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Comparative Analysis of Cellulose Preparation Techniques for Use with ^{13}C, ^{14}C, and ^{18}O Isotopic Measurements

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A number of operationally defined methods exist for pretreating plant tissues in order to measure C, N, and O isotopes. Because these isotope measurements are used to infer information about environmental conditions that existed at the time of tissue growth, it is important that these pretreatments remove compounds that may have exchanged isotopes or have been synthesized after the original formation of these tissues. In stable isotope studies, many pretreatment methods focus on isolating "cellulose" from the bulk tissue sample because cellulose does not exchange C and O isotopes after original synthesis. We investigated the efficacy of three commonly applied pretreatment methods, the Brendel method and two variants of the Brendel method, the Jayme−Wise method and successive acid/base/acid washes, for use on three tissue types (wood, leaves, roots). We then compared the effect of each method on C and O isotope composition (^{13}C, ^{14}C, ^{18}O), C and N content, and chemical composition of the residue produced (using ^{13}C nuclear magnetic resonance (NMR)). Our results raised concerns over use of the Brendel method as published, as it both added C and N to the sample and left a residue that contains remnant lipids and waxes. Furthermore, this method resulted in ^{18}O values that are enriched relative to the other methods. Modifying the Brendel method by adding a NaOH step (wash) solved many of these problems. We also found that processed residues vary by tissue type. For wood and root tissues, the ^{13}C NMR spectra and the ^{18}O and ^{13}C data showed only small differences between residues for the Jayme−Wise and modified Brendel methods. However, for leaf tissue, ^{13}C NMR data showed that Jayme−Wise pretreatments produced residues that are more chemically similar to cellulose than the other methods. The acid/base/acid washing method generated ^{13}C NMR spectra with incomplete removal of lignin for all tissues tested and both isotopic, and ^{13}C NMR results confirmed that this method should not be used if purified cellulose is desired.

Cellulose is the main constituent of plant cell walls and of the woody (xylem) tissues involved in water transport through trees and shrubs.[1] It also constitutes the majority of the plant fiber in animal diets and is the primary component of annual growth rings of trees.[2,3] Cellulose is a long-chain carbon-based polymer, composed of repeating β-1,4-anhydroglucose units. Once formed, the carbon, and oxygen atoms contained within the main cyclic ring of cellulose do not exchange with atoms in water or the myriad other compounds interacting with plant cells.[4−6] Because of this stability, the isotopes of cellulose keep a "record" of a host of different physiological and environmental signals and investigators have sought analytical methods to purify cellulose from other plant components.

To date, commonly applied methods are not completely successful at producing pure cellulose. The remaining residues always contain monosaccharide building units and other trace impurities such as lignin and other plant secondary chemicals.[7] Over the last century of research, several operationally defined

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procedures that leave a residue with properties close to pure cellulose have emerged. For example, one procedure involving chlorination and extraction with either sulfite or solvent[8] leaves a residue that is referred to as holocellulose. The portion of holocellulose that is insoluble in 17.5% solution of sodium hydroxide is referred to as α-cellulose. The fraction of holocellulose that dissolves in 17.5% NaOH solution is known as hemicellulose, which is composed of polysaccharides that are actually not cellulose at all. The misnomer stems from research by Cross and Bevan in the 1930s, who defined the compounds remaining in the NaOH solution as β- and γ-cellulose depending on whether they precipitated upon acidification (see ref 8).

Historically, most isotopic studies of plant tissues have concentrated on the analysis of wood, largely from tree rings. Therefore, the operationally defined methods to purify cellulose have been developed for wood as opposed to leaf or root tissues. More recently, however, leaf and root tissues, which vary considerably in chemical composition relative to wood, are also being used in isotope-based studies that require cellulose purification (e.g., refs 6 and 9−13). Researchers are often uncertain about how to pretreat these samples to remove compounds that may have undergone isotopic exchange or that formed after the synthesis of the original structural tissue.

Our study was focused on the isotopes of O and C (both ^{13}C and ^{14}C) because they have been widely used in a range of plant ecophysiological[13] and paleoecological studies[47] as integrators of plant function and recorders of the environments in which the plants grew. The ^{18}O content of wood, which varies with source water, is often used to infer changes in hydrologic regimes and thus past climate.[14] The ^{13}C content of tree rings varies with the amount of water stress to which the plant has been subjected and is typically used to infer plant growth responses to changing environmental conditions.[15,16] ^{14}C isotopes are used on prehistoric wood samples to determine the concentration of ^{14}C of CO_2 in the atmosphere. Additionally, use of the "bomb-^{14}C" technique, for samples that grew after 1950, allows dating of the year structural tissue was formed based on the ^{14}C signature of CO_2 fixed from the atmosphere.[17,18] This method also has many applications in global C cycle research.[19] The bomb-^{14}C approach is possible because thermonuclear weapons testing (primarily in the early 1960s) nearly doubled the global concentration of atmospheric ^{14}C of CO_2, which peaked in 1964, and has been decreasing at a known rate since that time.[17,19]

In this study, we examined three popular tissue pretreatment methods: (1) the Jayme−Wise pretreatment method,[8,20] (2) the Brendel method[21] and two variants, and (3) a method that uses a sequence of acid/base/acid washes. The Jayme−Wise method starts with a nonpolar solvent extraction followed by bleaching in sodium hypochlorite to isolate holocellulose. Thereafter, an additional step using NaOH isolates α-cellulose.[8,20] The Brendel method uses an acetic acid/nitric acid mixture to simultaneously remove lignin and noncellulose polysaccharides thereby leaving only α-cellulose.[21] The acid/base/acid method, a procedure used principally in radiocarbon analyses,[9,10] removes nonstructural carbohydrates, but does not claim to produce any type of cellulose product.

Sheu and Chiu[22] performed a test of tree ring pretreatment methods and recommended holocellulose produced by the Jayme−Wise pretreatment method as the standard for $\delta^{13}C$ measurements of tree rings over the acid/base/acid method. Since that paper, however, new methods including the Brendel method and the method of Loader et al.[23] have been developed for wood pretreatment with the aim of being quicker and allowing for processing of smaller sample sizes. Additionally, the Brendel method seems to be gaining attention from researchers for use in large-batch ^{18}O and ^{14}C applications (refs 24 and 25, Tom Guilderson personal communication, and authors' experience) despite the fact it was developed for ^{13}C analysis.

The advent of new pretreatment techniques, combined with their potential uses on tissues other than wood and for isotopes other than those originally intended, calls for a new comparative study of these methods. While it is unlikely that any single method will be best for all applications, it is nonetheless important to know the effectiveness of the different pretreatment methods and how each might interact with the different plant tissues used for isotope studies. In this study, we compared ^{18}O, ^{13}C, and ^{14}C measurements from wood, leaf, and root tissues treated with the Jayme−Wise, Brendel (plus two variants), and acid/base/acid methods. On a subset of samples, we also investigated the purity of the residue generated by each pretreatment procedure based on measurements of the chemical environment of organic carbon using cross-polarization magic-angle spinning ^{13}C nuclear magnetic resonance (CPMAS ^{13}C NMR) spectroscopy.[26,27]

EXPERIMENTAL SECTION

The three pretreatment methods were compared for use on three types of plant tissue: leaves, roots, and wood. Three different sample sets were used in our tests (Table 1). The first sample set (sample set 1) was a cross-tissue comparison (leaves, wood, roots) within one tree species (Redwood, *Sequoia sempervirens*). From these data, we analyzed tissues for yield (amount of unextracted fraction), C and N content, $\delta^{18}O$, $\delta^{13}C$, and carbon chemistry using

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Table 1. Sample Descriptions and Information for Each Sample Set Used in Comparisons of Pretreatment Methods

sample set	species/ ecosystem type	location	tissue sampled[a]	n^b	methods used (method abbrev)[c,d]	elemental analyses	^{13}C NMR
1	redwood[e]	Big Basin, CA	leaves roots coarse wood fine wood	6	Jayme−Wise (JW-alpha, JW-holo) Brendel (B, MB, WMB) acid/base/acid	% C % N $\delta^{18}O$ $\delta^{13}C$	yes
2	chestnut oak[f] tussock tundra[g] mixed deciduous[h] tiriwood B[i]	Harvard Forest, MA Toolik Lake, AK Oakridge, TN unknown	leaves roots roots fine wood	3	Jayme−Wise (JW-holo) Brendel (B, MB) acid/base/acid	% C % N $\delta^{13}C$ $\Delta^{14}C$	no
3	redwood[e]	Sonoma, CA	coarse wood	12	Jayme−Wise (JW-holo) Brendel (B, MB)	% C % N $\delta^{18}O$, $\delta^{13}C$	no

