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Methods Paper

An extractive removal step optimized for a high-throughput α-cellulose extraction method for $\delta^{13}C$ and $\delta^{18}O$ stable isotope ratio analysis in conifer tree rings

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Stable isotope ratios ($\delta^{13}C$ and $\delta^{18}O$) of tree-ring α-cellulose are important tools in paleoclimatology, ecology, plant physiology and genetics. The Multiple Sample Isolation System for Solids (MSISS) was a major advance in the tree-ring α-cellulose extraction methods, offering greater throughput and reduced labor input compared to traditional alternatives. However, the usability of the method for resinous conifer species may be limited by the need to remove extractives from some conifer species in a separate pretreatment step. Here we test the necessity of pretreatment for α-cellulose extraction in loblolly pine (*Pinus taeda* L.), and the efficiency of a modified acetone-based ambient-temperature step for the removal of extractives (i) in loblolly pine from five geographic locations representing its natural range in the southeastern USA, and (ii) on five other common coniferous species (black spruce (*Picea mariana* Mill.), Fraser fir (*Abies fraseri* (Pursh) Poir.), Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco), Norway spruce (*Picea abies* (L.) Karst) and ponderosa pine (*Pinus ponderosa* D.)) with contrasting extractive profiles. The differences of $\delta^{13}C$ values between the new and traditional pretreatment methods were within the precision of the isotope ratio mass spectrometry method used (±0.2‰), and the differences between $\delta^{18}O$ values were not statistically significant. Although some unanticipated results were observed in Fraser fir, the new ambient-temperature technique was deemed as effective as the more labor-consuming and toxic traditional pretreatment protocol. The proposed technique requires a separate acetone-inert multiport system similar to MSISS, and the execution of both pretreatment and main extraction steps allows for simultaneous treatment of up to several hundred microsamples from resinous softwood, while the need of additional labor input remains minimal.

Keywords: iWUE, pretreatment, resin removal, stable carbon isotope ratio, stable oxygen isotope ratio, water use efficiency.

Introduction

The stable isotope ratios of carbon and oxygen ($\delta^{13}C$ and $\delta^{18}O$) in tree rings are widely used in paleoclimatology, ecology, plant physiology and genetics (McNulty and Swank 1995, Dawson et al. 2002, McCarroll and Loader 2004, Barbour 2007, Treydte et al. 2007, Saurer et al. 2014, Bartholomé et al. 2015, Frank et al. 2015). The isotopic composition of α-cellulose provides an historical record of a number of environmentally and genetically controlled processes (e.g., Wei et al., 2014, and Baltunis et al. 2008), given that α-cellulose is abundant, is synthesized largely of newly assimilated carbon, and the C and O atoms in it do not exchange after its formation (Gaudinski et al. 2005). As the process of α-cellulose isolation from wood samples is usually labor-intensive and time-consuming, a number of different methods have been developed offering a different balance of speed, cost and purity.

Currently, there are over 10 different methods and method variants to choose from for α-cellulose extraction from wood samples, including variants of the Jayme-Wise type (Green 1963, Leavitt and Danzer 1993, Loader et al. 1997, Li et al.

2011, Wieloch et al. 2011, Kagawa et al. 2015, Table 1), Brendel type (Brendel et al. 2000, Evans and Schrag 2004, see variants in Brookman and Whittaker 2012) and the diglyme-HCl methods (Macfarlane et al. 1999, Cullen and MacFarlane 2005). The Brendel and diglyme-HCl methods are simple and fast (<24 h to complete around 100 samples), and do not need special glassware (Cullen and MacFarlane 2005, Brookman and Whittaker 2012). However, the purity of α-cellulose extracted by the Brendel method varies by species (Brookman and Whittaker 2012, see Gaudinski et al. (2005) and Dodd et al. (2008) for additional steps for improving sample quality). The diglyme-HCl method may not be effective with wood with high

Table 1. Comparison of major batch-wise Jayme-Wise α-cellulose extraction methods from wood samples.

