestrial reservoirs is used to infer the average age of mobilized organic carbon. For example, measurements of the $\Delta^{14}C$ of bulk particulate organic carbon (POC) and dissolved organic carbon (DOC) suggest that local geology and in situ biological production determine the average age of organic matter in rivers.[1−5] Still, terrestrial carbon is a complex mixture that integrates recent biological and ancient geological carbon sources into a complex mixture of carbon-based materials at various stages of decomposition. More in-depth knowledge about the age of various carbon sources can tell us how carbon from different sources is processed.

The advent of compound-specific radiocarbon analysis (CSRA) has presented the possibility of measuring the age of individual components within complex mixtures of organic carbon.[6,7] Some researchers have used the $\Delta^{14}C$ value of long chain fatty acids in river delta sediments to apportion sources of organic matter and, therefore, to understand the fate of terrestrial carbon.[8] However, the source of long chain fatty acids can be ambiguous, because they are trace components of terrestrial biomass and are principally thought to be transported via Aeolian processes. Thus, they are not necessarily representative of the bulk terrestrial carbon pool, and their low concentration requires sample sizes that have so far limited these analyses to sample types that can be collected in large quantities such as delta sediments where carbon can reside in the upper few centimeters for long time periods.

Lignin is the second most abundant biopolymer on earth. It is an unambiguous biomarker of higher plants comprising up to 40% of their biomass through its role as a structural component of cell walls. Analysis of lignin phenols (Figure 1) following CuO oxidation of plant-containing material reveals information about

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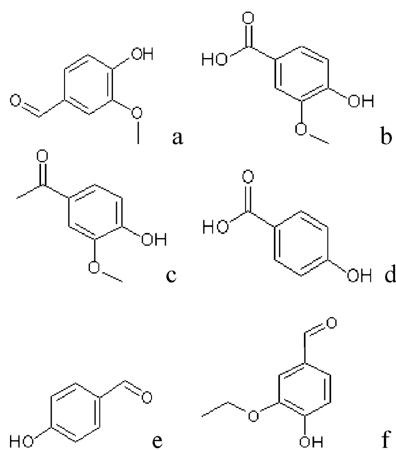

Figure 1. Structures of lignin phenols that were purified for radiocarbon analysis including (a) vanillin, (b) vanillic acid, (c) acetovanillone, (d) 4-hydroxybenzoic acid, (e) 4-hydroxybenzaldehyde, and (f) ethyl vanillin.

the source, quality, and quantity of vascular plant material.[9−14] Lignin is transported fluvially, and thus, fluxes are largely controlled by physical processes that control sediment transport.[12,15,16] All of these qualities make lignin phenols an attractive substrate to target for following terrestrial carbon from source to sink.[9]

CSRA techniques have yet to be applied to higher-plant derived lignin phenols in environmental samples, in part due to methodological challenges. McNichol et al.[17] used preparative capillary gas chromatography (PCGC) in an attempt to develop a purification method for lignin phenols. In their study, $\Delta^{14}C$ values of lignin phenols in a series of woods, lignin standards, and flavorings were compared with bulk values. They found that bulk and individual compound $\Delta^{14}C$ values agreed within 20‰, but concluded that natural sediment or water samples would present additional challenges due to the large number of interfering compounds present in natural samples and low yields of targeted compounds.[17] In addition, Ziolkowski and Druffel[18] used pure lignin phenols from flavorings as model compounds to quantify background carbon in the PCGC system in their effort to carry out CSRA of black carbon. The combination of significant advances in small-scale ^{14}C accelerator mass spectrometry (AMS) analysis,[19,20] high-pressure liquid chromatography (HPLC)-based approaches to lignin phenol analysis,[21] and HPLC-based approaches to compound purification,[22−25] suggest that an HPLC-based purification scheme for lignin phenol CSRA is feasible.

The main advantages of an HPLC-based method are that (1) lignin phenols can be purified without the need for derivitization and subsequent sample loss and correction for $\Delta^{14}C$ values of derivative carbon, (2) enhanced column capacity allows relatively large quantities to be purified with only a few injections on the HPLC, and (3) it employs multiple separation dimensions (reverse phase and normal phase separation protocols) allowing for higher purity of compounds separated from complex sample matrices.[25]

The technique presented here can be applied to many sample types including POC and DOC. Both natural and synthetic lignin phenols are used as flavorings, fragrances, and cleaning agents in foods and "green products".[26] There is active debate as to whether all of these lignin phenol-based products are synthesized from petroleum or are in fact isolated from modern plant materials. The technique presented here can be used to test the authenticity of natural products claims.

METHODS

Samples Analyzed. The greatest challenge in carrying out small scale CSRA from complex mixtures is maximizing the quantity of the compound of interest that is purified while minimizing the quantity of background carbon contamination or exogenous carbon (C_{ex}) that is added to the sample during purification and subsequent processing. Because some contribution from C_{ex} is inevitable, it is necessary to correct measured $\Delta^{14}C$ values for the contribution of C_{ex} and to propagate the error associated with the correction.[18−20] In order to assess the size and $\Delta^{14}C$ of C_{ex} associated with microwave assisted CuO oxidation and HPLC purification of lignin phenols, we carried out ^{14}C analyses of homogeneous bulk standard materials and individual compounds isolated from those bulk materials. The mathematical formulas chosen, described in detail in Santos et al.,[32] break C_{ex} into two carbon components referred to as modern carbon (MC) and dead carbon (DC). This technique for background correction is simpler compared to those of other approaches because MC and DC can be determined and verified separately.[18,20,27−29] However, to obtain reliable $\Delta^{14}C$ values of smaller "unknown" samples using this technique, they must be measured alongside independent sets of secondary materials (standards) of appropriate size and ^{14}C activity.