[a] All tissues were ground in a Spex Certiprep 8000M mixer mill to a very fine powder except for "coarse wood" samples, which were ground with a Wiley Mill to 20 mesh. [b] All samples came from the same, respective, homogenized sample container and thus are duplicates and not true replicates. [c] The Jayme−Wise method can produce α- or holo-cellulose. We abbreviate the two types as JW-alpha and JW-holo, respectively. See text for details. [d] We used the Brendel method and two modifications of this method: the modified Brendel and water-modified Brendel. In tables and graphs, we abbreviate the three methods as B, MB, and WMB, respectively. See text (where we refer to them as Brendel, MBrendel, and WMBrendel, respectively) and Supporting Information section for details. [e] Redwood (*S. sempervirens*). [f] Chestnut Oak (*Quercus prinus*). [g] Mixed species roots. Site is dominated by *Eriophorum vaginatum*, a tussock-forming sedge, with a smaller proportion of *Betula nana*, dwarf birch and several other shrub and moss species. [h] Mixed species roots. Site is dominated by white oak (*Quercus alba*), chestnut oak (*Q. prinus),* yellow poplar (*Liriodendron tulipifera),* and hickories (*Carya* spp.) with red maple *Acer rubrum*) as an understory tree. [i] This is an internationally recognized radiocarbon standard made from Scots (Belfast) pine (*Pinus sylvestris*).

^{13}C NMR (Table 1). The second sample set (sample set 2) compared tissue types (leaves, roots, wood) from four different tree species selected to maximize differences in ^{14}C content, including the wood sample (Tiriwood), which is 4500 years old and commonly used as a ^{14}C standard. Sample set 2 was used to analyze tissues for yield, C and N content, $\Delta^{14}C$, and $\delta^{13}C$ (Table 1). The third sample set (sample set 3) was also from one plant species (Redwood) and used wood only. These samples were analyzed for tissue yield, C and N content, $\delta^{18}O$, and $\delta^{13}C$ (Table 1). Not all sample sets were used to compare all methods (see Table 1).

As discussed in the introduction, cellulose is the compound researchers strive to isolate prior to isotope analysis. However, none of the methods available claim to isolate pure cellulose and instead produce a residue that is operationally defined (e.g., holocellulose, α-cellulose) or a residue that has nonstructural carbohydrates removed (acid/base/acid method). In this study, isotope ratios in the residues left by the different preparation methods were compared to those of the bulk sample. ^{13}C NMR analysis of the chemical compounds remaining in the residues from sample set 1 allowed us to determine which residues were most chemically similar to a commercially available cellulose (Aldrich, Catalog No. 31809-4; herein referred to as our cellulose standard).

PREPARATION METHODS

Jayme−Wise Method. This method was originally described by Jayme and Wise (see ref 8) and involves a solvent extraction (using benzene and ethanol) in a Soxhlet system followed by an acid sodium hypochlorite (bleach) delignification process, which leaves a residue designated as holocellulose. We used a modification of this method based on the work of Leavitt and Danzer,[20] who used toluene and ethanol rather than benzene/ethanol to reduce laboratory exposure to carcinogens. To obtain α-cellulose, a third step using NaOH is applied to the holocellulose purification.[8,28] Typically, researchers vary the timings and reagent concentrations of the protocol to fit the needs of their particular samples. For example, wood is fairly easy to bleach relative to roots so the bleach strengths and times are shorter for wood. Henceforth, we refer to the compounds purified by the Jayme−Wise method as JW-holo (holocellulose) and JW-alpha (α-cellulose), respectively. See Supporting Information for the exact description of the method used in this study.

Brendel, Modified Brendel, and Water-Modified Brendel Methods. The so-called Brendel method was developed by Brendel and colleagues[21] as a more rapid way to produce α-cellulose relative to the Jayme−Wise method. It uses an acetic acid/nitric acid mixture to simultaneously remove lignin and noncellulose polysaccharides. We tested the Brendel method as originally published, plus what we call the Modified Brendel (MBrendel), which adds a NaOH step at the end, followed by water rinsing and acidification. The water-modified Brendel (WMBrendel) method simply adds several more water rinses to the MBrendel method. The former (NaOH) modification was made because it was thought that the Brendel method only produced holocellulose, not α-cellulose, which by definition is that portion of cellulose that does not dissolve in a 17.5% solution of NaOH. The latter (water) modification was made because initial tests seemed to indicate that there was inadequate rinsing of reagents (samples often had a visible white residue remaining—likely salt). See Supporting Information for the exact description of the Brendel methods used in this study.

Acid/Base/Acid Method. The acid/base/acid treatment of plant material is commonly used to remove easily hydrolyzable carbon, such as carbohydrates. It consists of sequential washes of weak acids and bases and is thought to leave a residue consisting primarily of structural carbon components such as cellulose and lignin.[9,10] However, this method has never been

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described as a "cellulose" purification method. It is often used to treat organic samples prior to analyses for bomb-^{14}C with the goal of removing carbon constituents that do not reflect the atmospheric ^{14}C signature of the year the plant grew.[9,10] See Supporting Information for the exact description of the acid/base/acid method used in this study.

^{13}C NMR. Solid-state CPMAS ^{13}C nuclear magnetic resonance (NMR) was used to determine the chemical composition of the products of the different pretreatment methods. Measurements were made on a Bruker Avance 300 ($B_0 = 7.05$ T, $\nu_L(^{13}C) = 75.5$ MHz) NMR spectrometer using a 4-mm double-resonance MAS probe with high-power proton decoupling. Samples were packed into 4-mm (o.d.) zirconia (ZrO_2) rotors with end caps made of Kel-F. All ^{13}C NMR spectra were acquired using ramped-amplitude cross-polarization (RAMP-CP),[29] and were referenced to TMS ($\delta_{iso} = 0.0$ ppm) by setting the carbonyl resonance of solid glycine to 176.5 ppm.[30] The ^1H 90° pulse and Hartmann−Hahn matching conditions were also determined using this sample. All ^{13}C CP NMR spectra were acquired using a ^1H 90° pulse width of 4.0 μs, a pulse delay of 5.0 s, a contact time of 1.0 ms, an acquisition time of 17.1 ms, a sweep width of 60 kHz, and a spinning frequency of 5.0 kHz. The total number of transients collected was 1000 for each sample. Two-pulse phase modulation[31] with a ^1H decoupling field of 62.5 kHz was employed during the acquisition of all spectra. A Gaussian line broadening of 75 Hz was used to process all spectra. Bruker's WIN NMR package was used to estimate the relative integrated areas of various regions between 0 and 194 ppm. Many different spectral regions for the integration have been reported.[32,33] In this study, the spectral divisions were assigned based on local minimums of the spectra. The following regions were used for integration: 0−45 ppm attributed to alkyl carbons (ALK); 46−95 ppm attributed to methoxyl and *O*-alkyl carbons (O-ALK); 96−117 ppm attributed to di-*O*-alkyl and some aromatic carbon (DI−O-ALK); 118−164 ppm attributed to aromatic and phenolic carbons (AROPH); 165−194 ppm attributed to carboxylic and carbonyl carbons (CARB).

Data from solid-state CPMAS ^{13}C NMR experiments are considered "semiquantitative", primarily because of variability in $^1H-^{13}C$ cross-polarization efficiencies.[34] Thus, CPMAS ^{13}C NMR spectroscopy cannot be used to determine the quantities of different C types within a sample, but can be used to compare the relative abundance of different C types among similar types of samples, provided that they are analyzed under identical conditions,[35] as was the case in our study. For the purposes of this study, we further considered that integrated values were dependable within 1% and that differences in carbon distribution among samples could be assigned to differences in sample composition rather than to analytical errors when they exceeded 1% (i.e., a value of 1 in Table 2 since the total area in a given row sums to 100).