Methods	Special equipment[1]	Estimated capital cost[2]	Typical number of samples per batch	Typical processing time in days[3]		
				Main extraction	Pretreatment	Total processing time per batch/ per 1000 samples
Leavitt and Danzer (1993)[4,5]	Soxhlet extractors, special filter paper bags[6]	<$3,000	75–150	2	2–3	4–5/19–20[7]
Kagawa et al. (2015)[4,8]	Water bath, transmitted light microscope, PTFE[9] punch sheets and glass tubes	<$3,000	The number of rings varies due to the ring width. Usually hundreds to thousands rings can be processed in a batch	2	1	3/3
Loader et al. (1997)[4,10]	Customized Soxhlet extraction thimbles, ultrasonic bath and Soxhlet extractors	$5,000–10 000	100	1	1	2/20
Harada et al. (2014)[8]	Microscope, customized polyethylene filters[6], water bath, ultrasonic bath, PTFE[9] tube and glass container	$5,000–10 000	60	2	1	3/48
Wieloch et al. (2011)[4]	Customized multiple sample isolation system (MSISS drainage module), Büchner funnels, vacuum aspirator pump and water bath	>$15 000	≥320, expandable to higher numbers	5	Not equipped	5/15
Wieloch et al. 2011 + acetone pretreatment (current study)	Customized multiple sample isolation system (MSISS drainage module) and Delrin holders, Büchner funnels, vacuum aspirator pump and water bath	>$15 000	≥320, expandable to higher numbers	5	8	13/29[10]

[1]The equipment listed is specific for α-cellulose extraction, in a typical ecological wet laboratory setting as identified in the original publications when possible. Standard laboratory equipment like water purifier, centrifuge and hot plates are not included. The tools for wood sample preparation (grinding or slicing) are not included as well.
[2]Cost estimates are approximate, aiming to group the methods in broad categories rather than offer clear budgetary information. The exact costs will vary by country, vendor and existing infrastructure. Please see Supplementary Data for the cost estimates for the major equipment of each method. Although the reagent cost is proportional to reaction time and individual sample reaction volume, which differs up to 10-fold among the methods, their effect on overall cost is much smaller than that of the specialized equipment, and is not included here.
[3]The processing time is estimated based on literature reports except for the methods by Wieloch et al. (2011). The time estimates are approximate, and the exact time will vary by the researcher, species and availability of equipment. Processing time does not include sample preparation (slicing or grinding), loading and drying, or equipment clean-up and maintenance.
[4]The experimental protocol has been updated since publication. Interested researchers please contact the authors for the latest information. The number of samples per batch can be increased by having additional equipment. Please note that additional cost and labor input would be required in this case.
[5]The information related to this method has been provided by Dr S.W. Leavitt (personal communication, 2016).
[6]Items are disposed of after use.
[7]The extraction and pretreatment are staggered.
[8]The information related to this method has been provided by Dr T. Nakatsuka (personal communication, 2016).
[9]PTFE: polytetrafluoroethylene.
[10]The information related to this method has been provided by Dr N. Loader (personal communication, 2016).

resin and lignin contents (Cullen and MacFarlane 2005). A recent modification of the diglyme-HCl method was found to be successful for two conifer species (Li and Liu, 2013), but remains to be tested more broadly, especially with species with a high lignin content. However, the Jayme-Wise type methods have been found to produce consistently pure α-cellulose (Gaudinski et al. 2005, Kéri et al. 2015). Based on a blind inter-laboratory comparison study, Boettger et al. (2007) found that the different α-cellulose extraction methods (all Jayme-Wise type) used in nine European laboratories produced similar results within the precision of isotope ratio mass spectrometry (IRMS; ±0.2‰ for C and ±0.3‰ for O). The methods consist of two major steps: (i) delignification with acidified sodium chlorite solution (chlorination), and (ii) alkaline hydrolysis with 17% sodium hydroxide solution (purification). For conifers, an additional pretreatment step is usually required for removing extractives prior to extraction (Green 1963, McCarroll and Loader 2004).

As the stable isotope analysis becomes cheaper and faster, processing a large number of samples becomes able to reveal spatial and temporal complexities at a larger scale (Leavitt et al. 2010) and cellulose extraction becomes the rate-limiting step. Thus, new methods for batch-wise cellulose isolation (Table 1) have emerged in recent years. The method for extracting entire intact tree cores (laths; Li et al. 2011; Kagawa et al. 2015) offers the greatest throughput on a per tree-ring basis, but not on per sample basis (see Schollaen et al. (2015) for a complete guide with a costly but convenient semi-automated extraction system, Table 1). With this method, α-cellulose is extracted from intact cross-sectional laths, yielding 'cellulose laths' that retain their structural integrity. Cellulose fibers are pinched with forceps from the annual rings under transmitted light for stable isotope analysis (Li et al. 2011, Kagawa et al. 2015). This method eliminates the time- and labor-intensive peeling–grinding step to produce individual wood samples and made a breakthrough in the throughput of α-cellulose extraction methods to produce tree-ring chronologies. However, in genetic trials and many ecophysiological studies where individual years (rather than full chronologies) or other subsections from trees are of interest, the peeling–grinding step cannot be avoided and the whole lath extraction loses its advantage.

