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An optimized method for stable isotope analysis of tree rings by extracting cellulose directly from cross-sectional laths

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ABSTRACT

Stable isotopes in tree-ring α-cellulose are valued as environmental proxies and their use is steadily increasing; however, preparation of α-cellulose is a bottleneck in isotope analysis. Recent methodological breakthrough for extracting tree-ring α-cellulose directly from tree-ring cross-sectional laths drastically increased the throughput of tree-ring isotope data. In this paper, we evaluate our recently designed "cross-section" method. This method employs polytetrafluoroethylene (PTFE) cases, enabling direct extraction of α-cellulose from 1-mm thick tree-ring laths, in combination with fixation sheets to prevent disintegration of freeze-dried α-cellulose laths. Perforated PTFE cases are easily producible at an affordable cost. They are made of commonly available lab consumables in catalogs and do not require specially made PTFE parts. Freeze-dried α-cellulose laths preserved distinct anatomical structure, enabling precise separation at the tree-ring boundaries. Once separated from a lath, tree-ring α-cellulose can be weighed directly into silver or tin capsules for analysis. We checked chemical purity of α-cellulose prepared by the cross-section method from five tree species (larch, pine, spruce, beech, and oak). Residual lignin and hemicellulose contents were quantitatively assessed by Fourier transform infrared spectrometry and gas chromatography. The average chemical purity of α-cellulose laths from the five species was 94.5%, similar to the chemical purity of α-cellulose prepared with the standard Jayme-Wise method. Both oxygen and carbon isotope values of α-cellulose prepared by the cross-section method also closely matched those prepared by the standard method. We conclude that, by overhauling the method of α-cellulose preparation for tree-ring isotope analysis, we increased throughput of tree-ring oxygen and carbon isotope data without sacrificing sample purity.

1. Introduction

Stable isotopes in tree rings have a range of applications. They can be used to reconstruct paleoclimate (McCarroll and Loader, 2004; Grießinger et al., 2011; Xu et al., 2011; Sano et al., 2013) and study the expected responses of trees to climate change and elevated CO_2 (Kagawa et al., 2003; Saurer et al., 2004; Kirdyanov et al., 2008; Battipaglia et al., 2013), or used in place of tree-ring width measurements as alternative tools for cross-dating (Roden, 2008) or provenancing the geographical origins of timber (Kagawa and Leavitt, 2010). UV-laser micro dissection, a recent methodological breakthrough, enables analysis of tree rings at higher resolution (Schollaen et al., 2014). However, although stable isotope analysis has become cheaper and faster thanks to advances in mass spectrometry (Brenna et al., 1997; Farquhar et al., 1997; Saurer et al., 1998; Filot et al., 2006), the cellulose extraction process still remains the most laborious and time-consuming part of tree-ring isotope analysis.

Stable isotope ratios of tree-ring wholewood are typically measured after removing extractives. However, wholewood is composed of three major chemical components -cellulose, hemicellulose and lignin- and each of these has a different ratio of stable isotopes (Borella et al., 1998, 1999; Barbour et al., 2001; Loader et al., 2003; Verheyden et al., 2005). Accordingly, α-cellulose, which is valued for its immobility after tree-ring formation as well as its singular chemical composition, has become the preferred material for stable isotope analysis of tree rings (Wilson and Grinsted, 1977; Burk and Stuiver, 1981; Leavitt and Danzer, 1993; Macfarlane et al., 1999; McCarroll and Loader, 2004). For example, cellulose/lignin ratio differs between juvenile and mature wood, and between sapwood and heartwood (Saka, 1991; Leuenberger

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et al., 1998; Rowell et al., 2012). Wholewood and α-cellulose may therefore give different low-frequency isotope signals. To increase the efficiency of extracting α-cellulose and holocellulose, various methods have been proposed (Green, 1963; Leavitt and Danzer, 1993; Loader et al., 1997; Brendel et al., 2000; Cullen and MacFarlane, 2005; Gaudinski et al., 2005; Rinne et al., 2005; Wieloch et al., 2011), all of which have one point in common: Tree rings are first separated, and each separated ring is placed in a vial for the processing of cellulose. Intense effort is required, including grinding or slicing and then chemically processing each tree-ring sample in a separate vial. Because of the large number of samples generated with these "standard" methods, the cellulose extraction process remains the major bottleneck in development of large-scale tree-ring isotope network data.

The first attempt to chemically batch-process whole tree-ring strips and extract holocellulose before separation of tree rings was made by Loader et al. (2002), who immersed whole cores in an acidified sodium chlorite solution. However, the carbon isotope values obtained were significantly offset from values obtained with the standard method, suggesting insufficient purification of the holocellulose. Then Li et al. (2011) made a breakthrough with a method to extract α-cellulose directly from tree-ring laths of 3.5–4.0 mm thickness. Carbon and oxygen isotope values obtained with Li et al. (2011)'s method closely match those obtained with the standard Jayme-wise method (Leavitt and Danzer, 1993; Loader et al., 1997), but the resulting α-cellulose laths are fragile and require mechanical support. In our experience, this method has caused laths (1-mm thick teak, for example) to crack at ring boundaries, and we have also had difficulty keeping the tree rings in the right order. Therefore, in this study, we examined an improved method to overcome the difficulties inherent in Li et al. (2011)'s method.

To apply Li et al. (2011)'s method to teak and other tree species, we designed the first prototype of PTFE case that encloses a whole tree-ring lath and a "cross-section" method to chemically process and dry the laths within this case (Xu et al., 2011; Kagawa and Nakatsuka, 2012; Sano et al., 2013). We were thereby able to extract α-cellulose laths of satisfactory purity from teak without disintegration (Kagawa and Nakatsuka, 2012). Whereas Li et al. (2011) soak α-cellulose laths in acetone to prevent them from breaking while separating each tree ring from the lath under a stereomicroscope, we devised a simpler alternative method to fix dried α-cellulose laths onto sheets.