Table 2. Distribution of Carbon in ALK, O-ALK, DI-O-ALK, AROPH, and CARB[a] Regions of NMR Spectra Obtained from Bulk Samples and Residues from the Different Pretreatment Methods

	ALK 0−45 ppm	O-ALK 46−95 ppm	DI-O-ALK 96−117 ppm	AROPH 118−164 ppm	CARB 165−194 ppm
			Leaves		
bulk	20.5	45.8	12.9	15.1	5.7
JW-alpha	7.6	70.1	16.0	4.7	1.7
JW-holo	10.2	67.4	15.3	4.4	2.7
ABA	17.2	59.5	13.1	8.0	2.2
WMB	17.7	61.1	14.5	4.5	2.2
MB	20.9	61.6	13.5	2.4	1.6
B	22.0	59.6	13.3	2.9	2.3
			Coarse Wood		
bulk	4.2	61.1	15.0	18.0	1.8
JW-alpha	0.9	76.1	17.8	4.5	0.8
JW-holo	1.6	73.7	17.5	5.2	2.1
ABA	3.7	63.1	14.2	15.9	3.1
WMB	2.7	77.7	16.7	2.4	0.5
MB	1.8	76.6	17.1	3.8	0.7
B	3.0	75.5	16.4	3.7	1.5
			Fine Wood		
bulk[b]	4.6	64.9	14.2	15.2	1.1
JW-alpha	0.2	80.6	17.0	1.8	0.4
JW-holo	0.5	77.9	17.3	2.7	1.7
ABA	2.4	64.1	15.1	17.0	1.4
WMB	0.4	80.4	17.3	1.6	0.3
MB	0.4	80.0	17.1	2.0	0.6
B	3.7	76.2	15.6	3.0	1.6
			Fine Roots		
bulk	14.1	50.3	14.2	17.8	3.6
JW-alpha	7.2	73.2	15.5	2.8	1.3
JW-holo	6.9	72.1	15.6	3.4	2.0
ABA	6.1	61.9	14.4	14.5	3.1
WMB	3.8	74.8	16.4	3.9	1.1
MB	3.7	75.2	16.5	3.8	0.8
B	9.8	69.1	14.8	4.3	2.0

[a] ALK, alkyl C; O-ALK, methoxyl and O-alkyl C; DI-O-ALK, di-O-alkyl and some aromatic C; AROPH, aromatic and phenolic C; CARB, carboxylic and carbonyl C. [b] Represents peak area for bulk coarse wood.

Isotope, Carbon, and Nitrogen Analysis. The stable 13-carbon and 18-oxygen isotope ratios are expressed in standard delta (δ) notation, that is, relative to an internationally accepted reference standard as,

$$\delta^{XX}E = 1000\left(\frac{R_{sample}}{R_{standard}} - 1\right), ‰$$

in "per mil" or parts per thousand (‰), where E is the element of interest (e.g., C or O), XX is the mass of the rare and heavier isotope in the abundance ratio, and R is the abundance ratio of the heavier versus lighter isotope (e.g., $^{18}O/^{16}O$; see ref 13 for further details).

$\delta^{18}O$, $\delta^{13}C$, Percent C, and Percent N. The $\delta^{18}O$ values for sample sets 1 and 3 were obtained following the procedures of Brooks and Dawson[36] at the Center for Stable Isotope Bio-

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geochemistry (CSIB), UC Berkeley. This procedure follows a modified version of the pyrolysis method first published by Farquhar et al.[37] In brief, a standard elemental analyzer (Carlo Erba, NA1500, Milan, Italy) is modified as discussed in ref 37, but with ceramic combustion/reduction tubes, and interfaced with a gas-phase isotope ratio mass spectrometer (Finnigan MAT, Delta+XL, Bremen, Germany). Long-term precision for $\delta^{18}O$ analysis at CSIB is ±0.13‰. Percent C and N (by mass) and $\delta^{13}C$ data were obtained using a standard elemental analyzer interfaced with a gas-phase isotope ratio mass spectrometer (PDZ, Europa Scientific 20/20). Long-term precision for $\delta^{13}C$ and $\delta^{15}N$ are 0.12 and 0.20‰ respectively (see http://ib.berkeley.edu/groups/biogeochemistry/).

^{14}C. Samples analyzed for ^{14}C were first converted to graphite by sealed-tube zinc reduction[38] at the University of California, Irvine (UCI). The ^{14}C content of this graphite was measured at the Center for Accelerator Mass Spectrometry at Lawrence Livermore National Laboratory (LLNL CAMS). We express radiocarbon data as $\Delta^{14}C$, the difference in parts per thousand (per mil or ‰) between the $^{14}C/^{12}C$ ratio in the sample compared to that of an international standard (oxalic acid I, decay-corrected to 1950). All samples are corrected to a common $\delta^{13}C$ value of −25‰ (based on normalizing with each sample-specific measured $\delta^{13}C$ value) to correct for the effects of mass-dependent isotope fractionation on measured ^{14}C values. This accounts for photosynthetic discrimination of atmospheric ^{14}C in CO_2 (^{14}C is assumed to fractionate twice as much as ^{13}C). Zinc-reduced targets produced at UCI have an accuracy of ±5‰ on modern samples (i.e., samples post-1950) when measured at LLNL CAMS, based on repeated measures of secondary standards.

Statistical Analysis. ANOVA was used to test for differences among methods (JW-alpha, JW-holo, Brendel, MBrendel, WMBrendel, acid/base/acid) within each unique tissue type (Sample set: 1 redwood roots, redwood leaves, redwood coarse wood, and redwood fine wood. Sample set 2: HF leaves, AK roots, WB roots, and TIRI wood. Sample set 3: redwood coarse wood). There were three to six replicates within each method for each tissue type. A post hoc Tukey's HSD multiple comparisons test was subsequently done to determine significant differences between specific pretreatment methods ($\alpha = 0.05$).

We also analyzed $\delta^{13}C$ and $\delta^{18}O$ and differences among methods (JW-alpha, JW-holo, Brendel, MBrendel, WMBrendel, acid/base/acid) across all tissue types in sample sets 1 and 2. Each tissue type had three to six pretreatment replicates analyzed for each variable, with the number depending on the variable in question. We tested for significant differences between pretreatment methods by analyzing each variable using a nested general linear model ($\alpha = 0.05$) and a post hoc Tukey's HSD multiple comparisons to determine significant differences between specific pretreatment methods ($\alpha = 0.05$).

Differences in methods across tissue type were analyzed slightly differently for $\Delta^{14}C$ measurements because there were no replicates within tissue type within a pretreatment (i.e., $n = 1$). Therefore, we normalized the data within each tissue type in order to compare across tissue types. We determined the mean value, for each variable, for each tissue type value (across all methods) and then calculated the difference between each pretreatment method and this overall tissue-type mean. This normalization procedure standardized the values irrespective of tissue types. We then used one-way ANOVA to quantify significant differences among pretreatment methods using the normalized tissue types as replicates for treatment ($\alpha = 0.05$). In the case of $\Delta^{14}C$, we reported the less conservative Student's t test for post hoc analysis in order to pinpoint the treatment differences indicated by the ANOVA that were not revealed by Tukey's HSD. It should be noted that using Student's t test increases the probability of a type I error for this analysis.

All statistical analyses were performed using the SYSTAT 10.2 statistical software package. Hereafter, use of the term "significant" indicates that there was a difference between mean values of the measured variables at the $P < 0.05$ level.

RESULTS AND DISCUSSION

Unextracted Fraction. The amount of original sample mass remaining after pretreatment (yield) varied significantly among methods (Figure 1A and B). In sample set 1, the fraction of mass remaining after pretreatment varied for leaf tissue from 12 to 69%, for fine wood from 22 to 35%, for coarse wood from 14 to 46%, and for fine roots from 11 to 69%. In sample set 2, mass remaining varied from 8 to 33% for leaf material, from 8 to 68% for wood, and from 6 to 52% for fine roots with the lowest values (<10%) resulting from the MBrendel method.

Based on other studies, the expected cellulose content values (by weight) were 5−30% for leaves and 40−80% for stems and roots.[39] For herbaceous roots (e.g., AK roots), the expected yield was 15−30% since herbaceous roots have total structural carbon values that range from 15 to 30%.[39] Other studies using the Brendel and a variant of the JW-alpha method had residual mass values from wood of 30−41%.[21,23,24] Leavitt and Danzer[20] reported slightly higher yields from wood of 49−73% for the JW-holo method, depending on the amount of time samples spent in bleach solution.

Our data fall within the expected yield ranges for leaves, in sample sets 1 and 2 (Figure 1A and B), and for wood and roots in sample set 2 (except for the MBrendel method). The values for wood and roots in sample set 1, however, ranged from 14 to 35% and were often below the expected values. The variation seen among methods is expected. For example, the mass remaining after the JW-alpha pretreatment should be less than that using the JW-holo pretreatment because hemicellulose has been removed in the NaOH step. Similarly, MBrendel and WMBrendel methods should have lower unextracted fractions than that from the Brendel method, again due to the addition of the NaOH step. These trends are indeed seen in sample sets 1 and 2 (Figure 1A and B). The acid/base/acid method tended to remove less mass than the other methods in most cases.