Currently, the highest throughput for the separated wood samples can be achieved using the Multiple Sample Isolation System for Solids (MSISS) developed at the Potsdam Dendro Laboratory, German Research Centre for Geosciences, Germany (Wieloch et al. 2011). It was designed to be modular and extendable to over 300 samples per batch. Although the extraction process takes relatively longer compared to other methods (Table 1), the minimized reaction volume, modular design and vacuum-operated evacuation of consumed chemicals in MSISS allow for greater throughput and significant labor savings.

However, the design considerations of MSISS were based on the chlorination and purification steps described above, but not on the pretreatment step for extractive removal from conifer wood. As the extractives have an isotopic signature distinct from α-cellulose (Harlow et al. 2006), their presence can bias $\delta^{13}C$ and $\delta^{18}O$, pointing to the need to ensure the purity and homogeneity of the sample material (Tao et al. 2010). The need for pretreatment appears to be species-dependent and remains to be debated. Some authors have argued that extractives are removed during the main extraction steps of the Jayme-Wise protocol (Rinne et al. 2005, Boettger et al. 2007), whereas others concluded that an explicit pretreatment was required (Tao et al. 2010). As each of these studies has focused on a few species, the need for the pretreatment step as a general protocol remains a matter of discussion.

The current study was set up to develop and test a pretreatment step for the MSISS-based extraction system to expand the usability of this powerful method to resinous species. The traditional pretreatment technique used in dendrochronological studies (Loader et al. 1997) requires refluxing wood slivers in a mixture of toluene and denatured alcohol for at least 6 h in Soxhlet extractors, which is not compatible with MSISS. An alternative protocol of pretreatment is achieved by soaking wood slivers in acetone at ambient temperature for 8 days (Yokoyama et al. 2002). This technique, popular in wood science but less known in dendrochronology and ecology, was tested for effectiveness in extracting tree-ring α-cellulose samples from different conifer species with contrasting extractive profiles. Additional tests for method sensitivity were performed on loblolly pine (*Pinus taeda* L.), the most important commercial tree species in the USA, as the work was carried out as a part of the PINEMAP project (http://pinemap.org; Will et al. 2015). The current report presents a potential alternative pretreatment step for extractive removal using the high-throughput MSISS apparatus. Thus, the specific objectives of the current study were to (i) test if a pretreatment step to remove extractives is necessary for α-cellulose extraction in loblolly pine growing in contrasting environments; and (ii) test if the modified acetone pretreatment can produce comparable results of isotopic signatures to those produced by traditional toluene-based pretreatment method, using six conifer species with different profiles of extractives.

Materials and methods

Materials and experimental design

Six species with contrasting resin profiles were chosen for the study. Wood samples (10–30 mg, allowing for α-cellulose yield of 30%) were collected from middle-aged to mature loblolly pine, Norway spruce (*Picea abies* (L.) Karst.), Fraser fir (*Abies fraseri* (Pursh) Poir.), ponderosa pine (*Pinus ponderosa* D.), Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) and black spruce (*Picea mariana* Mill.) trees (Table 2). Loblolly pine was

subsampled to evaluate the resolution of the method for detecting range-wide variance, and inter-annual differences between wet and dry years. The additional species were selected to span conifer species with a range of extractive contents in the xylem. For deriving plant water status and intrinsic water use efficiency in a given year, α-cellulose was extracted from the latewood portion of the rings because earlywood may be partly produced using carbohydrate reserve produced in the previous year (McCarroll and Loader 2004). In each species except black spruce, whose wood materials were obtained from tree cores, entire wood disks were cut at breast height and latewood slivers were sampled from selected growth rings. As the occurrence of extractives is expected to be higher under drought stress (Lautner, 2013), and the goal of the current study was to critically evaluate the effectiveness of a new extractive removal step, samples from dry years were selected when possible. However, in the case of loblolly pine from Florida, wood from a wet year was analyzed because the growth rings in dry years were too narrow to sample. In addition, multiple adjacent annual growth rings were combined in black spruce, because the core material in one annual ring did not provide the minimum required sample weight (10 mg, according to the yield rate and guideline on sample weight from Cornell Stable Isotope Laboratory where the stable isotope analysis was conducted).

Meteorological dry years were identified using the US Drought Monitor (http://droughtmonitor.unl.edu/) for samples from the USA, and site-specific meteorological records from the Estonian Meteorological and Hydrological Institute (http://www.ilmateenistus.ee) for samples in Estonia.