Using an indirect method to quantify MC and DC contributions in our purified compounds, we chose bulk materials that spanned a range of $\Delta^{14}C$ values from postbomb (∼+80‰) to radiocarbon dead (−1000‰). Bulk materials and the rational for choosing

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Table 1. Bulk Materials and Standards Used for Method Development and the Rational for Choosing Each Material

goal		sample name	rational
blank assessment	^{14}C dead set	USGS coal (USGS/Argonne premium coal)	Used to determine the modern C contamination component (MC) for combustion and graphitization background procedures (AMS sample preparation, results not shown).
		synthetic ethyl vanillin (EV)	Purchased from Aldrich (Lot #01618PC) to determine the MC contamination component from HPLC and AMS sample preparation. It was also used to produce mixed samples with modern vanillin (MV) to verify HPLC purification capabilities. Bulk material was also analyzed to determine its precise Δ^{14}C value (Δ^{14}C = −1000.5 ± 0.4‰; Table S-1, Supporting Information).
		"Patagonia" spruce wood (PW)	^{14}C-free wood originally donated by S. Stine to KCCAMS/UCI. Although it is commonly called Patagonia wood, it is not from Patagonia, since no spruce species grow in South America. It was used as a blank to determine the MC component from microwave, HPLC, and AMS sample preparation steps. Bulk material was A/B/A chemically pretreated and analyzed to determine its precise Δ^{14}C value (Δ^{14}C = −998.1 ± 0.4‰; Table S-1, Supporting Information).
	^{14}C modern set	OX-I and OX-II (oxalic acid I and II)	Used as ^{14}C-modern blank to determine the dead C contamination component (DC) for combustion and graphitization procedures and for normalization of all samples (results not shown).
		modern vanillin (MV)	^{14}C-modern material purchased from SAFC Supply Solutions (Lot #05930KC) to determine the DC component from microwave, HPLC blank, and AMS blanks. It was also used to produce mixed samples with ethyl vanillin (EV) to verify HPLC purification capabilities (Δ^{14}C = 81.5 ± 2.3‰; Table 2, Supporting Information).
		Pacific Northwest (PNW) leave	Pacific Northwest (PNW) leaves collected in March 2009 in Seattle, WA. Bulk material was A/B/A chemically pretreated and analyzed (Δ^{14}C = 35.5 ± 2.3‰; Table S-2, Supporting Information.
accuracy tests	^{14}C known value	IAEA-C7 and C8 oxalic acids	Oxalic acids of intermediate ^{14}C values (49.53 and 15.03 pMC, respectively) were produced by Groningen Laboratory.[35] They were used to evaluate accuracy of ^{14}C results from small samples that undergo AMS sample preparation only (results not shown).
		IAEA-C5 wood	Subfossil wood from the IAEA ^{14}C Intercomparison Exercise 1990 (consensus value = 23.05 pMC; equivalent to a Δ^{14}C of −769.5).[36] It was used to evaluate accuracy of ^{14}C results from the CSRA method described in this study (Δ^{14}C value = −770.8 ± 1.1‰; Table S-2, Supporting Information).

them in this work are summarized in Table 1. We measured the ^{14}C content of lignin phenols purified from Patagonia wood including vanillin, vanillic acid, acetovanillone, 4-hydroxybenzoic acid, and 4-hydroxybenzaldehyde; from IAEA-C5 wood including vanillin, vanillic acid, and acetovanillone; from Pacific Northwest (PNW) leaves including 4-hydroxybenzaldehyde. We measured the ^{14}C content of a bulk modern vanillin (MV) standard and then measured this standard after HPLC purification as well as after microwave digestion and HPLC purification. We mixed modern vanillin with synthetic ethyl vanillin (Table 2) to determine how effectively two components of widely variable ^{14}C content could be separated. We also used a direct method to assess the size and Δ^{14}C value of C_{ex} associated with HPLC purification by measuring a solvent carrier without sample (sample identification NP solvent; Table S-1, Supporting Information). Since the sample was produced by a large number of injections (15 injections in which a total of 26 mL were collected by the fraction collector), in order to compare the size of the HPLC MC and DC blank obtained using the indirect method, we normalized the amount of C_{ex} quantified to the volume of HPLC effluent collected, assuming that this carbon contribution is mostly from column bleed that is directly correlated with the volume of solvent.[20,25] Finally, one unknown "artificial vanilla" flavoring was purchased at a supermarket (Kroger brand), and ethyl vanillin and vanillin were purified from this sample to determine the origin of the vanilla flavoring. Here, we report the method for purification of these compounds and the resulting uncorrected and corrected Δ^{14}C values of the purified compounds.