Variation of yield between coarse and fine wood samples was expected owing to differences in tissue particle sizes. Coarse wood was expected to have higher yields because the larger relative particle size would lead to less efficient chemical processing and less mass loss during processing. However, consistently higher yields were not found for coarse wood. In both JW methods, the coarse wood had higher remaining mass values than the fine

(37) Farquhar, G. D.; Henry, B. K.; Styles, J. M. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1554−1560.
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(39) Poorter, H.; Villar, R. In *Plant resource allocation*; Bazzaz F. A., Grace, J., Eds.; Academic Press: London, 1997; pp 39−72.

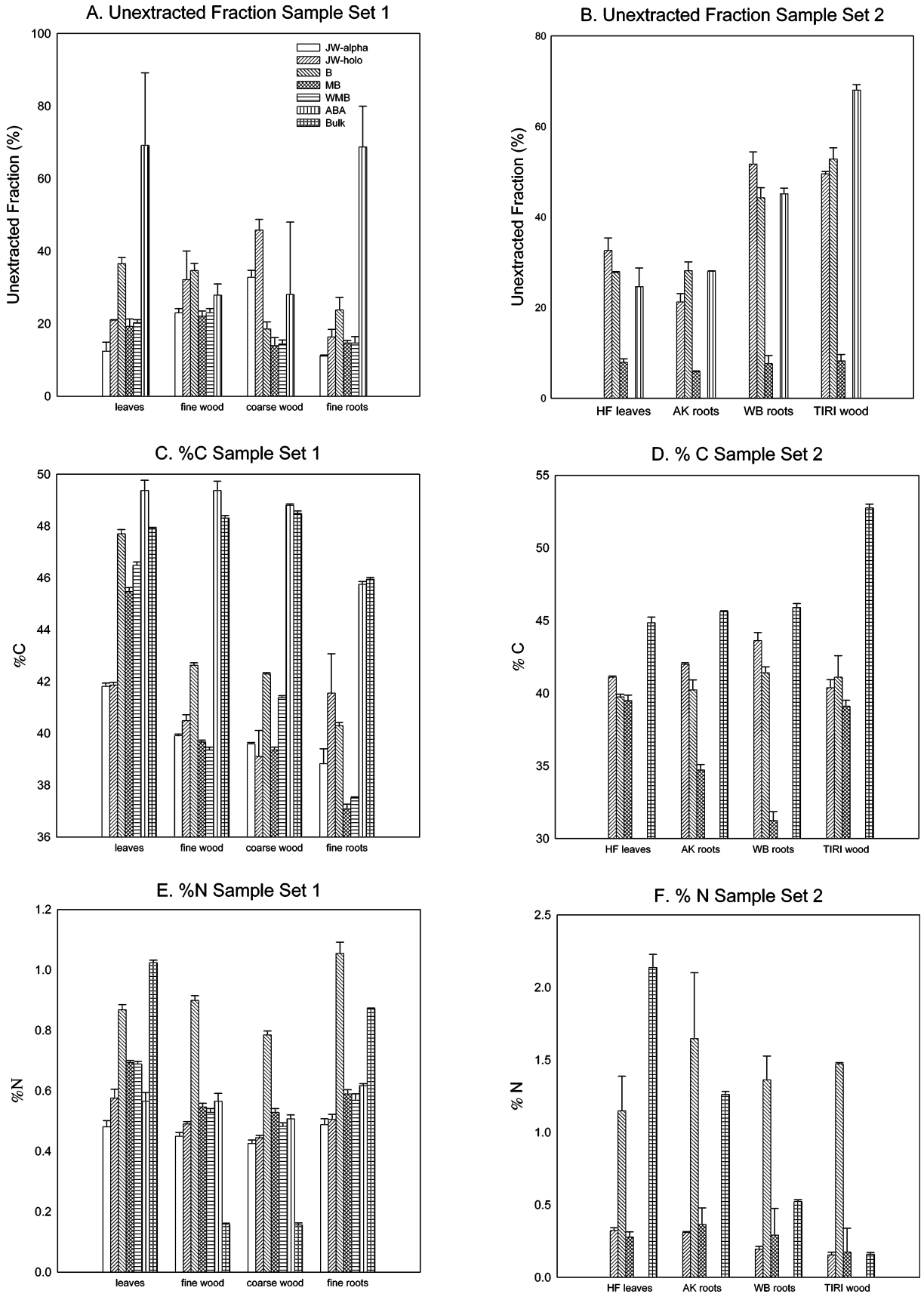

Figure 1. Means (±1 SE) for percent yield (unextracted fraction), percent carbon and percent nitrogen of various tissue samples used in sample sets 1 ($n = 6$) and 2 ($n = 3$), as described in text. Values are for bulk tissue samples and six pretreatment methods: Jayme−Wise isolation of holocellulose and α-cellulose (JW-holo and JW-alpha, respectively), Brendel (B), modified Brendel (MB), water-modified Brendel (WMB), and acid/base/acid wash (ABA).

wood, but for all other methods, the coarse wood had lower or similar remaining mass relative to fine wood (Figure 1A).

C and N Data. Bulk carbon contents for untreated materials were between 45 and 53% (Figure 1C and D). Following treatments, the values in residues were between 37 and 49% C. All methods showed a lower % C content compared to the untreated bulk sample with the exception of the acid/base/acid method. Bulk nitrogen contents were highest in leaves and roots (0.5−2.0%) and lowest in wood (0.16%) (Figure 1E and F) as expected because living leaf and root tissues possess more N-rich compounds relative to wood. For N-rich leaves and roots, all treatments, except Brendel, resulted in residues that were depleted in N relative to bulk tissue, whereas all treatments left N-poor wood residues with increased N relative to bulk tissue. The Brendel method, among all pretreatment methods, had the highest residual N contents (Figure 1E and F).

Pure cellulose is ~44.5% C on a mass basis[21] while its N content is zero. Relative to the C content of an untreated (bulk) sample, isolated cellulose should be lower because many of the impurities removed during cellulose purification are secondary plant metabolites that possess a range of C-rich compounds (e.g., lignin, phenolics, resins.[40] Similarly, the N content of purified cellulose should also be lower than unpurified "bulk" samples, which contain some nitrogen. In general, our data show lower C and N content of treated samples relative to bulk tissue; however, there are some important exceptions.

The higher C content in residues of the acid/base/acid method (Figure 1C) suggests the presence of lipid compounds such as monoterpenes. In sample set 1, and in three of four cases in sample set 2, the Brendel method yielded significantly higher % C values than the MBrendel and WMBrendel methods. Higher % C content in these residues could have resulted from the failure to remove C-rich plant compounds, as suggested above, or from the addition of C through the acetic acid used in the pretreatment method. In sample set 1, the JW-alpha and JW-holo methods yielded % C values that were similar to each other in leaves and coarse wood, but for roots and fine wood, JW-holo was significantly higher in % C content than JW-alpha (P = 0.05 and 0.02, respectively).

For wood samples that were initially low in N, all pretreatment methods left residues with higher N concentrations than the original material, with the highest % N values, in all cases, resulting from the Brendel method (Figure 1e). The Brendel method also had significantly higher % N in high-N tissues (leaves and roots). Higher %N values in all residues from the Brendel method suggest that this method adds N to samples in the nitric acid step. Furthermore, the MBrendel and WMBrendel methods always gave significantly lower % N than the Brendel method. These latter results are consistent with the idea that removal of nitric acid occurs via the addition of the NaOH step, used in both modified Brendel methods. Brendel et al.[21] did investigate the possibility of nitric acid contamination causing elevated and variable N contents in their wood samples (Scots pine). They found that the % N content of 14 samples treated with the Brendel method varied between 0.072 and 0.198, which was within ~0.15% of the minimum sensitivity of their elemental analyzer. They thus concluded residual nitric acid was not a problem. Our data urge caution with this conclusion.