In the standard α-cellulose extraction method based on the Jayme-wise method, wood is either ground to a particle size of less than 30 mesh (Green, 1963; Leavitt and Danzer, 1993) or sliced into thin slivers (Nakatsuka et al., 2004; Laumer et al., 2009; Wieloch et al., 2011; Harada et al., 2014), then treated with bleaching solutions and alkali solutions. Fourier transform infrared analysis (FTIR) can be used for checking the purity of α-cellulose (Brendel et al., 2000; Anchukaitis et al., 2008; Li et al., 2011; Harada et al., 2014). However, although more quantitative methods, such as the alditol-acetate method – which determines residual hemicellulose content using gas chromatography – exist, we find no study that quantitatively determines how much residual lignin and hemicellulose is present in the α-cellulose and the extent of possible isotopic ratio offset. This is important because extraction of α-cellulose may be incomplete, especially when tree-ring laths of 1 mm thicknesses – instead of wood particles or slivers – are processed in chemical solutions, which may not soak through to the inside of the tree-ring lath. In this study, in order to quantitatively assess the effect of such impurities on oxygen and carbon isotope values, we used acid hydrolysis and the alditol acetate method (Borchardt and Piper, 1970; Blakeney et al., 1983) to determine the amount of Klason lignin and hemicellulose in chemically processed tree-ring laths.

The objectives of this study were as follows:

(1) To examine whether tree-ring oxygen and carbon isotope values obtained with our cross-section method match those obtained with the standard method (Leavitt and Danzer, 1993; Loader et al., 1997; Harada et al., 2014).
(2) To quantitatively evaluate the effects of impurities in α-cellulose and holocellulose laths on oxygen and carbon isotope values.
(3) To assess if any contamination is caused by adhering α-cellulose or holocellulose laths (hereafter collectively called "cellulose laths") to fixation sheets.
(4) To find the optimum drying method to minimize shrinkage and cracking of cellulose laths.

2. Material and methods

2.1. Preparation of tree-ring laths

As test samples for our cross-section method, we used disk samples taken from five tree species (*Larix gmelinii*, *Picea abies*, *Pinus koraiensis*, *Fagus crenata*, and *Quercus crispula*) at the Botanical Garden of Hokkaido University in Sapporo, Japan. These were sliced by diamond saw microtome (SP1600, Leica microsystems, Diamond wheel saws by Buehler or Presi can also be used. Slicing by diamond saw reveals distinct anatomical structure at the cutting surface, but a circular (twin) saw could also suffice.) into cross-sectional laths of 1 mm (longitudinal length) × 12 mm (tangential) × less than 83 mm (radial), as shown in Fig. 1a and weighed. Prices of diamond saws range 9000–30,000 USD in Japan and are typically used for cutting geological and medical samples, such as rock and bones. To check the minimum thickness for extracting α-cellulose without disintegration, we prepared cross-sectional laths of 0.2-0.9 mm thickness from oak and teak. Furthermore, to test suitability of the cross-section method for archeological wood, we also prepared 1-mm thick laths from archeological wood of seven species (*Chamaecyparis*, *Cryptomeria*, *Pinus*, *Sciadopitys*, *Cinnamomum*, *Quercus* and *Zelkova*) with various magnitudes of decomposition.

2.2. Polytetrafluoroethylene (PTFE) cases

Each lath was enclosed in a custom-designed PTFE case and sealed with cotton thread (Fig. 1b). This required about 5 min per case. The case consisted of a rectangular frame cut from a 1.5-mm thick PTFE sheet, sandwiched between two perforated PTFE sheets (Teflon punching sheet, 0.5 mm thick, hole size φ1.5 mm, pitch 3.0 mm, part #: TCF07027-3, TIC Co. Ltd.) to cover both sides of the rectangular frame. The gap between the two perforated cover sheets was adjusted to 1.5 mm, to create a 0.5 mm gap after enclosing a 1.0 mm-thick lath so that chemical reagents could flow through and reach all surfaces of the lath. The gap allowed the laths to shrink freely within the case during chemical treatment and drying, but was narrow enough to prevent the laths from overlapping and shifting out of order.

For our routine analysis of 5-mm cores, we used 1.5-mm thick PTFE sheets with two rectangular spaces of 5.2 × 83 mm to enclose two laths.

2.3. Chemical treatment

For chemical treatment of the tree-ring laths, we followed the general methodology of Jayme-Wise (Green, 1963; Loader et al., 1997; Li et al., 2011).

To remove extractives, the laths in the PTFE case were first treated with a toluene/ethanol mixture (1:1) in a 2-L Soxhlet extractor for at least 48 h, dried in a fume hood for 1–2 h, and then boiled in hot water for at least 48 h. A portion of the laths were set aside to be used as wholewood samples for lignin and hemicellulose analysis. Prior to the analysis, the wholewood was ground in a ball mill.