To minimize C_{ex}, samples are only allowed to contact glass cleaned by baking in an oven at 500 °C for 5 h to remove organic contaminants. The only exception is the microwave step that uses Teflon vessels followed by centrifugation in

Table 2. Amount of Radiocarbon Dead ($\Delta^{14}C = -1000‰$) and Modern[b] ($\Delta^{14}C = 0‰$) Exogenous Carbon (C_{ex}) Introduced during Each Sample Preparation Step[a]

sample preparation step	samples	dead contamination (μg C)	modern contamination (μg C)
AMS (indirectly)	USGS coal, OX-I and -II	0.35 ± 0.18	0.35 ± 0.18
microwave (indirectly)	PW and MV	0.80 ± 0.40	0
HPLC (indirectly)	EV and MV	0.65 ± 0.33	0
HPLC (directly)	NP solvent	~0.64	~0.04[c]
total (ΣC_{AMS}, $C_{chemistry}$, C_{HPLC})		1.80 ± 0.90	0.35 ± 0.18

[a] An uncertainty of ±50% was assumed for the C_{ex} introduced during individual sample processing steps based on variability in blank values. [b] The term "modern" is used loosely here to refer to near contemporary $\Delta^{14}C$ values. [c] These values were obtained from sample size (4.1 μgC) and ^{14}C ratio to normalizing standard (0.1289; not shown in Table 2) and then normalized to the number of injections necessary to produce this sample. Note that the C_{HPLC} assessed indirectly and directly are indistinguishable.

polycarbonate centrifuge tubes and HPLC system. Teflon microwave vessels, connector tubing, and polycarbonate centrifuge tubes are acid-washed overnight and then rinsed with distilled water. Immediately prior to use, the vessels and tubes are rinsed with dichloromethane and methanol. The entire HPLC system, including the fraction collector, is extensively flushed with fresh solvent of the highest purity between sample types to eliminate memory from prior sample injections and leaching from HPLC components. We do this by making injections of pure solvent just prior to sample purification with all HPLC settings exactly as they are during sample purification.

CuO Oxidation of Lignin and Quantification of Lignin Phenols. Because lignin phenols exist in plants as complex polymers, the first step of the method is to liberate lignin-derived phenols from their polymers by CuO oxidation. Individual lignin phenols in each sample were quantified[30] to determine AMS sample size requirements. Briefly, freeze-dried and homogenized samples were transferred to Teflon microwave vessels containing CuO powder and ferrous ammonium sulfate. After adding 2 N NaOH, the samples were subjected to oxidative hydrolysis by CuO in a microwave. The microwave held sample temperatures at 144 °C for 90 min, with an optimal pressure ranging between 45 and 70 PSI, depending on the number of microwave vessels used. Upon completion, the samples were acidified and extracted in ethyl acetate. Resulting lignin phenols were quantified by gas chromatography flame ionization detection (GC-FID) after derivitization with trimethylsilyl ethers and esters using bis(trimethyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS). For samples destined for CSRA, batch oxidations were needed. Because each Teflon vessel is optimized for 3−5 mg of organic carbon, six Teflon vessels containing a given sample type were microwaved and pooled in order to obtain enough material. This means that the size of C_{ex} associated with the Teflon vessels should increase with the number of vessels used.

HPLC Purification. Purification of lignin-derived phenols was carried out using a reverse phase chromatography purification step followed by a normal phase purification step. For reverse phase,[21] typically between 2 and 5 injections were required to obtain adequate material from potentially complex sample matrices, although 15 injections were used for the PNW leaves. In normal phase, individual compounds were purified from between 2 and 5 injections of this relatively simple sample matrix. It is important to minimize variability in the number of injections and total volume of effluent collected from sample to sample so that any C_{ex} from the HPLC effluent and subsequent sample processing is the same for all samples and standards. Impurities that may be introduced during reverse phase purification are likely removed during normal phase purification, so it is most important for the volume of normal phase effluent collected to be constant.

For reverse phase HPLC analysis, ethyl acetate extracted CuO oxidation products were dried down under a gentle stream of nitrogen at 45 °C and then dissolved in a 1:1 solution of 7.4 mM phosphoric acid in water and methanol. Samples were filtered through a 0.45 μm PTFE filter prior to injection on an Agilent 1100 HPLC. The HPLC was equipped with a Zorbax Eclipse XDB-C18 column (4.6 × 150 mm; 5 μm pore size). Lignin phenols were eluted from the column using a gradient program of 7.4 mM phosphoric acid in water (Solvent A) and 4:4:3 by volume 7.4 mM phosphoric acid/methanol/acetonitrile (Solvent B). The column was held at 55 °C, and the initial conditions were 5% Solvent B at a flow rate of 0.8 mL/min. The gradient program ramped to 10% Solvent B by 2 min, 51% Solvent B by 25 min, and 100% Solvent B by 26 min, and was held for 2 min at 100% solvent B, and then, the column was re-equilibrated in 5% Solvent B for 10 min between injections. Lignin phenols were detected using a diode array detector (DAD) set to monitor 280 nm, and individual lignin phenols were identified using an Agilent XCT ion trap electrospray ionization-mass spectrometer (ESI-MS). The MS scanned from 75 to 450 m/z, and the spray chamber conditions were as follows: nebulizer pressure, 60 psi; dry gas, 7 L/min; drying temperature, 350 °C. During purification of lignin phenols, only the DAD was used to detect compounds and the effluent was collected with a fraction collector using time-based fraction collection in which the fraction collector was programmed to collect from the beginning to the end of the time interval over which each UV peak eluted. When peaks were baseline separated, additional fractions were collected before and after each peak to avoid sample loss in the event that peak retention times varied. Every attempt was made to collect the entire peak so that if isotope fractionation occurs during chromatography our samples would not be affected. In some cases, collected fractions were reinjected in order to verify that lignin phenols were only present in the expected fractions.