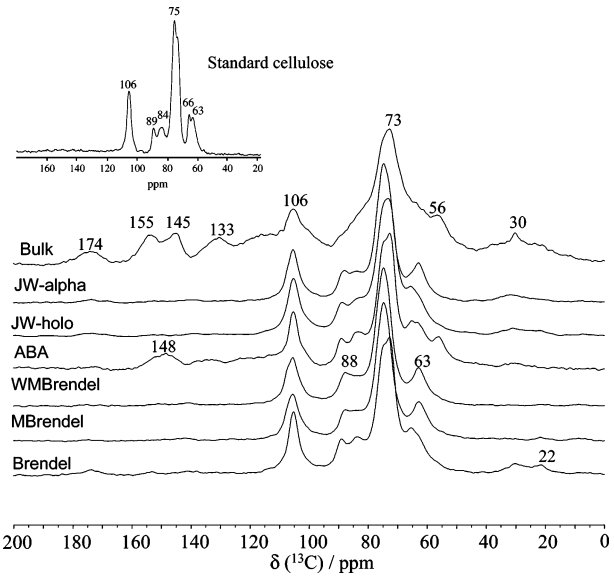

Figure 2. CPMAS ^{13}C NMR spectra for fine roots (sample set 1; Redwood) and standard cellulose. Method abbreviations are the same as in Figure 1.

^{13}C NMR. Carbon-13 NMR spectra from residues of all methods were compared to the original bulk sample and to a spectrum of standard cellulose (Figure 2). The commercial cellulose spectrum showed peaks at 63 and 66 (C-6), 73−75 (C-2, C-3, and C-5), 84−89 (C-4), and 105−106 ppm corresponding to anomeric (C-1) carbons.[26] No peaks are present in the alkyl C (ALK, 0−45 ppm), aromatic and phenolic C (AROPH, 118−164 ppm), or carboxylic and carbonyl C (CARB, 165−194 ppm) spectral regions.

Spectra from the untreated bulk samples were dominated by peaks at 73 and 106 ppm assignable to cellulose (Figure 2). Additionally, all bulk samples exhibited signals in the AROPH spectral region, indicating the presence of aromatic and phenolic carbons from lignins and tannins (Table 2). In particular, the peak at 133 ppm probably originated from C-substituted aromatic carbons, such as the C-1 carbon of guaiacyl and syringyl units or the C-1, C-2, and C-6 carbons of *p*-hydroxyphenyl lignin moieties.[41] The methoxyl carbon signal of lignins was noticeable at 56 ppm in spectra from all bulk samples. Furthermore, the C-3 carbons of guaiacyl units and the C-3 and C-5 carbons of syringyl units typically contribute a broad signal centered around 150 ppm, which was apparent in spectra from bulk fine wood and coarse wood. On the other hand, bulk leaves and bulk fine roots exhibited a distinct split peak at 145 and 155 ppm in the phenolic region, which is a characteristic marker for condensed tannins.[42] The contribution of the alkyl C region (ALK), typically arising from carbons in long-chain fatty acids and waxes, was small in spectra from bulk fine wood and coarse wood but exceeded 10% for the bulk samples of leaves and fine roots (Table 2, Figure 2). In this spectral region, the main peaks occurred around 30 ppm, sug-

(40) Boutton, T. W. In *Mass Spectrometry of Soils*; Boutton, T. W., Yamasaki, S.-i., Eds.; Marcel Dekker Inc.: New York, 1996; pp 47−82.

(41) Landucci, L. L.; Ralph, S. A.; Hammel, K. E. *Holzforschung* **1998**, *52*, 160−170.

(42) Preston, C. M.; Trofymow, J. A. *Can. J. Bot.* **2000**, *78*, 1269−1287.

gesting that alkyl carbons were mainly of the polymethylene type.[43] Finally, the peak at 174 ppm, which was most apparent in spectra from bulk leaves and bulk fine roots, is indicative of the carbonyl carbon in acetyl and ester moieties.

For the fine root extracts, the MBrendel and WMBrendel methods yielded NMR spectra that were similar to the standard cellulose sample (Figure 2). Specifically, these two spectra showed four main peaks assignable to cellulose carbons at 63 (C-6), 75 (C-2, C-3, and C-5), 88 (C-4), and 106 (C-1) ppm (Figure 2). Contamination by noncellulose moieties was significant on spectra from the JW-holo, JW-alpha, acid/base/acid, and Brendel residues, all of which exhibited a peak at 31 ppm that can be assigned to polymethylene chains. Contribution of the alkyl C region for these four residues exceeded 5% of the total spectral area (Table 2). For the Brendel residue, an additional peak at 22 ppm is indicative of methyl carbons from acetyl groups in hemicelluloses (Figure 2). Since the spectrum from the bulk fine root sample did not show any peaks near 20 ppm, this may also be indicative of cellulose acetylation, which could have occurred during the pretreatment procedure. In the *O*-alkyl C region, these four spectra showed a peak at 84 ppm, which may arise from the presence of amorphous cellulose or other noncellulose polysaccharides. Finally, the spectrum from the acid/base/acid residue exhibited significant signals in the aromatic and phenolic C region, with two peaks centered around 134 and 148 ppm.

All pretreatment methods applied to coarse wood showed a reduction in the alkyl peak area relative to bulk tissue in the order, bulk > acid/base/acid ≥ Brendel ≥ WMBrendel ≥ MBrendel ≥ JW-holo ≥ JW-alpha (Table 2). For the fine wood extracts, contribution of the alkyl C region was less than 0.5% of the total spectral area, and peak differences were less than 1% among the JW-alpha, JW-holo, MBrendel, and WMBrendel spectra. These four methods yielded NMR spectra that were dominated by peaks attributable to cellulose and, based on the NMR analysis, may be considered acceptable pretreatment techniques for wood samples. On the other hand, the Brendel and acid/base/acid residues showed significant contamination. The coarse and fine wood spectra obtained from the Brendel method had a peak at 21 ppm, similar to the one apparent on the spectrum from the fine roots (Figure 2). The aromatic and phenolic C region was prominent on both fine and coarse wood spectra for the acid/base/acid method (Table 2), with peaks at 116 ppm corresponding to C-substituted aromatic carbons and phenolic carbons appearing at 133 and 149 ppm. Both spectra also exhibited a peak at 56 ppm, characteristic of the presence of lignins.

For the residues obtained from leaf tissues, spectra from all pretreatment methods showed a signal in the alkyl C region, although this peak was markedly smaller for the JW-alpha and JW-holo residues relative to the others (Table 2). Contribution of the alkyl C region increased in the order, JW-alpha < JW-holo < acid/base/acid ≤ WMBrendel < bulk < MBrendel ≤ Brendel. On all spectra, signals in the alkyl C region resulted from a broad peak centered around 30 ppm. The acid/base/acid spectrum again exhibited signals in the AROPH region.

In summary, the JW-alpha, JW-holo, MBrendel, and WMBrendel pretreatment techniques yielded NMR spectra that were dominated by peaks attributable to cellulose, while the Brendel and acid/base/acid techniques produced spectra with significant contamination from noncellulose components. In particular, the Brendel method was not efficient at removing noncellulose constituents such as fatty acids and waxes (with signals in the alkyl C region), while the acid/base/acid treatment left significant amounts of lignin in the sample. The purity of the cellulose produced by the JW-alpha, JW-holo, MBrendel, and WMBrendel methods varied among tissue types. This is not surprising given the different chemical compositions, particularly for alkyl carbon content, of the starting tissues (Table 2; column 1). Leaves had the highest alkyl carbon content (20.5%), and none of the methods totally removed this constituent from the samples, although the JW-alpha and JW-holo were clearly best at doing so. Fine roots had the second highest alkyl C content (14.1%), and for this tissue, the WMBrendel and MBrendel methods were the most efficient at removing alkyl C, although the JW-alpha and JW-holo were also effective. Fine and coarse wood had the least alkyl C (4.2−4.6%) and any of the JW-alpha, JW-holo, MBrendel, and WMBrendel methods effectively removed it.

Oxygen Isotope Ratio. In sample set 1, all methods resulted in residues that were enriched (heavier) in ^{18}O by 0.3−8.0‰ relative to the bulk samples (Figure 3A). In general, the relative ranking of methods was the same across the four tissue types. Notably, the JW-holo $\delta^{18}O$ values were significantly depleted (lighter) compared to all other methods by 1−5‰ except the acid/base/acid method for which residues were consistently the least enriched. In fact, the $\delta^{18}O$ of acid/base/acid residues were significantly heavier than the bulk sample in only two cases. Both sample sets 1 and 3 showed that JW-alpha samples were significantly lighter than Brendel samples in all but one case (fine roots in sample set 1). However, JW-alpha values were never significantly different from those of MBrendel samples (sample sets 1 and 3). Reproducibility of $\delta^{18}O$ values across all tissue types is shown by our standard deviations, which ranged between 0.17 and 1.28‰ (average 0.51‰; including sample sets 1 and 3, for 31 batches ($n = 3$ or 6 per batch) of samples run).