Each PTFE case, still wet, was transferred into a "fruit fly vial" (Fig. 1c), a glass vial with a flat bottom (inner diameter 33.3 mm, height 130 mm) able to hold up to four PTFE cases. Deionized water (DI water) was preheated in a large beaker and sodium chlorite and acetic acid were added to achieve the same chemical reagent concentration as described in Loader et al. (1997) and Leuenberger et al. (1998). Each vial

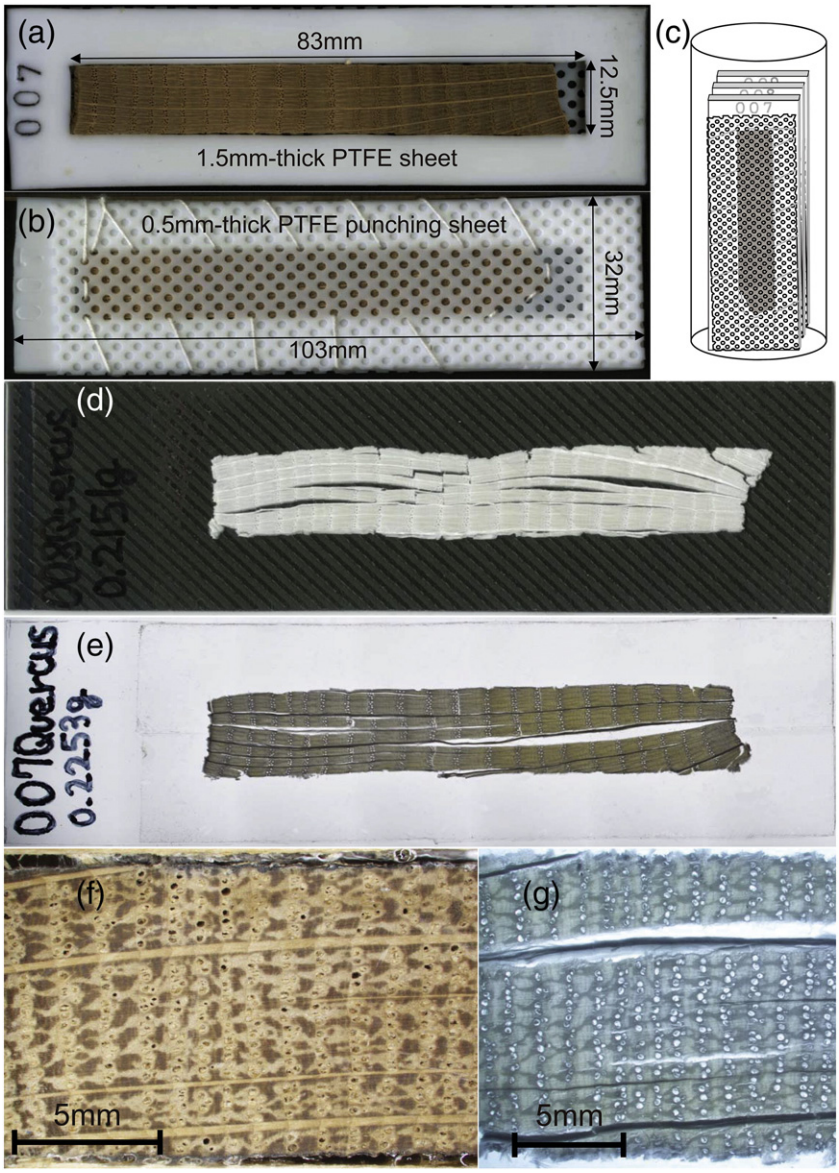

Fig. 1. The PTFE container, vial, fixation sheets and tree-ring laths: (a) PTFE container before enclosing tree-ring lath, (b) PTFE container after enclosing tree-ring lath with cotton thread, (c) vial with flat bottom for chemical treatment in water bath, (d) α-cellulose lath of *Quercus* pasted on album mount sheet and on (e) transparent plastic sheet with removable double-sided tape, (f) microscopic photos of *Quercus* tree rings before and (g) after α-cellulose extraction.

was filled with the solution (about 75–100 ml per vial) and placed in a stainless steel vial rack. The rack was then transferred to a 70 °C water bath and the bleaching reaction was allowed to continue for 1 h before the solution in the vials was replaced. This process was repeated four times for a total reaction time of 4 h. When laths retained a yellowish tinge after the fourth reaction, they were subjected to a fifth reaction period.

After completion of the bleaching reaction, hot DI water was poured into the vials to rinse the laths in their PTFE cases, and they were left for at least 5 min in an ultrasonic bath. This rinsing process was repeated more than three times until electron conductivity of rinsing water became lower than 10 μS/cm. Then, the vials were filled with DI water and left to soak overnight. Again, a portion of the laths (holocellulose laths) were set aside for lignin and hemicellulose analysis.

The following day, sodium hydroxide solution (17.5% wt/wt) was prepared in a large beaker and poured into the vials carefully, since the tree-ring laths are very fragile at this stage. The vials were transferred in the rack to a water bath set at 80 °C and left for 1 h. The sodium hydroxide solution was then renewed and the vials left for another hour. This process was repeated once more for a total reaction time of 3 h.

After the sodium hydroxide treatment, cold DI water was poured into the vials and they were placed in a vacuum desiccator for 5 min to remove air bubbles trapped in the pores of the PTFE cases and the water was drained. This rinsing process was repeated until the decanted solution reached a pH of less than 10. For neutralizing, hydrochloric acid was added to adjust the solution pH to 2. This rinsing/vacuuming process was repeated until the pH of the decanted solution fell to between 5 and 7 to obtain laths of α-cellulose.

2.4. Drying, fixation and dissection of α-cellulose laths

To find the optimal method of drying the α-cellulose laths, we tested three methods: (1) freeze-drying for more than 24 h, (2) dehydration by replacing water in the α-cellulose laths with graduated series of ethanol (Jansen et al., 1998), and (3) oven drying overnight at 70 °C. Throughout the drying processes, the α-cellulose laths were kept within the PTFE cases. Among the three drying methods, freeze-drying showed

the best results and all cellulose laths used in isotope analysis were freeze-dried. The only disadvantage of freeze-dried laths was their fragility, which we overcame by adhering them to fixation sheets.