Four separate experiments were conducted (Table 3). To reach our first objective, α-cellulose was extracted from two loblolly pine latewood samples produced in a dry and a wet year from GA, USA, with and without traditional pretreatment (three replicates, Experiment 1). Experiments 2–4 were designed to test if the modified acetone pretreatment by Yokoyama et al. (2002) (acetone pretreatment hereafter) can produce comparable results of isotopic signatures to those produced by traditional pretreatment method. Because composition and content of extractives may vary with locations, we extracted α-cellulose with traditional and acetone pretreatments using wood samples

Table 2. Samples used for evaluating the effectiveness of acetone pretreatment in a multiport extraction system for analyzing the isotopic composition of α-cellulose.

Species	Location	The year of latewood sampled	Number of replicates
Loblolly pine (*Pinus taeda* L.)	Clarke County, Georgia, USA	2010 (wet year), 2008 (dry year)	3 and 10[3]
Loblolly pine	Washington County, North Carolina, USA	2008 (dry year)	3
Loblolly pine	Buckingham County, Virginia, USA	2002 (dry year)	3
Loblolly pine	McCurtain County, Oklahoma, USA	2011 (dry year)	3
Loblolly pine	Alachua County, Florida, USA	2004 (wet year)[1]	3
Norway spruce (*Picea abies* (L.) Karst.)	Elva, Estonia	2011 (dry year)	3
Fraser fir (*Abies fraseri* (Pursh) Poir.)	Boone County, North Carolina, USA	2008 (dry year)	3
Ponderosa pine (*Pinus ponderosa* D.)	Klamath County, Oregon, USA	1994 (dry year)	3
Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco)	Klamath County, Oregon, USA	1994 (dry year)	3
Black spruce (*Picea mariana* Mill.)	Saskatchewan, Canada	Multiple years[2]	3

[1]The latewood produced during dry years was too thin for separation. Thus latewood produced in a wet year was used.
[2]Because the wood material in one annual ring of black spruce did not meet the minimal weight requirement for α-cellulose extraction, wood from multiple rings was used.
[3]The wood samples were used for two studies with different number of replicates.

Table 3. The purposes of experiments and statistical analysis methods used.

Experiment	Purpose	Plant material	Replicates	Factors and levels for two-way ANOVA
1	Evaluate the need for extractive removal pretreatment for loblolly pine	Loblolly pine (GA)	3	Pretreatment: with/without traditional pretreatment Climate: wet/dry
2	Evaluate the effectiveness of acetone pretreatment for loblolly pine	Loblolly pine (FL, GA, NC, OK and VA)	3	Pretreatment: traditional/acetone pretreatments Location: five states in southeastern USA
3	Evaluate the utility of the acetone pretreatment for capturing the variation in the isotopic composition of α-cellulose	Loblolly pine (GA)	10	Pretreatment: traditional/acetone pretreatments Climate: wet/dry
4	Evaluate the effectiveness of acetone pretreatment across five conifer species with contrasting resin profiles	Norway spruce, Fraser fir, ponderosa pine, Douglas fir and black spruce	3	Pretreatment: traditional/acetone pretreatment Species: five conifer species

of loblolly pines from five states (VA, NC, GA, FL and OK) across the southeastern USA with three replicates (Experiment 2). Specifically, a fraction of latewood samples from GA was selected for a full factorial analysis of wet and dry year difference in 10 replicates (Experiment 3) to capture any variation of the stable isotope analysis beyond the precision of the IRMS method used. Finally, we tested acetone pretreatment on five coniferous species: black spruce, ponderosa pine, Douglas fir, Fraser fir and Norway spruce (Table 2) for wider application of this method with three replicates (Experiment 4).

The ^{13}C and ^{18}O stable isotope ratios of all extracted α-cellulose were determined at the Cornell University Stable Isotope Laboratory (http://www.cobsil.com), using Thermo Delta V isotope ratio mass spectrometer interfaced to a NC2500 elemental analyzer and to a Temperature Conversion Elemental Analyzer. The within-run isotopic precision of the methodology using quality control standards is 0.2‰ for carbon and 0.4‰ for oxygen.

Extraction apparatus (MSISS and Delrin holders)

With the goal of increased sample throughput, and with minimum labor input, the Potsdam Dendro Laboratory (Wieloch et al. 2011) developed the MSISS. Following their technical drawings, we manufactured a set of MSISS drainage modules at the North Carolina State University Precision Instrument Machine Shop (http://www.engr.ncsu.edu/machineshop). We modified the unit dimensions to accommodate the available Pyrex® 2 ml Büchner funnels with 10 mm-diameter coarse porosity fritted discs.