Removal of noncellulose compounds should, in general, increase the $\delta^{18}O$ value of plant tissue samples because lipids and lignins, important constituents of bulk tissues, tend to be isotopically lighter in ^{18}O than cellulose,[40] with lignin typically 13‰ lighter.[44] Removal of nonstructural carbohydrates will have less of an effect relative to lignin and lipid removal and can be either lighter or heavier than cellulose.[44] As expected, the treated samples in sample set 1 are all significantly more enriched in ^{18}O than the bulk samples with the exception of fine and coarse wood samples treated with the acid/base/acid method (Figure 3A).

α-Cellulose is typically the preferred substrate for oxygen isotope analysis compared to holocellulose because it does not contain exchangeable oxygen moieties (these are removed by the additional NaOH step). Samples isolated with the JW-alpha method were always significantly heavier than those done with the JW-holo method (sample set 1) as expected since the additional NaOH step removes isotopically lighter lignin and bound proteins. Unexpectedly, the Brendel samples were, on average, heavier than the MBrendel samples (except sample set 1 fine roots). The addition of the NaOH step in this case (MBrendel) decreased the $\delta^{18}O$ values relative to the Brendel method, but with subsequent

(43) Keeler, C.; Maciel, G. E. *J. Mol. Struct.* **2000**, *550−551*, 297−305.

(44) Schmidt, H.-L.; Werner, R. A.; Rossmann, A. *Phytochemistry* **2001**, *58*, 9−32.

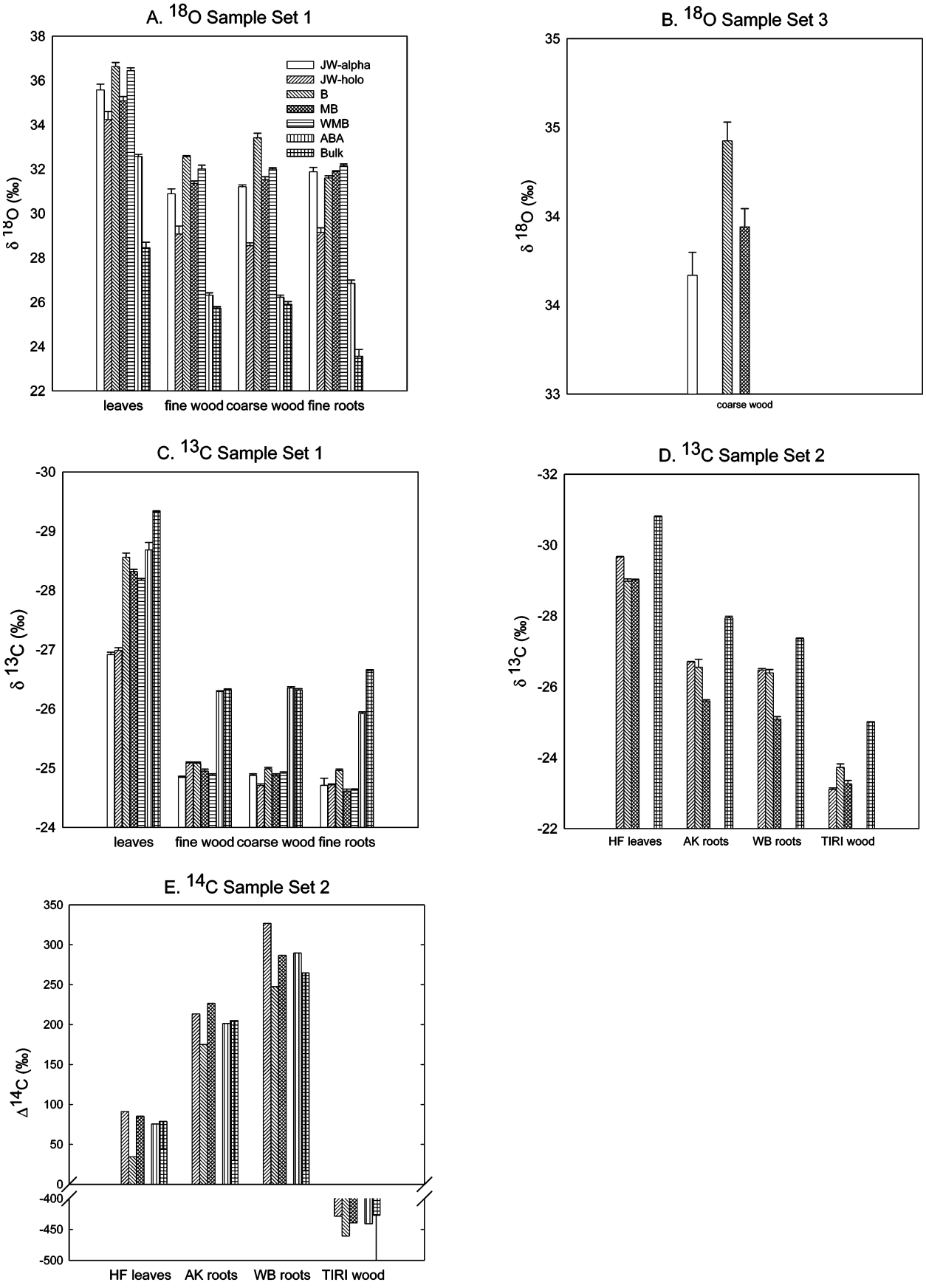

Figure 3. Oxygen isotope ratios ($\delta^{18}O$), carbon isotope ratios ($\delta^{13}C$), and carbon-14 contents ($\Delta^{14}C$) for various untreated (bulk) tissues and the same tissues treated with six pretreatment methods (abbreviations as given in Figure 1). Panels show results for sample sets 1 ($n = 6$), 2 ($n = 3$), and 3 ($n = 12-15$), as described in text, and represent mean values $\pm$ 1 SE (except for ^{14}C data, for which $n = 1$).

Table 3. Results of Pairwise Comparisons among All Pretreatment Methods and Bulk Tissue Samples across All Tissue Types for Mean Oxygen Isotope Ratios ($\delta^{18}O$), Mean Carbon Isotope Ratios ($\delta^{13}C$), and Mean Carbon-14 Concentrations ($\Delta^{14}C$)[a]

method	$\delta^{18}O$[b]	$\delta^{13}C$[b]	$\Delta^{14}C$[c]
bulk	a	a	a
JW-alpha	b	b	*d*
JW-holo	c	c	b
ABA	d	d	a
Brendel	e	e	c
MBrendel	b	f	a b
WMBrendel	f	f	*d*

[a] Significant differences ($P < 0.05$) among methods are expressed as different letters within each isotope analysis (i.e.. compare letters within the same column). [b] Tukey's pairwise comparison. [c] Student *t* test. [d] This method was not analyzed for $\Delta^{14}C$.

water washes (WMBrendel method), samples again became heavier. We are not sure why.

Acid/base/acid residues had the smallest difference in $\delta^{18}O$ values relative to bulk samples, indicating they were modified the least compared to the residues produced by all other methods.

An evaluation of differences in methods across all tissue types was done using a nested general linear model and Tukey's pairwise comparisons to determine significant differences among specific pretreatment methods ($\alpha = 0.05$). All methods significantly modify the $\delta^{18}O$ values relative to untreated bulk samples. Furthermore, JW-alpha, JW-holo, acid/base/acid, Brendel, and WMBrendel methods all produced results significantly different from each other (Table 3). The MBrendel results, however, were not significantly different from those of the JW-alpha method.

Our finding of a significant difference in $\delta^{18}O$ values between the Brendel and JW-alpha methods is among only a few published data of this kind. Evans and Schrag[24] made reference to unpublished data that showed no significant difference between the two methods for $\delta^{18}O$. However, those data, belonging to John Roden (a coauthor here), did not show the expected $\delta^{18}O$ value for their source location. Technically, this should not matter if one applies different methods to the same initial material; nevertheless, given this inconsistency we feel those data should be utilized with caution. Again, however, when we compare the JW-alpha pretreatment procedure with the NaOH-modified Brendel (MBrendel), the methods are not significantly different. Oxygen isotopes are used in a wide range of studies (see ref 45). Researchers wishing to use the Brendel or MBrendel method for ^{18}O analysis should do a careful comparison with the JW-alpha method for their particular application.