Ethanol-dried samples showed the second lowest shrinkage (Table 1). We suggest this drying method as a good alternative for laboratories without freeze-drying equipment. Oven-dried α-cellulose laths showed the highest shrinkage, and separating these tree rings by knife was difficult because they were the hardest among the three.

After freeze-drying the α-cellulose laths in the PTFE cases overnight, we removed cotton threads from each PTFE case and carefully removed one PTFE cover sheet to reveal the α-cellulose lath. A rectangle cut from an album mount sheet (A-LDR-5, Nakabayashi Co. Ltd.) was pushed against each lath to adhere the lath onto the sheet (Fig. 1d). The weight of the lath was measured to calculate the yield. While album mount sheets were used for samples with clear ring boundaries, in cases of ambiguous ring boundaries, we wanted to observe tree-ring samples with transmitted light, so such laths were instead pasted onto 0.5 mm-thick transparent plastic sheets with removable double-sided tape (CW-D15, Nichiban Co. Ltd., Fig. 1e).

To check for possible contaminants left behind by the adhesive on cellulose laths (Fig. 1d and e), nine rectangles the size of the sample laths (12 mm × 83 mm, Fig. 1a) were cut out from silver foil with very low blank oxygen and carbon (150 × 150 mm, 4 μm thick, part #: 32210–102, Nittokagaku Co. Ltd.) to create mock sample laths. Three of these mock laths were weighed and pressed against the album mount sheet (Fig. 1d), and three more were weighed and pressed against the transparent plastic sheet (Fig. 1e) in the same manner as cellulose lath samples were pressed onto fixation sheets for mounting. The foil was then peeled off the sheets and weighed again to an accuracy of ± 1 μg and the weight increase was calculated to find out how much, if any, residual adhesive remained on the foil. The amount of blank oxygen and carbon in the peeled-off foil and their isotope ratios were also measured in the same manner as tree-ring samples. The final three mock laths were used as a control group for comparison.

Individual tree rings were separated by ophthalmologic knife (MF-100 and K-730, Feather safety razor Co. Ltd.) from the freeze-dried laths under stereomicroscope equipped with incident light (for laths pasted on album sheet, Fig. 1d) or transmitted light (for laths pasted on transparent plastic sheet, Fig. 1e). Tree rings formed during 1975–1984 in heartwood zone (if any) and 1995–2004 in sapwood zone were chosen for oxygen and carbon isotope analysis. After nicking cellulose laths by knife at ring boundaries, fibers were pulled off with sharp-tipped tweezers. Separating tree rings from soft α-cellulose lath was much easier than from hard wholewood lath. All of our test laths had ring width average of more than 2 mm and each separated ring was put into an Eppendorf tube and ultrasonically homogenized (Laumer et al., 2009) before aliquots were weighed into silver or tin capsules.

2.5. Stable isotope analysis

For isotope analysis of α-cellulose, portions of the processed material were weighed into capsules. For carbon isotope analysis, 0.9 − 1.1 mg were weighed into commercially available tin capsules. For oxygen isotope analysis, 225 − 275 μg were weighed into prepared silver capsules. These capsules were shaped from circles (φ8 mm) punched from the above-mentioned silver foil sheets and weighed one-fourth the weight of the smallest commercially available silver capsules (φ3.2 × 4 mm, Luedi Swiss AG). Since the number of samples that can be analyzed with one pyrolysis tube (or crucible) is limited by silver accumulation, the light-weight capsules allowed us to analyze four times as many samples with one pyrolysis tube, compared to use of commercial silver capsules.

Isotope analysis was conducted with a Thermo-Finnigan MAT252 continuous flow mass spectrometer coupled to a high-temperature pyrolysis system (Hekatech HTO) with Costech Zero-blank 100-position autosampler for oxygen isotope analysis and an elemental analyzer (CE instruments NC2500) for carbon isotope analysis.

To remove moisture from hygroscopic cellulose, samples for oxygen isotope measurement were dried overnight in a vacuum-oven at 70 °C, and then loaded onto a carousel. The carousel chamber was vacuumed for 5 min, then was filled with helium to the same pressure as the carrier helium gas and left for 15 min. This vacuum/helium process was repeated three times, after which the isolation valve, separating the carousel chamber from the pyrolysis tube, was opened. Background mass spectrometer readings originating from the carousel chamber were monitored (mass = 28, 29, 30), a leak check was performed around the cover lid, and the vacuum/helium process was repeated. After stabilization of background readings originating from the autosampler was confirmed, the analysis sequence was initiated. Instead of flushing the carousel chamber with helium as done by Brand et al. (2009) we found that repeating the vacuum/helium processes improved analytical accuracy for oxygen isotope analysis, possibly by increasing the efficiency of removal of water bound to hygroscopic cellulose.

At least two replicates were measured for both oxygen and carbon isotopes and we herein report tree-ring $\delta^{18}O$ and $\delta^{13}C$ using the δ notation in per mil units (‰) with respect to the international stable oxygen and carbon isotope standards Vienna Standard Mean Ocean Water (VSMOW) and Vienna Peedee belemnite (VPDB), respectively. Two standards, Merck cellulose and IAEA-C3 cellulose, were used to calibrate sample oxygen isotope ratios and three standards (CERKU-07, 08, and 09) were used to calibrate carbon isotope ratios (Tayasu et al., 2011). Standard deviation of the repeated measurements of the standards within a sequence was better than 0.15‰ for $\delta^{18}O$ and 0.10‰ for $\delta^{13}C$.