Like fractions from multiple reverse phase injections were pooled either by hand or by the fraction collector and then either dried under a gentle stream of nitrogen at 45 °C or extracted into ethyl acetate and then dried under N_2 at 45 °C. Evaporation from ethyl acetate is recommended because it avoids lignin phenol evaporation during sample drying from less volatile solvents (see Results and Discussion below). Pooled, dried fractions were dissolved in 50%

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isopropyl alcohol (IPA) in hexane (v/v) for normal phase chromatography. In normal phase, peaks were detected by DAD only since the lignin phenols did not ionize well under the conditions employed. The HPLC was equipped with a LiChrospher Diol column (4.6 × 150 mm, 5 μm) held at 45 °C. Lignin phenols were eluted from the column using a gradient program of 1% IPA in hexane (Solvent A) and 1:1 IPA/hexane (Solvent B) and a flow rate of 0.2 mL/min. The gradient ramped from 5% Solvent B (initial conditions) to 45% Solvent B by 30 min and to 100% Solvent B by 31 min, where it was held for 2 min. The column was re-equilibrated in 5% Solvent B for 10 min between injections at a flow rate of 0.5 mL/min. The fraction collector was programmed for time-based collection. Like fractions from between 2 and 5 injections were pooled, and the volume was reduced to ~0.5 mL under a gentle stream of N_2 at 45 °C and then transferred into a quartz tube (6 mm o.d.). The vial was then rinsed 3× with methanol to ensure complete transfer to the quartz tube. Quartz tubes are precombusted (850 °C, 5 h) the night before they are used. To minimize surface area carbon contamination, they are kept in a "wet" box in the presence of 1 N NaOH vapor (to trap carbon from the environment). The remaining solvent was completely evaporated from the quartz tube. Due to the volatility of lignin phenols, overdrying results in significant sample loss.[26]

Radiocarbon Analysis by Accelerator Mass Spectrometry. Bulk samples of wood and leaves were acid−base−acid (A/B/A) washed to remove any labile carbon. Bulk vanillin and ethyl vanillin were weighed and transferred directly into prebaked quartz tubes and 60 mg of CuO and 15 mg of silver wire were added (these reagents were also prebaked before use). Quartz tubes containing samples were evacuated on a vacuum line after immersion in a slurry of IPA and dry ice (−78 °C) for 1 min (to avoid sample loss from volatilization in the vacuum line). The tubes were then evacuated for 3 min and flame-sealed while still being cooled in the slurry. Sealed tubes were combusted at 850 °C for 5 h. The CO_2 from combustion was cryogenically cleaned, quantified manometrically, trapped, sealed in Pyrex tubes (6 mm o.d.), and then sent to the Keck Carbon Cycle AMS Facility (KCCAMS) at University of California Irvine (UCI) for further analyses. Encapsulated clean CO_2 was converted to graphite in small sets of 12 targets according to standard procedures.[31] Samples that yielded <0.1 mgC were graphitized in smaller graphitization reactors, designed especially to handle small and ultrasmall samples.[32] A total of 73 samples (as CO_2) ranging in size from 0.0009 to 1 mgC were graphitized and analyzed.

Final $\Delta^{14}C$ values must be corrected for mass-dependent fractionation. This fractionation is determined from the measured $\delta^{13}C$ value of a sample. At the KCCAMS/UCI, the AMS has the capability to measure all the three carbon isotopes. Thus, each $\Delta^{14}C$ value reported is corrected for isotopic fractionation using its own online $\delta^{13}C$ value. Correction with online $\delta^{13}C$ values is essential when measuring ultrasmall samples to account for spectrometer-induced isotopic fractionation.[33]

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RESULTS AND DISCUSSION

A purification method that is appropriate for CSRA must obtain pure compounds from mixtures while minimizing contamination. The separation method described uses reverse phase chromatography to obtain semipure fractions of lignin phenols. The subsequent normal phase purification of these fractions provides highly pure single lignin phenols (Figure 2). Care must be taken to ensure that solvents are thoroughly removed because they are the most common known source of C_{ex} contamination.[6,33,34]

Purified compounds on which $\Delta^{14}C$ values are measured ($\Delta^{14}C_{measured}$) contain carbon from several sources including: carbon from the sample or compound of interest (C_{sample}), carbon introduced during chemical processing such as the microwave ($C_{chemistry}$), carbon introduced during HPLC purification (C_{HPLC}), and carbon introduced during AMS sample preparation, e.g., combustion, graphitization, and graphite target handling (C_{AMS}). The sum of $C_{chemistry} + C_{HPLC} + C_{AMS}$ is equal to the total exogenous carbon (C_{exT}) introduced. Each sample processing protocol and lab instrument that comes in contact with samples to be measured have distinct C_{exT}, and so adaptation of any CSRA method in a new lab requires testing for the size and ^{14}C signature (age) of C_{exT}. The approach presented here allows any lab to determine C_{exT} values specific to their lab/instruments/protocols.