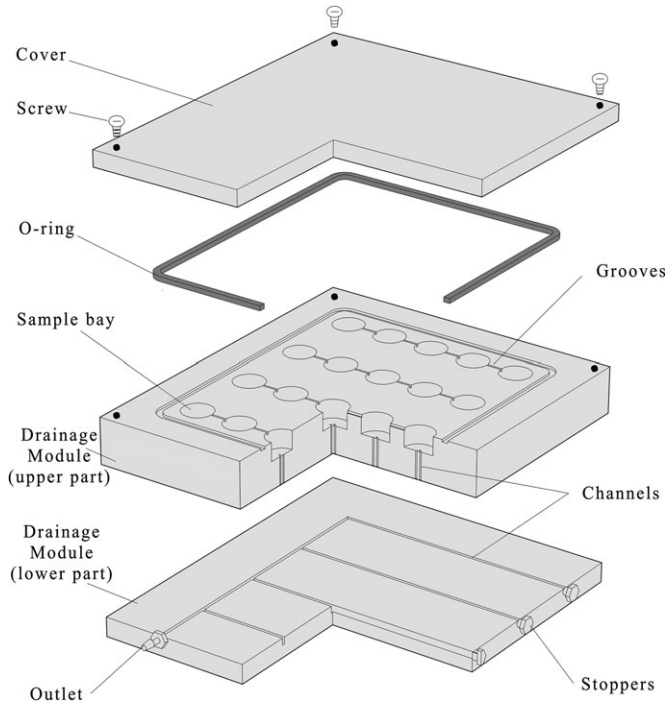

Figure 1. The schematic drawing of Delrin holder for acetone pretreatment.

Due to the different corrosiveness of acetone (used in pretreatment) and sodium hydroxide and sodium chlorite (used in the main extraction steps), we have developed a separate MSISS-like module out of Delrin (polyoxymethylene; Figure 1), which is resistant to acetone and cheaper than polytetrafluoroethylene (PTFE) used for MSISS.

The body of the Delrin holder is made of a solid Delrin® block which encases a network of channels. The main difference of the pretreatment module compared to the MSISS module is that the samples are treated in drilled-out sample bays rather than in Büchner funnels. Twenty sample bays are drilled in the same 4 × 5 arrangement as funnel holes on MSISS. Each bay is enlarged to 2 ml in volume so that it can hold wood slivers, while its bottom is connected to the inter-linked channel system inside the block by four 0.5 mm holes. A thin Delrin plate is used as a cover to prevent the evaporation of acetone with the help of an acetone-resistant O-ring. Once wood slivers are loaded into the sample bays, water or acetone is added, the cover is attached to the block with screws. The draining of extractant is done with a vacuum aspirator pump similar to MSISS.

α-Cellulose extraction

Each latewood sample was cut into ~0.3 mm thick slivers using a razor blade. Wood slivers of each sample were then mixed and divided into two fractions. For Experiment 1, one half of the slivers was prepared using the traditional pretreatment, that was carried out in a Soxhlet extractor using a 2:1 mixture of toluene and denatured alcohol, with 8 h of refluxing (Loader et al. 1997) while the other half was not treated. For Experiments 2–4, the other half was prepared with acetone pretreatment, that was completed by an overnight soaking in deionized water followed by 8-day-soaking in acetone (acetone was replaced every 2 days), modified from Yokoyama et al. (2002).

The samples were treated identically after pretreatment and tree-ring α-cellulose was extracted using MSISS. The extraction protocol was adopted from Wieloch et al. (2011) except that each step of chlorination was shortened to 7 h from 10 h due to the evaporation of solution from funnels and we repeated chlorination until cellulose became pure white. After extraction, α-cellulose samples were homogenized using a Branson 450 Sonifier Analog Cell Disruptor, similar to Laumer et al. (2009). The main steps of sample processing and α-cellulose extraction are illustrated in Figure 2.

Statistical analysis

Statistical analyses were performed using the R software (Version 3.2.2; R Core Team, 2015). Two-way analysis of variance (ANOVA) was conducted for Experiments 1–4 (Table 3). Data of $\delta^{13}C$ and $\delta^{18}O$ were analyzed the same way but separately except for $\delta^{18}O$ values in Experiment 3, where an outlier was detected (>5 times the interquartile range above the third quantile). We estimated a value to replace the outlier and

corrected the bias according to Ott and Longnecker (2001), and then applied two-way ANOVA in this case.

Results

The necessity of pretreatment for α-cellulose from wood samples of loblolly pine

The need of a pretreatment step to remove extractives prior to chlorination and purification was tested in Experiment 1 (Tables 3 and 4). Compared to the traditional toluene-based pretreatment, the omission of the pretreatment step resulted in 0.28‰ higher $\delta^{13}C$ estimates in the dry year, and 0.62‰ lower estimates in the wet year. The effect was statistically significant ($P < 0.01$), and exceeded the precision uncertainty of the IRMS method 0.2‰. For $\delta^{18}O$, the difference (−0.94‰ in the dry year and 0.54‰ in the wet year) exceeded the IRMS uncertainty threshold (0.4‰) but was not statistically significant ($P = 0.26$).