2.6. Residual hemicellulose and lignin analysis

FTIR analysis was conducted on α-cellulose, holocellulose and resin-extracted wholewood samples of less than 10 mg each. Each sample was placed on the ZnSe crystal of an attenuated total-reflectance (ATR) accessory of a Thermo Scientific NICOLET 6700 spectrometer. After scanning the background, twelve spectra of each sample were taken over 1800–650 cm^{-1} with a resolution of 1 cm^{-1}, eight scans per sample, and the spectra were then smoothed to a resolution of 4 cm^{-1}.

Hydrolysis of cell wall polysaccharides was conducted to isolate Klason lignin. About 0.5 g of each sample was treated with 5 ml of 72% sulfuric acid for 4 h, subsequently diluted with 186 ml of DI water and autoclaved for 1 h at 121 °C. The solution was then filtered (φ8 mm or φ21 mm filter paper) and dried at 105 °C. In order to accurately weigh the residue as Klason lignin, the filters were left overnight in a room held at constant temperature and humidity (20 °C, 65%) before being weighed to an accuracy of ± 1 μg.

We followed the alditol-acetate method (Borchardt and Piper, 1970; Blakeney et al., 1983) to determine the neutral sugar composition of α-cellulose and hemicellulose. After the filtrate was cooled, inositol was added as an internal standard. Neutral sugars in the solution were processed to alditol acetates, and determined by gas chromatography with flame ionization detector (Agilent Technologies 6890 N, Fig. 2).

Table 1
Shrinkage of α-cellulose laths treated with three drying methods.[a]

Species	Oven-dry		Ethanol-dry		Freeze-dry	
	Radial	Tangential	Radial	Tangential	Radial	Tangential
Larix	64.7	59.8	79.7	72.1	94.4	90.0
Picea	68.3	67.8	72.4	69.5	82.4	84.5
Pinus	69.8	67.1	70.3	65.9	80.6	84.4
Fagus	67.8	65.0	75.7	77.5	81.7	92.9
Quercus	70.0	67.0	73.0	84.1	82.0	95.9
Average	68.1	65.3	74.2	73.8	84.2	89.5

[a] Shrinkage was measured as the percentage ratio of α-cellulose lath size to the original wholewood lath size (n = 2).

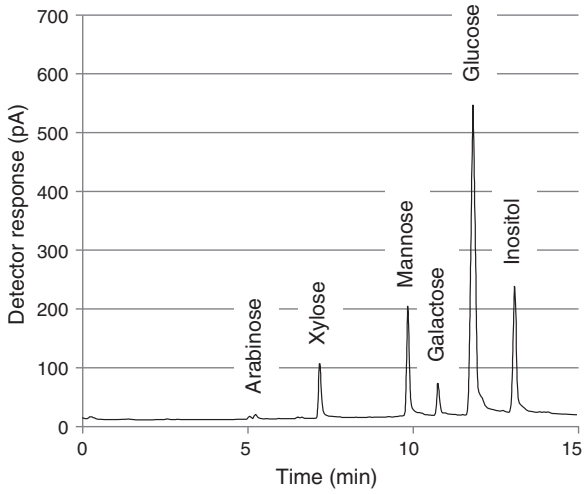

Fig. 2. An example of chromatographic trace for neutral sugar analysis of *Larix* wholewood.

Inositol peak area was used to calculate neutral sugar concentrations as given in Table 2.

2.7. Alpha-cellulose preparation by the standard method

Each tree ring was separated from the tree-ring lath by stationary knife (D-400, NT Cutter Inc., Osaka, Japan) under stereomicroscope. Each separated ring was further sliced by stainless razor blade (FHS-5, Feather Co. Ltd.) into thin slivers (thickness 50–80 μm). A cylindrical-shaped filter (o.d. 6.2 mm) made of sintered polyethylene was inserted into the bottom of a transparent PTFE tube (i.d. 6.0 mm, length 150 mm; Harada et al., 2014; Nakatsuka et al., 2004). Resin-extracted slivers of 10–20 mg were put into the tube and up to 60 tubes were put into a glass jar with airtight lid. During the chemical treatment (Leavitt and Danzer, 1993; Loader et al., 1997), the jar was placed in a water bath for heating. The resultant α-cellulose was ultrasonically homogenized (Laumer et al., 2009) and weighed. Samples for oxygen and carbon isotope analysis were prepared as described in Section 2.5.

3. Results and discussion

3.1. Evaluation of drying methods and fixation sheets

Among the three drying methods, freeze-drying yielded α-cellulose laths with the least shrinkage in both radial and tangential directions (Table 1), i.e. these laths showed the most well-preserved anatomical structure with clearly recognizable ring boundaries, especially visible with transmitted light (Fig. 1e and g). Although α-cellulose laths are entirely white and therefore difficult to view with incident light, transmitted light, which reflects density variation, allowed easy recognition of even ambiguous ring boundaries with small density variation (such as diffuse-porous *Fagus*), and earlywood/latewood boundaries, often examined for intra-annual isotope analysis. Some laths from species possessing broad ray parenchyma cells (*Fagus* and *Quercus*) showed a split along broad rays (Fig. 1d, e and g).

As for the potential contamination of cellulose laths with adhesive from the fixation sheets, there was negligible weight increase in silver foil peeled off from the album sheet (3 μg) and transparent sheet (1 μg). The amount of blank oxygen and carbon in the control foil measured by the elemental analyzer was less than 2 μg for both elements. The silver foil peeled off from both the album sheet and transparent sheet showed negligible increase in blank oxygen and carbon (less than 3 μg increase). We estimated the effect of residual adhesive from the fixation sheet, if any, on isotope ratios. When analyzing tree-ring cellulose sample of 250 μg (about 1 mm^2 surface area in contact with the fixation sheets), the expected isotopic shifts were less than 0.016 and 0.003 ‰, for oxygen and carbon isotope ratios, respectively. These values are negligible compared to the analytical accuracy of oxygen and carbon isotope analyses (0.15‰ and 0.10‰, respectively). Because of these practical advantages of the freeze-drying and negligible contamination from fixation sheet, we chose to freeze-dry laths and adhere them to fixation sheets for our tree-ring isotope analysis.