The ^{13}C NMR spectra provide some insights that can help explain differences in $\delta^{18}O$ in residues left by different pretreatment methods. First, the ^{13}C NMR spectra generally showed that the acid/base/acid method did not effectively remove lignin. Accordingly, the $\delta^{18}O$ values for acid/base/acid treatments were least modified relative to the bulk samples and lightest of all treatments. This is consistent with lignin (isotopically lighter than cellulose) being left in the sample. Second, the ^{13}C NMR spectra also showed that the Brendel method did not efficiently remove lipids and waxes (also isotopically lighter compared with cellulose). While the Brendel method generally left residues that were the most enriched in ^{18}O of all of the treatments (despite incomplete removal of lignins and waxes as indicated by ^{13}C NMR), addition of the NaOH step in the MBrendel method tended to decrease the $\delta^{18}O$ values, and according to the ^{13}C NMR results, the MBrendel method had fewer waxes and lipids relative to the Brendel. These findings put at odds a clear interpretation; that is, our ^{18}O data appear to contradict the ^{13}C NMR data, although these could be explained based on partial cellulose acetylation during the Brendel extraction. Acetylation is possible because peaks from acetyl methyls at 20 ppm could be observed on the NMR spectra of the Brendel extracts, especially for the fine root samples, and these contributed to the stronger contribution of the alkyl C region as compared to the MBrendel extracts. Additionally, Anchukaitis et al. (The University of Arizona, unpublished work, 2005) have demonstrated that for wood the Brendel method results in a small degree of partial acetylation of the cellulose which may affect isotopic results and account for the discrepancy between our ^{18}O and ^{13}C NMR data.

While trends in $\delta^{18}O$ results were consistent across tissue types, the ^{13}C NMR data showed only small differences in the C chemistry of residues from the JW-alpha and MBrendel methods for fine roots and coarse and fine wood. The largest difference was 3.3% more alkyl C in JW-alpha fine roots relative to MBrendel fine roots. However, for leaves, the JW-alpha and JW-holo samples clearly had the purest cellulose (7.5−13.3% less alkyl C than the MBrendel methods) with JW-alpha having 2.4% less alkyl carbon than JW-holo (Table 2; column 1). In agreement with the ^{13}C NMR results, the $\delta^{18}O$ of leaf samples treated by the JW-alpha method were heavier than those from the JW-holo method. This result is as expected if complete removal of lipids, waxes, and lignins increases the $\delta^{18}O$ value.

Carbon Isotope Ratio. In sample set 1, samples from all methods were enriched in $\delta^{13}C$ relative to the bulk tissue by about 1.0−2.25‰ with the exception of samples from the acid/base/acid method, which were not significantly different from bulk tissues for both the fine and coarse wood comparisons (Figure 3C and D). Treatment groups in sample set 2 were 1.0−1.5‰ heavier for $\delta^{13}C$ than the respective bulk samples (Figure 3D). Across tissue types, in both sample sets, the only consistent trend was that samples treated by the Brendel method were lighter than those isolated via the MBrendel method for seven out of eight cases. Repeatability of $\delta^{13}C$ values across all tissue types and methods was 0.02−0.31‰ (average 0.07‰; including sample sets 1 and 3) for the 31 batches ($n = 3$ or 6 per batch) of samples run. This compares well with published standard deviations for $\delta^{13}C$ studies using similar methods[20−23] and the long-term precision for the mass spectrometer on which these samples were run (±0.20‰).

Removal of noncellulose compounds should, in general, increase the $\delta^{13}C$ content of plant tissue samples because the majority of these compounds are lipids and lignin, which are depleted in ^{13}C relative to cellulose.[40] In fact, lignin can be up to 3‰ lighter than cellulose.[22] As expected, all treated samples in

(45) Barbour, M. M.; Cernusak, L. A.; Farquhar, G. D. In *Stable isotopes and biosphere-atmosphere interactions. Process and biological controls*; Flanagan, L. B., Ehleringer, J. R., Pataki, D. E., Eds.; Elsevier Academic Press: San Diego, 2005; pp 9−28.

sample set 1, with the exception of the acid/base/acid samples, were ~1.0−2.5 ‰ heavier than the bulk samples (Figure 3C).

In sample set 1, the JW-alpha and JW-holo treated samples were ~2.25‰ heavier than the bulk sample for leaves and 1.0−1.5‰ heavier than the bulk samples for coarse wood, fine wood, and roots. There was a statistically significant difference ($P < 0.02$) between JW-alpha and JW-holo samples, only in the case of the fine wood samples (which differ by only 0.24‰) and the coarse wood samples (which differ by only 0.17‰), though both these differences are similar to the precision of the mass spectrometer (0.20‰). In general, little difference between these treatments was expected because the extra NaOH step in the JW-alpha method primarily removes the molecular moieties that contain exchangeable oxygen and thus should have little effect on C isotopes. Similarly, Sheu and Chui[22] found the $\delta^{13}C$ of α- and holocellulose of tree rings to be 1.6‰ heavier than bulk tissue and only 0.04‰ different from each other. The three Brendel methods were all 1.0−1.5‰ heavier than the bulk tissue. The Brendel-treated samples were the lightest of the three Brendel-related methods in sample sets 1 and 2, with the exception of HF leaves. The acid/base/acid-treated samples were 0.75−1‰ heavier than bulk tissues of leaves and roots, but were not significantly different from the bulk tissue for both wood samples. These findings are also consistent with those of Sheu and Chui,[22] who found no significant differences between the $\delta^{13}C$ values for bulk versus acid/base/acid-treated wood.

Investigation of the methods across all tissue types using sample sets 1 and 2 showed that JW-alpha, JW-holo, acid/base/acid, Brendel, and MBrendel methods all produced $\delta^{13}C$ results that differed significantly from the bulk $\delta^{13}C$ values and from each other (Table 3). The WMBrendel was significantly different from the bulk but not significantly different from the MBrendel.

As with the ^{18}O isotope data, the ^{13}C isotope data viewed across all tissue types are in agreement with the broad trends observed in the ^{13}C NMR spectra. First, the acid/base/acid method had $\delta^{13}C$ values close to those of the original bulk material (unlike all other methods whose residues show much larger ^{13}C enrichment). This pattern is consistent with evidence of incomplete removal of lignin (which is more depleted in ^{13}C than cellulose) as shown by the ^{13}C NMR spectra. Trends among the remaining methods are less distinct; however, the Brendel method left residues that were the most depleted in ^{13}C. This finding is also consistent with less efficient removal of lipids and waxes (isotopically lighter than cellulose), as indicated by the ^{13}C NMR spectra. As mentioned above, isotopic and NMR results for tissue samples processed via the Brendel method may be affected by some degree of partial acetylation of the cellulose (also noted by Anchukaitis et al.; University of Arizona, unpublished work, 2005).

Leaves were the only tissue for which the ^{13}C NMR spectra indicated large differences among treatment methods. The $\delta^{13}C$ results were concordant with this variation in that the methods that resulted in the lowest $\delta^{13}C$ values (i.e., JW-alpha and JW-holo) had ^{13}C NMR spectra closest to those of the cellulose standard (leaf spectra not shown but compare alkyl C contents for leaves in Table 2). Relative to all other tissue types tested, leaves had a much higher alkyl C content. Thus, for leaves, more so than wood and roots, more negative $\delta^{13}C$ values were likely caused by the failure of the pretreatment methods to remove all alkyl C moieties. Differences in ^{13}C and ^{18}O results as a function of tissue type have also been recently reported for the diglyme−HCL pretreatment method.[46] These authors found the diglyme−HCL method satisfactory for ^{13}C and ^{18}O isotope analysis of hardwoods (e.g., three *Eucalyptus*, species), but for softwoods (*Pinus pinaster* and *Callitris glaucophylla*) and foliage samples, the efficacy of the method for both isotopes was species dependent.

Radiocarbon (^{14}C) Isotope Composition. During the late 1990s and early 2000s, the concentration of ^{14}C of CO_2 in the atmosphere was decreasing at a rate of 6‰/year.[19] Therefore, any differences found between the radiocarbon content of untreated bulk samples collected in the 1990s (i.e., leaf and root tissues) and residues from the isolation methods could signify either (1) the presence of compounds in the bulk sample that postdate the production of the cellulose in the sample or (2) the addition of C-based reagents to the sample during processing (for the Brendel methods). Tiriwood, which is a radiocarbon standard material distributed by the International Atomic Energy Association, was also used in this study because its radiocarbon content is known and the wood was produced at a time when ^{14}C content of the atmosphere was fluctuating very little (4500 yb) relative to the post "bomb" period (i.e., after 1950). As such, the pretreatment methods were not expected to have a significant affect on the $\Delta^{14}C$ value of these residues.