Comparison of two pretreatments using stable isotope ratios

The $\delta^{13}C$ and $\delta^{18}O$ values in Experiments 2–4 were examined to test the effectiveness of the acetone pretreatment compared to the traditional pretreatment (Table 5). The mean difference between the traditional and acetone pretreatments was 0.01‰ for $\delta^{13}C$ (ranging from −0.07‰ to 0.16‰), and 0.12‰ for $\delta^{18}O$ (ranging from −0.45‰ to 0.74‰). If the data of Fraser fir are excluded, the mean difference was 0.06‰ for $\delta^{18}O$, ranging from −0.45‰ to 0.32‰.

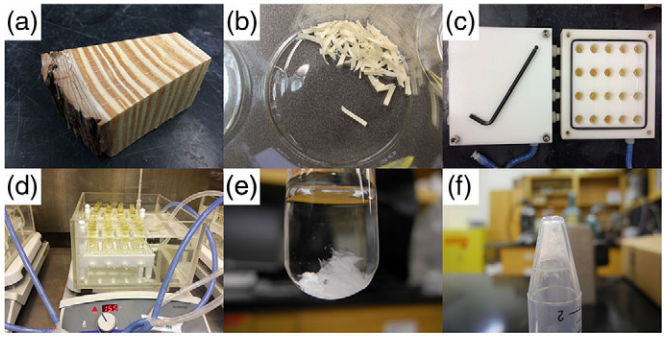

Figure 2. The main steps of α-cellulose extraction: (a) a surfaced wood wedge for sampling; (b) wood slivers for α-cellulose extraction; (c) slivers in Delrin holders with/without the lid (pretreatment); (d) α-cellulose extraction by MSISS; (e) extracted α-cellulose in chips and fibers; (f) homogenized and dried α-cellulose. (All photos by W. Lin. The glass tube in (e) and plastic vial in (f) are for demonstration only and were not used in the experiment.)

The two-way ANOVA on loblolly pine samples from five locations in southeastern USA (Experiment 2) indicated that the main effect of pretreatment was statistically significant for $\delta^{13}C$ ($P = 0.03$), while insignificant for $\delta^{18}O$ ($P = 0.99$). When the sample size increased to 10 (Experiment 3 with loblolly pine samples from GA, Figure 3), we obtained similar results ($P < 0.01$ for $\delta^{13}C$ and $P = 0.77$ for $\delta^{18}O$). However, the 95% confidence intervals ([0.01‰, 0.08‰] for the wet year and [0.05‰, 0.18‰] for the dry year) between means of $\delta^{13}C$ from Experiment 3 were smaller than the ±0.2‰, the resolution of the IRMS method.

Unlike in loblolly pine, the differences between the two pretreatments were not statistically different in the other species ($P = 0.59$ for $\delta^{13}C$ and $P = 0.09$ for $\delta^{18}O$, Table 5). With Fraser fir excluded, the *P*-value for the main effect of pretreatment on $\delta^{18}O$ increases to 0.32.

Discussion

The necessity of pretreatment for α-cellulose from wood samples

The need for an explicit extractive-exclusion treatment prior to α-cellulose extraction remains open to debate. Most Jayme-Wise methods include this step (Green 1963, Leavitt and Danzer 1993, Loader et al. 1997, Li et al. 2011, Kagawa et al. 2015). Yet, some studies argued that the extractives in at least some conifer species are removed in the regular two-step α-cellulose extraction (e.g., Rinne et al., 2005). However, it is also recognized that contamination by lipids may be possible if the pretreatment step is omitted (Rinne et al., 2005; Tao et al., 2010). Our current findings lend support to this argument.

Compared to primary carbohydrates, lipids are generally more depleted in ^{13}C (Melzer and Schmidt 1987), whereas the reported $\delta^{13}C$ values of α-cellulose are usually higher than other wood components (e.g., Loader et al., 2003). However, our study found that the $\delta^{13}C$ values of α-cellulose extracted from wood samples produced in a wet year without pretreatment (presumably with more remaining lipids) were also enriched compared to those with pretreatment (Table 4). This is in agreement with the study reported by Taylor et al. (2007). The authors compared the $\delta^{13}C$ values of extractives and α-cellulose

Table 4. Carbon and oxygen stable isotope ratios (±1σ) of latewood α-cellulose extracted from samples of loblolly pine from GA, USA with and without traditional pretreatment in 2 years with contrasting precipitation profiles ($N = 3$).