3.2. Comparison of the cross-section and standard methods

Average yields of α-cellulose and holocellulose laths were 34.6% and 58.9%, respectively, similar to reported yields of the standard method (Leavitt and Danzer, 1993; Loader et al., 1997; Cullen and MacFarlane, 2005). The average yield of α-cellulose prepared from thin slivers of wood (Harada et al., 2014) was 36.8%. Because the cross-section method produced yields similar to the standard method, we hypothesize that the chemical purity of α-cellulose laths is also similar to that of the standard method.

Tree-ring oxygen and carbon isotope ratios measured for samples from each species prepared by the cross-section method also closely matched those of samples prepared by the standard method (Fig. 3), confirming our initial test results (Kagawa and Nakatsuka, 2014). The correlation coefficient, R^2, between the ratios obtained from the cross-section method and the standard method was 0.961 for oxygen isotopes and 0.977 for carbon isotopes (Fig. 4). In another study, Li et al. (2011) reported an average difference between tree-ring isotope ratios from

Table 2
Composition of neutral sugars and Klason lignin in the α-cellulose, holocellulose and resin-extracted wholewood laths.

Species	Sample category	Klason lignin %	Glucose %	Xylose %	Mannose %	Arabinose %	Galactose %
Larix	Wholewood	34.0	46.0	6.2	10.4	0.6	2.9
	Holocellulose	0.5	77.7	7.7	12.5	0.6	1.0
	α-Cellulose	0.1	95.3	0.0	3.9	0.7	0.0
Picea	Wholewood	27.8	51.2	6.4	12.4	0.9	1.3
	Holocellulose	2.1	73.1	7.8	16.2	0.8	0.0
	α-Cellulose[a]	0.8	-	-	-	-	-
Pinus	Wholewood	29.9	49.8	5.3	11.3	0.9	2.8
	Holocellulose	1.5	75.9	6.4	14.5	0.6	1.1
	α-Cellulose	0.2	93.9	1.2	3.9	0.8	0.0
Fagus	Wholewood	23.0	45.7	27.0	2.9	0.7	0.7
	Holocellulose	1.6	65.3	29.4	3.3	0.5	0.0
	α-Cellulose	0.6	94.4	1.5	2.8	0.7	0.0
Quercus	Wholewood	21.8	47.9	26.6	2.6	0.4	0.6
	Holocellulose	2.2	89.3	2.4	5.4	0.7	0.0
	α-Cellulose	0.9	94.4	1.8	2.3	0.6	0.0
IAEA-C3	Cellulose	0.1	81.6	10.6	7.1	0.7	0.0

[a] Neutral sugar content data for *Pica* α-cellulose are missing due to experimental failure.

the cross-section method and the standard method of 0.20‰ for oxygen isotopes and 0.06−0.12‰ for carbon isotopes. In our study, we found average difference of only 0.046‰ and 0.001‰ for oxygen and carbon isotopes, respectively. We believe this discrepancy between isotope ratios produced from the two methods is marginal, and that the cross-section method can be used as an attractive alternative to the standard method for preparing samples for tree-ring oxygen and carbon isotope analysis.

3.3. Purity of α-cellulose and holocellulose

The FTIR spectra did not reveal any clear peaks indicative of the presence of residual lignin or hemicellulose in α-cellulose laths prepared from *Larix* (Fig. 5) and the other species (see Online Supplementary information), suggesting successful removal of lignin and hemicellulose. On the other hand, wholewood spectra show peaks at 1269 cm^{-1} (assigned to G ring plus C = O stretching vibration of lignin) and at 1510 cm^{-1} and 1596 cm^{-1} (assigned to aromatic skeletal vibration of lignin) related to lignin (Faix, 1992), and another peak in holocellulose spectra at 1732 cm^{-1} is assigned to the carbonyl stretching vibration of hemicellulose (Marchessault, 1962). All of these lignin- and hemicellulose-related peaks were absent or significantly reduced in the spectra collected from α-cellulose laths of the five species in our study. The spectra of α-cellulose laths appeared highly similar to spectra of IAEA-C3 cellulose. Holocellulose laths also showed successful removal of lignin, although, as we expected, a peak at 1732 cm^{-1} indicates the presence of hemicellulose.

Inspection of the FTIR spectra therefore revealed no clear traces of residual lignin or hemicellulose in α-cellulose laths. However, the presence of hemicellulose was detectable in the spectra of holocellulose laths.

3.4. Effect of residual hemicellulose and lignin on isotope ratios

According to our quantitative analysis of lignin and neutral sugars, wholewood consisted of 21.8-34.0 % of lignin and 20.1-31.3 % of hemicellulose-specific sugars (Table 2), which matches reported values for the genera (Timell, 1967). Hemicellulose of conifers mainly consisted of mannose and xylose, and that of broad-leaves consisted of xylose.