C_{exT} is always a mixture of carbon sources that range in their ^{14}C content from modern (MC) to dead (DC). For conventional ^{14}C-AMS sample processing, DC is usually smaller than MC and becomes detectable only when sample sizes are very small ($\ll$0.100 mgC).[33] It is difficult to directly measure the size and $\Delta^{14}C$ value of C_{exT} and to determine which step(s) add MC and DC. Santos et al.[32] showed that the masses of MC and DC contaminants are generally constant for a given lab carrying out a particular protocol. In this approach, a mass balance correction is used to account for deviation of a measured $\Delta^{14}C$ value from the true value of ^{14}C-free blanks and standards. This same correction is then applied to correct $\Delta^{14}C$ values of unknown samples produced using the same protocol. In our case, to determine the C_{exT} from lignin phenol purification, we measured small aliquots of standards or bulk materials whose $\Delta^{14}C$ values were known a priori (Table 1) and treated these samples as unknowns.

Materials that have a ^{14}C-dead signal are mostly affected by the MC component of C_{exT}, e.g., the uncorrected $\Delta^{14}C$ values increase systematically as sample sizes decrease. Likewise, materials that have a ^{14}C modern or near-modern signal are mostly affected by DC contamination and, consequently, their associated uncorrected $^{14}C/C$ ratios decrease systematically as sample sizes decrease.[32,33] One way to represent this is to plot the deviations of the uncorrected $\Delta^{14}C$ values of purified compounds from those of the 1 mgC bulk materials/standards ($\Delta\Delta^{14}C$) versus sample sizes (Figure 3 and 4). Deviations from this expected trend are apparent with very small sample sizes (<10 μgC) and in samples that are very old and, therefore, unaffected by DC contamination.

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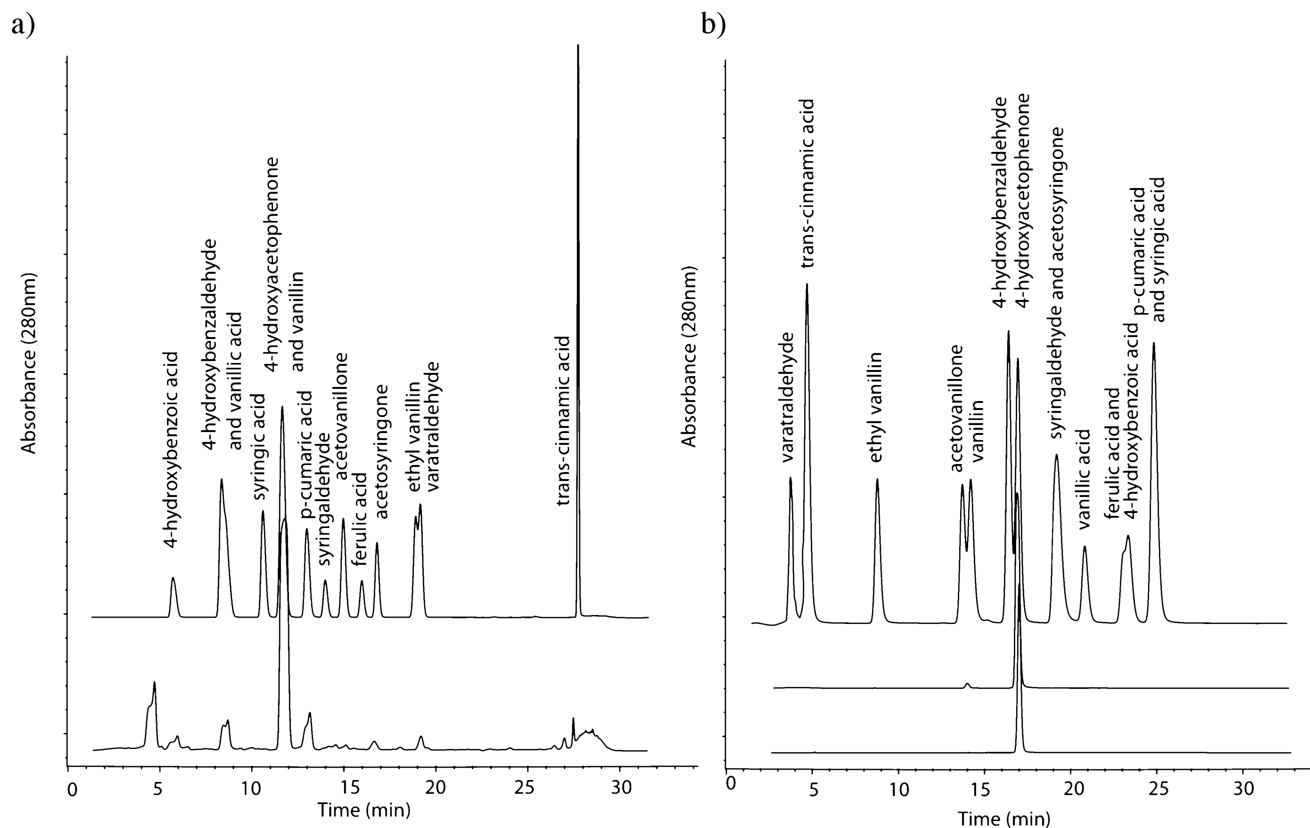

Figure 2. High-pressure liquid chromatography traces showing UV absorption at 280 nm: (a) reverse phase chromatogram of lignin phenol standard mixture (upper) and CuO oxidation products of PNW leaves (lower); (b) normal phase chromatogram of lignin phenol standard mixture (upper), 4-hydroxyacetophenone and vanillin from PNW leaves after reverse phase purification (middle), and 4-hydroxyacetophenone from PNW leaves after normal phase purification (lower).