Samples	$\delta^{13}C$ (‰, VPDB)			$\delta^{18}O$ (‰, VSMOW)		
	Traditional pretreatment	No pretreatment	Mean difference	Traditional pretreatment	No pretreatment	Mean difference
Loblolly pine, GA, dry year	−24.74 ± 0.02	−25.01 ± 0.04	0.28	32.9 ± 0.25	33.84 ± 0.09	−0.94
Loblolly pine, GA, wet year	−26.81 ± 0.05	−26.19 ± 0.05	−0.62	32.45 ± 0.13	31.90 ± 0.16	0.54

of Douglas fir, and some of the former were enriched compared to the latter. Thus, the pattern of $\delta^{13}C$ of extractives and α-cellulose appears to be more complicated, probably due to the different components of extractives produced in a specific year and those that remain after the extraction processes.

Comparison of two pretreatments

The acetone pretreatment step arguably removes over 95% of the nonvolatile extractives from the wood of loblolly pine (Yokoyama et al. 2002). In the current study, we found that the acetone pretreatment adapted for the multiport system produced comparable results to those by traditional toluene-based pretreatment. Although the $\delta^{13}C$ signatures were significantly different for loblolly pine samples following these two pretreatments (Table 5), the differences were smaller than the accuracy of the subsequent IRMS. However, the difference of $\delta^{18}O$ values of Fraser fir samples between the two pretreatments is unexpected. As a species without resin canals, the pretreatment was expected to have no effect on Fraser fir samples.

Given the success of this acetone-based pretreatment in most tested species, in terms of stable isotope ratios, we propose this pretreatment as a viable replacement for the more labor-consuming and toxic traditional toluene-based pretreatment in applications where individual annual rings are to be analyzed. For the laboratories that apply less highly equipped variants of the Jayme-Wise method using Teflon filter bags like Leavitt and Danzer (1993), the acetone pretreatment can be easily adopted by allowing multiple bags soaking in deionized water and acetone within a sealed container. Although the technique worked reliably in five out of six species, the unexpected result in Fraser fir for the difference of $\delta^{18}O$ suggests that validation with new species is advisable.

Sample preparation and further methodological suggestions

The MSISS method (Wieloch et al. 2011) is recommended for small samples (2.5–50 mg). Given that the 2 ml well size in the pretreatment module is sufficient for extracting this amount of sample with acetone (H. Chang, personal communication, 2012), our proposed pretreatment system is well suited for coupling with MSISS.

Directions on both grinding and slicing are available from Wieloch et al. (2011). Delrin holders with the design as shown in Figure 1 work best with sliced wood samples. Wiley mill, ball mill or Wig-L-Bug grinding mill would cause major or complete sample loss if the wood material is <10 mg. In such situations, slicing wood samples becomes the only option.

However, when sample size allows homogenization by grinding (see Borella et al. (1998) for a theoretical calculation and discussion on pooling and milling for sample homogeneity), the powder of ground samples may block the channels at the bottom of sample bays of the Delrin holders, complicating sample transfer from the Delrin to MSISS module. If wood powder is used for α-cellulose extraction, we recommend adding a layer of molded stainless steel mesh to each access point and increasing the wall height of the Delrin holder so that the cover can still seal well after this addition. As containers for individual wood samples, the mesh layers would also make transferring wood samples to MSISS from Delrin holders more convenient.

Table 5. Carbon and oxygen stable isotope ratios (±1σ) of α-cellulose extracted from samples of loblolly pine, ponderosa pine, black spruce, Douglas fir, Norway spruce and Fraser fir with acetone pretreatment and traditional pretreatment (VPDB, Vienna Pee Dee Belemnite; VSMOW, Vienna Standard Mean Ocean Water).