Residual lignin content in α-cellulose was less than 0.9%. This drastic decrease compared to wholewood indicates successful removal of

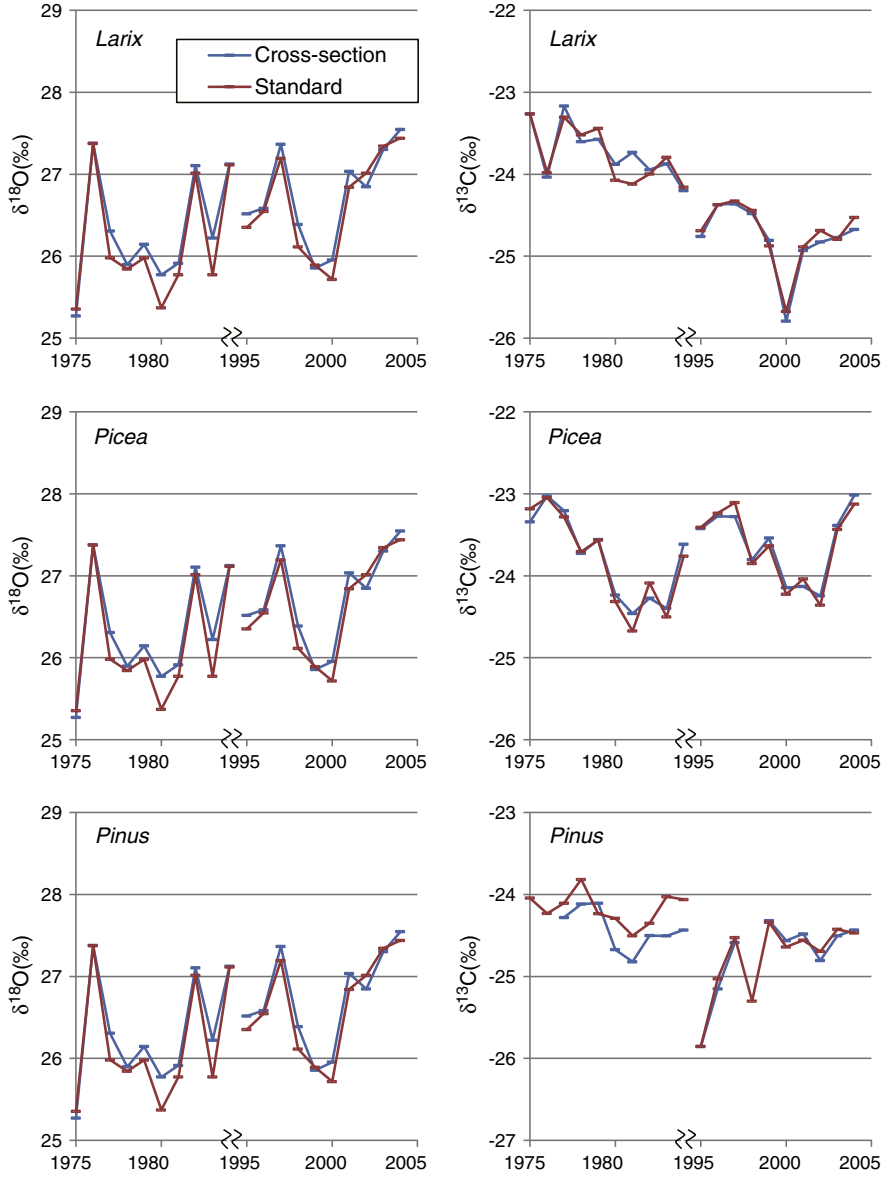

Fig. 3. Oxygen and carbon isotope ratios of tree-ring α-cellulose for five tree species prepared with the cross-section method (blue line) and the standard method (red line).

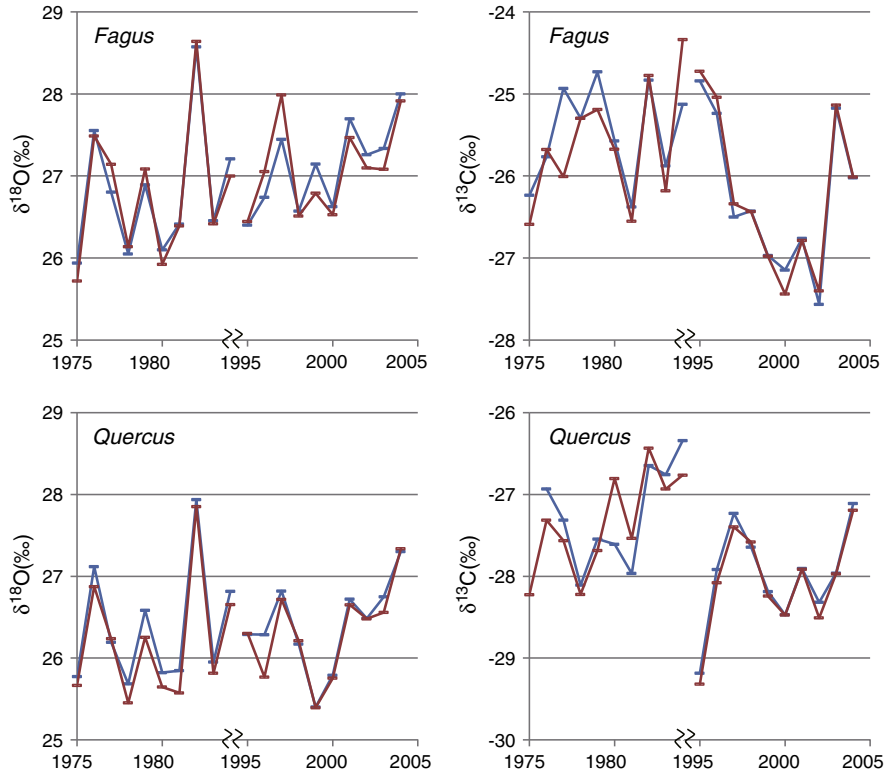

Fig. 3 (*continued*).

lignin. However, the residual hemicellulose (non-glucose sugar) content in α-cellulose was as much as 5.9%, which was not detectable in the FTIR spectra (Fig. 5, See Online Supplementary information).