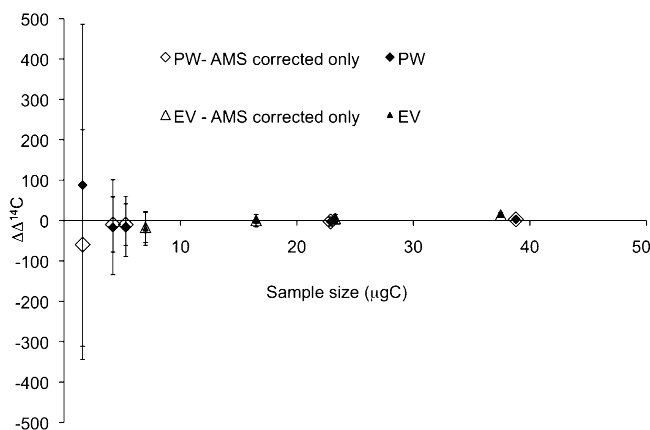

Figure 3. Δ^{14}C values of processed samples minus those of bulk samples ($\Delta\Delta^{14}$C corrected for C_{ex}) from ^{14}C-free samples. Open symbols are corrected for combustion, graphitization, and graphite target handling only (C_{AMS}), and filled symbols are corrected for $C_{chemistry}$ and/or C_{HPLC} as well as C_{AMS}. All data were corrected for isotopic fractionation with online δ^{13}C-AMS values as stated in the text. Samples containing <2 μgC are plotted here and shown in italics in Tables S-1 and S-2 (Supporting Information) for illustration only. The Δ^{14}C values of these samples are not meaningful because the mass of the total blank correction applied is bigger than the mass of the samples, as mentioned in the text.

By selecting the appropriate set of bulk materials/standards (Table 1) and by repeatedly carrying them through various stages of sample preparation, the Δ^{14}C value for each component of C_{exT} (e.g., $C_{chemistry}$, C_{HPLC}, and C_{AMS}) can be assessed (Table 2). Repeated measurements of this type can lead to an understanding of the variability in C_{exT} over time. Batches of these materials prepared in our laboratories over the course of 9 months yielded very similar values for the size and ^{14}C signature of C_{exT} components. In this work, we chose to report radiocarbon analyses as Δ^{14}C (‰) values (Table S-1 and S-2, Supporting Information).

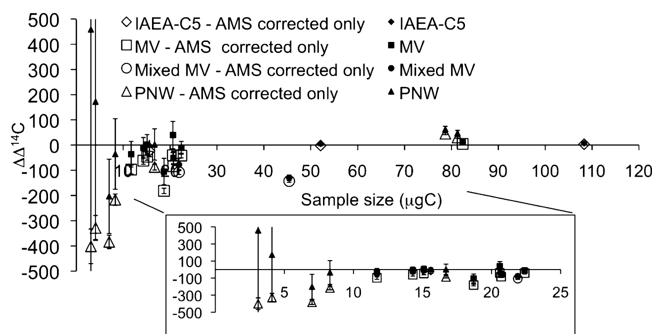

Figure 4. Δ^{14}C values of processed samples minus those of bulk samples ($\Delta\Delta^{14}$C corrected for C_{ex}) from ^{14}C-modern or near modern and subfossil samples. Open symbols are corrected for combustion, graphitization, and graphite target handling only (C_{AMS}), and filled symbols are corrected for $C_{chemistry}$ and/or C_{HPLC} as well as C_{AMS}. All data were corrected for isotopic fractionation with online δ^{13}C-AMS values as stated in the text. Samples containing <2 μgC are plotted here and shown in italics in Tables S-1 and S-2 (Supporting Information) for illustration only. The Δ^{14}C values of these samples are not meaningful because the mass of the total blank correction applied is bigger than the mass of the samples, as mentioned in the text.

C_{ex} from AMS Sample Preparation (C_{AMS}). To assess C_{AMS}, we used standards that ranged from radiocarbon dead to modern (i.e., USGS coal, IAEA-C7 and -C8, OX-I and OX-II; Table 2). All samples were prepared for combustion in the same fashion so that C_{AMS} is similar for all samples and standards processed within assigned error bars. For example, using the same size quartz tube and the same amount of CuO and Ag wire for each sample, the CO_2 gas produced is exposed to the same surface area and contribution from CuO and Ag. By comparing the measured $\Delta^{14}C$ values of purified compounds with consensus values from blanks and standards (such as, USGS coal, small aliquots of OX-I and OX-II, and IAEA-C7 and -C8), we are able to quantify that for combustion in the Ingalls lab and graphitization in the Keck AMS lab, 0.35 μg of MC and 0.35 μg of DC are introduced during the AMS sample preparation (Table 2; $\Delta^{14}C$ values of these samples are not shown in Tables S-1 and S-2, Supporting Information). Note that C_{AMS} may vary among batches. Therefore, these standards were quantified on multiple occasions in multiple batches, and the amount of modern and dead C contamination was consistent across time and sample types.

HPLC Separation. In the first chromatographic dimension (reverse phase), phosphoric acid is used to modify the mobile phase. High purity volatile organic solvents are used in normal phase. This 2-dimensional approach is needed for several reasons.[25] First, several lignin phenols either coelute or are not baseline separated by reverse-phase chromatography (see Figure 2). Second, even in a leaf sample that is likely to be significantly less complex than a soil or sediment sample, the baseline contains numerous small peaks of unknown origin. This complexity suggests that it is likely that unknown components also coelute with lignin phenols (Figure 2). The order of lignin phenol elution is different in the two phases, and compounds that coelute in reverse phase are baseline separated in normal phase. For example, 4-hydroxyacetophenone and vanillin coelute in reverse phase, but are well separated in normal phase allowing complete purification of these compounds with a two-dimensional approach (Figure 2). The likelihood of a contaminant compound having the exact same chromatographic behavior as a lignin phenol in both reverse phase and normal phase is remote. Examination of fractions containing compounds purified from standard materials shows that they are >99% pure (Figure 2).