Samples	*N*	$\delta^{13}C$ (‰, VPDB)			$\delta^{18}O$ (‰, VSMOW)		
		Acetone pretreatment	Traditional pretreatment	Mean difference	Acetone pretreatment	Traditional pretreatment	Mean difference
Loblolly, GA, dry year	10	−24.25 ± 0.02	−24.36 ± 0.02	0.11	32.39 ± 0.06	32.34 ± 0.07	0.05
Loblolly, GA, wet year	10	−26.74 ± 0.01	−26.79 ± 0.01	0.05	30.01 ± 0.15	30.14 ± 0.21[1]	−0.13
Loblolly, NC	3	−25.47 ± 0.02	−25.44 ± 0.05	−0.03	31.73 ± 0.16	31.49 ± 0.11	0.24
Loblolly, OK	3	−24.84 ± 0.03	−24.77 ± 0.02	−0.07	33.30 ± 0.10	33.12 ± 0.20	0.18
Loblolly, VA	3	−25.14 ± 0.02	−25.30 ± 0.05	0.16	32.04 ± 0.16	32.49 ± 0.33	−0.45
Loblolly, FL	3	−26.25 ± 0.03	−26.29 ± 0.07	0.04	32.11 ± 0.21	31.97 ± 0.17	0.14
Ponderosa pine	3	−22.96 ± 0.02	−22.99 ± 0.01	0.03	30.77 ± 0.12	30.48 ± 0.15	0.29
Black spruce	3	−23.88 ± 0.03	−23.83 ± 0.15	−0.05	25.03 ± 0.10	25.16 ± 0.02	−0.13
Douglas fir	3	−22.93 ± 0.02	−22.87 ± 0.02	−0.06	30.25 ± 0.17	29.93 ± 0.11	0.32
Norway spruce	3	−26.67 ± 0.02	−26.65 ± 0.00	−0.02	23.59 ± 0.38	23.47 ± 0.52	0.12
Fraser fir	3	−24.26 ± 0.15	−24.22 ± 0.06	−0.04	27.94 ± 0.34	27.20 ± 0.27	0.74
Average				*0.01*			*0.12/0.06*[2]
Standard deviation				*0.07*			*0.29/0.23*[2]

[1]An outlier was excluded.

[2]The average and standard deviations of $\delta^{18}O$ mean differences between acetone and traditional pretreatments for α-cellulose extraction were calculated with and without data from Fraser fir.

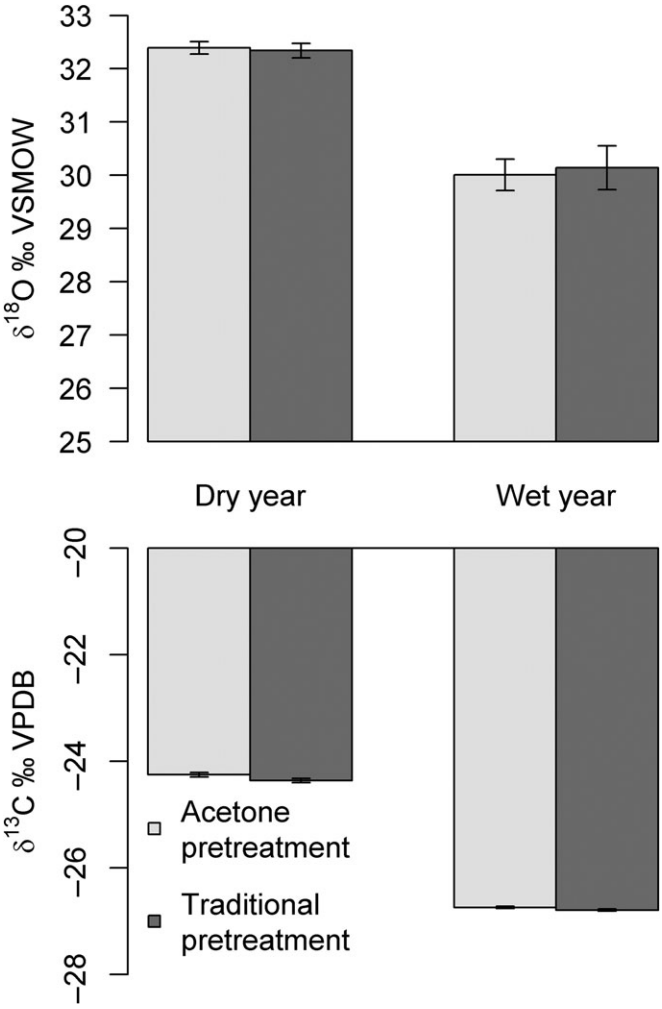

Figure 3. Mean carbon and oxygen stable isotope ratios (with 95% confidence intervals) of α-cellulose extracted from wood samples of loblolly pine from GA, USA in 2 years (a wet and a dry year) with acetone pretreatment and traditional pretreatment ($n = 10$, see Table 3).

Conclusions

Our results suggest that the chlorination and purification steps may not remove the majority of extractives in wood of loblolly pine, and that an explicit pretreatment step may be necessary for conifer species. The modified acetone pretreatment based on Yokoyama et al. (2002) was as effective as the traditional toluene-based methods for removing extractives from the wood of five widely spread conifer species. The method is easy and safe to apply to MSISS and other Jayme-Wise variants using Teflon filter bags. When combined with MSISS, the labor savings from the standardized and MSISS-compatible system quickly offset the upfront equipment costs for a different solvent-resistant sample processing apparatus (e.g., Delrin holder). Although this pretreatment method worked well with five out of six common and contrasting conifer species, we recommend that additional tests be performed with new species to confirm efficacy.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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