Leaf and root residues from the JW-holo and MBrendel methods had $\Delta^{14}C$ signatures that were 7−22‰ greater than those of the original bulk tissues (Figure 3E). Residues from the Brendel method were 17−45‰ depleted relative to bulk samples, and residues produced by the acid/base/acid method were within one standard deviation (i.e., ±5 ‰) of the original material for all tissues except the WB root samples, which were enriched by 39‰. $\Delta^{14}C$ values of Tiriwood treated by the JW-holo method were within one standard deviation of those for bulk samples, whereas the Brendel, MBrendel and acid/base/acid residues were depleted by 12−13‰ relative to bulk values.

The higher $\Delta^{14}C$ values of JW-holo leaf and root residues relative to original bulk material (Figure 3E) are consistent with the expected removal of recently formed carbohydrates that should have lower $\Delta^{14}C$ signatures than $\Delta^{14}C$ of structural components—those formed in previous growing seasons (this also assumes that the lifetime of the tissue being sampled is longer than several months). Values seen from the Brendel method, which were depleted relative to bulk samples (Figure 3E), must represent contamination by the pretreatment method. This is likely a result of the acetic acid step. Acetic acid is derived from fossil fuels when industrially manufactured. Since fossil fuel carbon contains no ^{14}C, any acetic acid residue would reduce the total measured $\Delta^{14}C$ values. The fact that residues left by the MBrendel method had higher $\Delta^{14}C$ values compared to the original bulk material argues that the addition of the NaOH step removed the added acetic acid carbon. However, we cannot conclusively confirm whether the acetic acid was completely removed. Carbon contents generally decreased for the MBrendel residues relative to the Brendel residues (Figure 1D), but we cannot independently determine if it was the ^{14}C-enriched carbon that was removed. Thus, we conclude that the Brendel and MBrendel methods are

(46) Cullen, L. E.; MacFarlane, C. *Tree Physiol.* **2005**, *25*, 563−569.
(47) Leavitt, S. W. *Can. J. Forest Res.* **1993**, *23*, 210−218.

not acceptable pretreatment methods for ^{14}C isotopic analysis because they are capable of adding old carbon to the sample. The addition of the NaOH step improves the problem but the contamination risk, and possibility of inconsistent results, makes all variants of the Brendel method unreliable for ^{14}C analysis. Such an interpretation is further supported by the work of other researchers who also detected significant fossil fuel depression of ^{14}C contents in measurements of Brendel processed wood samples in a Cordia (species not known) sample from Costa Rica and a *Miliusa velutina* sample from Thailand (ref 24 and Pascale Poussart, personal communication) but not in a *Samanea saman* sample from Indonesia.[25]

The Tiriwood JW-holo residue was within one standard deviation of the bulk sample values as expected since the atmospheric ^{14}C of CO_2 was changing relatively little during the period of Tiriwood production. The Brendel and MBrendel methods both resulted in residues that had lower $\Delta^{14}C$ values relative to the bulk values (by greater than two standard deviations). This again indicates probable contamination by older carbon in the Brendel method that is then ameliorated, in part (but not completely), with addition of the NaOH step in the MBrendel method. The acid/base/acid method also returned residues that were depleted in ^{14}C relative to bulk samples by greater than two standard deviations, but the reason for this remains unknown since no fossil fuel-derived reagents were used in this method.

Across all tissue types, the JW-holo and Brendel methods produced $\Delta^{14}C$ results that were significantly different from the bulk values while the acid/base/acid and MBrendel methods were not (Table 3). The fact that the Brendel and MBrendel methods added old carbon, via acetic acid, makes any Brendel method (even modified with the NaOH step) a risky choice for ^{14}C analysis. It should be noted that the JW-holo treatment does contain an acetic acid step (see step 2). However, at its strongest, the concentration is 19 times weaker than that used in the Brendel methods, and the water rinsing in the JW-holo method is quite lengthy (six discrete rinses with DI in a sonicator followed by at least 4 h of continuous DI rinse). In contrast, the Brendel and MBrendel methods provide few rinses. The acid/base/acid method does not appear to have a consistent affect on $\Delta^{14}C$ values and is therefore an inferior choice relative to the JW-holo method for ^{14}C analysis.

Significance to Community. In recent years, the number of biological studies using stable isotopes has increased tremendously.[13] The need for information comparing the efficacy and chemical purity of different methods with varying tissue types and isotope application has similarly increased. To our knowledge, our study is the first rigorous comparison of the Brendel method (and two variants), Jayme−Wise methods (JW-alpha and JW-holo) and acid/base/acid washes on multiple tissue types (leaves, wood, roots) and for many isotope analyses ($\delta^{13}C$, $\Delta^{14}C$ and $\delta^{18}O$).

Our results showed that for the use of the Brendel method on leaves, roots, and wood (1) an NaOH step should be added, (2) caution (and testing) should be exercised before using a NaOH-modified Brendel method (MBrendel) for ^{18}O analyses, (3) attention should be paid to the N content of the residue relative to the untreated bulk, and (4) the method is a risky choice for use with ^{14}C analyses. While we point out potential problems with the Brendel method as published, simple addition of the NaOH step rectifies most of the problems (except for use with ^{14}C). Addition of further rinsing steps (WMBrendel) does not appear to add additional benefit for any tissue type or isotope analysis.

The Jayme−Wise methods appear to be good sample pretreatments for all tissue types and isotope applications studied here (according to both ^{13}C NMR and isotope data). Although, the ^{13}C NMR results showed that the MBrendel method yielded residues of a purity similar to the Jayme−Wise methods for wood and fine roots, leaf samples appeared to have the least alkyl C residues when processed by the Jayme−Wise methods (particularly the JW-alpha). The same interpretation can be made from the ^{14}C,^{13}C, and ^{18}O isotope data. Of all the methods tested, and across all tissue types and isotope analyses, the acid/base/acid washing was the least effective for removing noncellulose components (particularly lignin) of a sample and generated isotope values that were the least modified relative to the bulk.

In assessing a method to use for sample pretreatment, removal of noncellulose compounds and the quality of the resulting isotope data is of paramount importance. However, other factors also play into the final decision of which method to use. Much of the desire to use the Brendel method was due to its rapid (same day) processing of large sample batches. The Brendel has also been modified to handle sample sizes of <1.5 mg.[24] Since all other methods presented here require a minimum of 20−50 mg of dry sample, the Brendel may be the only possible choice for samples where only a very small amount of material is available. However, for samples of ≥20−50 mg, decreased processing time (per sample) is possible by adding batch processing and sample bagging to the Jayme−Wise method. These modifications have increased sample throughput for this method to many hundreds of samples per week. Furthermore, the many steps of the Brendel method require constant attention by the practitioner and are therefore more likely to produce errors in pretreatment processing and thus errors in the isotope results. As such, any added benefits of same-day cellulose preparation using the Brendel methods may be offset by the many potential sources for error. Similarly, processing of samples using the acid/base/acid method allows for large batch processing within a few hours but this method is inadequate for some analyses.

CONCLUSIONS

A ^{13}C NMR spectral analysis showed that each of the pretreatment methods tested resulted in residues that differ from standard (pure) cellulose; much of this variation is related to differences in alkyl C content, and the degree of discordance varied based on tissue type (leaf, fine root, or wood).

The ^{13}C NMR spectra, $\delta^{18}O$, and $\delta^{13}C$ data of wood and root samples showed small differences between residues resulting from the Jayme−Wise methods and modified Brendel methods. For leaf tissues, the Jayme−Wise methods clearly remove more of the alkyl C moieties than the modified Brendel methods.

Our analyses raised concerns about using the original Brendel method as a cellulose preparation method in general and for ^{18}O and ^{14}C isotope analyses in particular. The ^{13}C NMR spectral analysis showed that the Brendel method did not sufficiently remove lipids and waxes from tissue samples. $\delta^{18}O$ values for the Brendel method were the most enriched relative to all other methods (despite incomplete removal of lipids and waxes). The

strong nitric and acetic acid steps in the Brendel methods also appeared to add carbon and nitrogen to the final residue, and these additional constituents may not be completely removed by subsequent NaOH or water treatments. The presence of acetic acid-derived carbon was clearly evident in the $\Delta^{14}C$ results.

Addition of the NaOH step to the Brendel method (as in the modified Brendel methods) rectified many of these problems. Addition of further rinsing steps did not further improve the method.

The acid/base/acid method generated ^{13}C NMR spectra that showed incomplete removal of lignin for all tissues tested. This result is consistent with finding $\delta^{13}C$ and $\delta^{18}O$ values that were greatly depleted relative to all other treatments. $\Delta^{14}C$ results using the acid/base/acid method were inconsistent.

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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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