As with any CSRA method, care must be taken to limit loss of compounds of interest during sample processing. Our largest losses of compounds are due to the volatile nature of lignin phenols. Some compound purification runs resulted in extremely small sample sizes (0.9−10 μg C; Table S-1 and S-2, Supporting Information). During the initial development of this method, we took fractions from the reverse phase separation and dried down the water/methanol/acetonitrile and phosphoric acid mobile phase under a stream of N_2 at 45 °C. We later determined that this step resulted in extremely low sample yields due to evaporation of lignin phenols. Subsequently, lignin phenols collected in reverse phase were extracted into ethyl acetate and then dried. The increased volatility of ethyl acetate relative to aqueous solvents allowed their complete vaporization with much less sample loss. For samples extracted into ethyl acetate prior to drying, our overall recovery is ~50% of the original lignin phenol. Solvent dry down is still the most likely source of sample loss and should be monitored carefully.[26] Because the KCCAMS spectrometer measures ^{14}C, ^{13}C, and ^{12}C (as mentioned earlier), we can correct for any isotope fractionation that might occur during evaporation.

In this study, we focused on the most abundant lignin phenols in wood and leaf samples (vanillin, vanillic acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, 4-hydroxyacetophenone, and acetovanillone) and two purchased lignin phenol standards (ethyl vanillin (EV) and vanillin from a modern plant source (MV); Table 1). We assessed the influence of C_{HPLC} by subjecting bulk materials (EV and MV) and wood and leaf samples of known ^{14}C ages to HPLC purification. The difference in $\Delta^{14}C$ values between bulk and HPLC purified standards is a result of the addition of C_{HPLC}. Likewise, the difference in $\Delta^{14}C$ values between bulk wood and leaf samples and compounds purified from these materials represents $C_{chemistry}$ plus C_{HPLC}. Note that both bulk and purified materials are affected by similar C_{AMS} contamination. The observed $\Delta^{14}C$ value of −1000.5 ± 0.4‰ (n = 2) for bulk synthetic ethyl vanillin (Table S-1, Supporting Information) agrees with the expectation based on the likely petroleum source of this xenobiotic. The observed modern or postbomb $\Delta^{14}C$ value of +81.5 ± 2.3‰ (n = 2) for vanillin supports a modern source, likely from wood pulp (Table S-2, Supporting Information). The $\Delta^{14}C$ value of the HPLC-purified modern vanillin (MV) demonstrates that a mass balance correction of 0.65 μg of C with a $\Delta^{14}C$ value of −1000‰ is needed to correct $\Delta^{14}C$ values for C_{HPLC}. A similar mass for this C_{ex} was quantified directly by measuring the carbon obtained from combustion of a process blank in which a solvent carrier (without sample) was taken through the HPLC purification procedure (after proper normalization for the volume of effluent collected; Table S-1, Supporting Information, and Table 2). Analysis of the PNW leaves confirms that ~0.8 μg C with a $\Delta^{14}C$ value of −1000‰ is needed to correct these samples for the microwave step ($C_{chemistry}$) (Table S-2, Supporting Information, and Table 2).

When we mixed MV and EV together (1:1 molar masses) and purified them from the mixture, the $\Delta^{14}C$ value of the purified MV after correction for C_{HPLC} was depleted (replicates were 4 ± 24‰ and −48 ± 11‰; Table S-2, Supporting Information) relative to those that had not been mixed prior to purification (+66 ± 24‰; n = 6) and bulk MV values (+81.5 ± 2.3‰; n = 2). Examination of the HPLC chromatograms of the two source materials prior to mixing revealed that there was a small amount of vanillin in the synthetic ethyl vanillin. We hypothesize that the vanillin in the ethyl vanillin standard was of petroleum origin and was, therefore, the source of ^{14}C-free contamination to the vanillin purified from the mixture of the two.

The artificial vanilla extract (Kroger brand) contained a mix of synthetic ethyl vanillin (35%) and vanillin (65%). The vanillin had a negative ^{14}C age ($\Delta^{14}C$ = +168.4 ± 1.5‰), suggesting its source is wood that contains bomb carbon and is less than several decades old. The ethyl vanillin had a $\Delta^{14}C$ value of −957.7 ± 2.1‰ prior to C_{HPLC} correction, suggesting a petroleum source. Apparently, this manufacturer of artificial vanilla flavoring is mixing modern and petroleum-based starting materials to make their flavoring. Although there are no

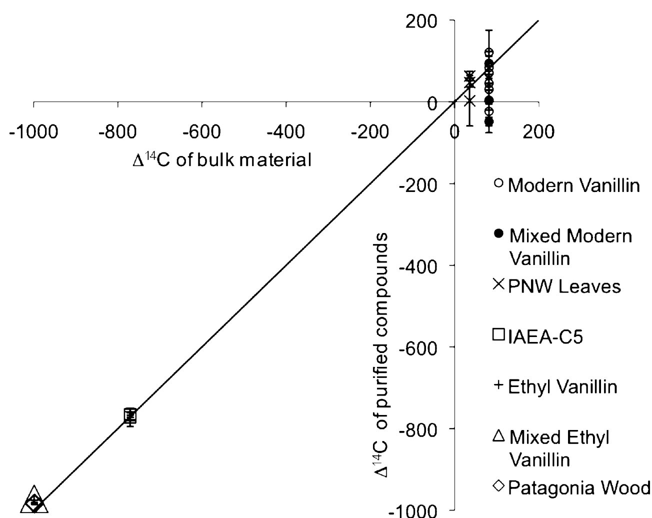

Figure 5. Δ^{14}C values of bulk materials and lignin phenols purified from bulk materials. All bulk values are corrected for combustion and graphitization. All purified compounds are corrected for HPLC and chemistry when appropriate. All data were corrected for isotopic fractionation with online δ^{13}C-AMS values as stated in the text. The line shows the 1:1 ratio that is expected if all purified compounds agree exactly with bulk values.

consensus values for this unknown sample, the large difference in the Δ^{14}C values of vanillin and ethyl vanillin purified from the artificial vanilla confirms that vastly different Δ^{14}C signatures can be obtained from a mixture.

Microwave Digestion. In order to assess the contribution of $C_{chemistry}$ (CuO oxidation and sample extraction and drying) to wood and leaf samples, we subjected natural vanillin to HPLC purification alone and also to microwave digestion followed by HPLC purification. The difference between the Δ^{14}C values of these two sample preparation methods represents the contribution from $C_{chemistry}$. A correction of 0.8 μg of carbon with a Δ^{14}C value of -1000‰ is required to make the Δ^{14}C value of the microwaved and HPLC-purified modern vanillin agree with the HPLC-purified vanillin that was not microwaved (Table S-2, Supporting Information). This result does not require that the entire $C_{chemistry}$ is in fact radiocarbon dead but only that it can be accounted for using this correction. The actual amount of $C_{chemistry}$ could be greater and have a less negative Δ^{14}C value. The most likely source of this carbon is sample carryover and leaching in the microwave vessels and polycarbonate centrifuge tubes. These containers cannot be baked at 500 °C prior to use and traces of lignin phenols and other sample components may remain in the cups and be freed during the microwave process. In the future, we will attempt to minimize this source of C_{ex} by cleaning Teflon vessels as well as possible and using glass centrifuge tubes instead of polycarbonate tubes.

Agreement of Δ^{14}C Values of Processed Materials with Bulk Values and Sample Size Limitation. The Δ^{14}C values of lignin phenols purified from MV, Pacific NW leaves, and IAEA-C5 wood generally agreed with bulk consensus values after proper background corrections for C_{ex} were applied (Table S-2, Supporting Information, and Figure 5). This agreement was best for samples that were >80 μgC (the difference between corrected and uncorrected values are <6‰, demonstrating that the introduction of the dead C contamination prior to AMS sample preparation is negligible; Figure 4 and Table S-2, Supporting Information). For sample sizes between 10 and 80 μgC, evaluation of the background signal associated with full sample processing is necessary in order to correct final results and determine the appropriate error. This mass balance correction is done such that all processed small samples are forced to agree with consensus values for bulk samples/standards within $\pm 2\sigma$ (Figures 3 and 4). Additionally, note that, in this size range, the Δ^{14}C values of lignin phenols purified from IAEA-C5 wood (a standard of intermediate radiocarbon content; Table 1) agreed with the consensus value (Figure 4), because most of the exogenous carbon introduced by our method is from a DC source (Table 2). As a consequence, DC contamination from this method should be even less evident in future ^{14}C analyses of unknown lignin phenols with Δ^{14}C values similar to IAEA-C5 or even more depleted (older).

While the errors on samples smaller than 10 μgC often overlapped with the consensus value for that material, the error bars are so large that the values are not meaningful (Figures 3 and 4). These results suggest that sample sizes of lignin phenols purified using this protocol that are smaller than 10 μg are not suitable for Δ^{14}C analysis. Note that MC and DC values quantified as shown in Table 2 were used to mass balance correct all Δ^{14}C values shown in Tables S-1 and S-2 (Supporting Information), including the small aliquots produced from IAEA-C7 and -C8 (AMS sample processing only, results not shown) and IAEA-C5 (full procedure, Table S-2, Supporting Information, and Figure 4). Therefore, we were able to verify the accuracy of our method as well as the background calculations applied. A comparison of Δ^{14}C values of bulk and purified compounds suggests good agreement between the two and that the more depleted the Δ^{14}C value, the closer the agreement between the bulk and compound results (Figure 5).

The method developed here demonstrates that samples can be prepared for CSRA of individual lignin phenols with carbon contamination from "modern" (0.35 ± 0.18 μg; from AMS sample preparation only) and "dead" (1.8 ± 0.9 μg; $C_{chemistry}$, C_{HPLC} and C_{AMS}) sources. When properly characterized using a series of bulk materials and standards with a range of sizes and Δ^{14}C values, this C_{exT} can be corrected for in samples as small as 10 μgC. The overall yield of the two-dimensional HPLC purification procedure is ~50%, provided that loss during solvent evaporation steps is minimized. The method successfully separated lignin phenols from both modern and dead sources in the same sample. One unknown sample of artificial vanilla flavoring demonstrated that it contained a mixture of vanillin from modern sources (likely wood pulp) with ethyl vanillin from petroleum sources.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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