Lignin has lower $\delta^{18}O$ values than α-cellulose, by 10.5 ± 0.4‰ for white spruce wood (Gray and Thompson, 1977), and 6.4-6.8 ‰ for oak and pine (Barbour et al., 2001). Lignin also has lower $\delta^{13}C$ values than cellulose by 3.25‰ on average from measurements of beech, birch, oak and spruce (Borella et al., 1998) and by 3 ‰ for oak (Loader et al., 2003). In order to calculate the effect of residual lignin and hemicellulose on isotope ratios, we assumed $\delta^{18}O$ and $\delta^{13}C$ offsets between cellulose and lignin to be 10.5 ‰ and 3.25‰, respectively, and offsets of hemicellulose (non-glucose sugars) to be half of offsets of lignin. The offset of isotope ratios of α-cellulose due to residual lignin and hemicellulose was estimated by mass balance calculation based on the data in Table 2. The isotopic offsets of the α-cellulose from ideal cellulose made of 100% glucose were in the range of 0.25 − 0.34‰ for $\delta^{18}O$ and 0.08 − 0.11‰ for $\delta^{13}C$.

In reality, the chemical purity of the α-cellulose (percentage of glucose) prepared from wood particles with the Jayme-Wise method (Green, 1963) is around 95% (Fengel and Wegener, 1983; Green, 1963; Tsutomu Ikeda, unpublished data) and the purity of commercially available cellulose (Avicel PH-101) is 97% (Qing and Wyman, 2011). The chemical purity of α-cellulose prepared with the cross-section method was 93.9 − 95.3%, similar to the purity of α-cellulose prepared with the standard Jayme-Wise method, and we can therefore expect the isotopic offset produced by residual lignin and hemicellulose to be smaller than the offsets estimated above.

The wholewood was initially composed of 21.8 − 34.0% lignin, while holocellulose prepared with the cross-section method was composed of only 0.5 − 2.2% residual lignin (Table 2). In other words, more than 90%

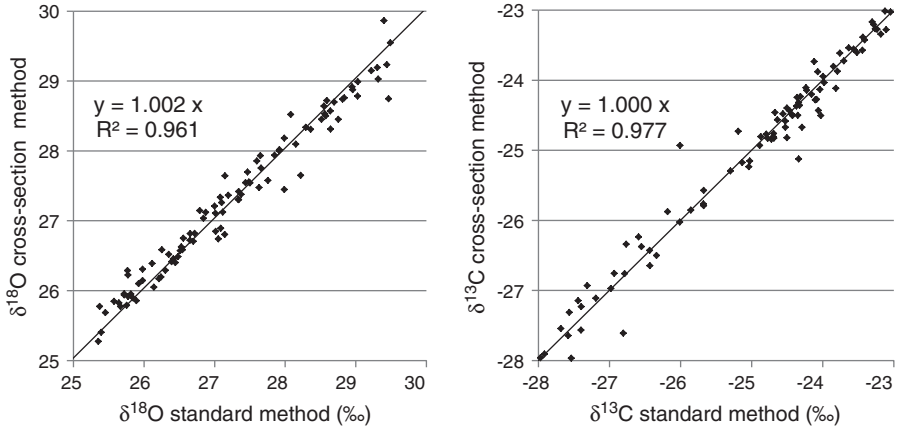

Fig. 4. Correlation between the isotope values obtained with the standard and the cross-section method. Data for all tree species are plotted.

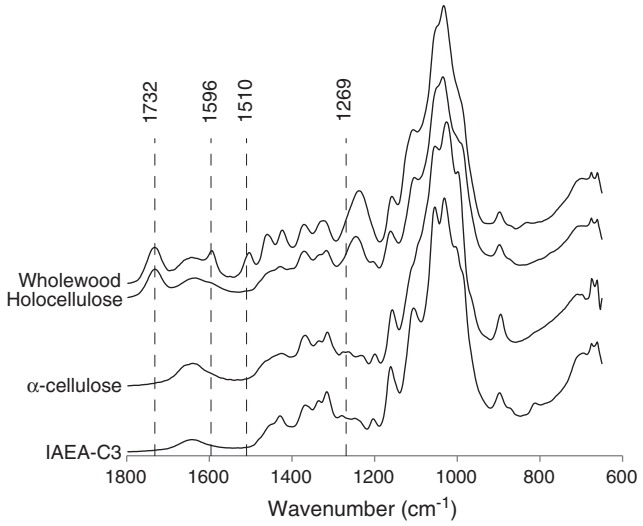

Fig. 5. FTIR spectra of α-cellulose, holocellulose, and resin-extracted wholewood prepared from *Larix* tree-ring laths, and cellulose standard (IAEA-C3). For the spectra of other species, see Online Supplementary information. The vertical dotted lines show the wave lengths related to lignin or hemicellulose.

of lignin was successfully removed by the bleaching process. However, as expected, the holocellulose lath contained significant amount of xylose and mannose, the main constituents of hemicellulose (Green, 1963; Fengel and Wegener, 1983). The shift in isotopic values of the holocellulose due to residual lignin was estimated to be in the range of 0.05 − 0.23 ‰ for $\delta^{18}O$ and 0.02 − 0.07 ‰ for $\delta^{13}C$. We therefore found the purity of the holocellulose prepared with the cross-section method to be sufficient for oxygen and carbon isotope analysis.

3.5. Optimization of the cross-section method

Small modifications are necessary when applying the cross-section method to different types of samples. For example, for analysis of increment cores, cores (diameter 5 mm or 12 mm) should be mounted in wooden grooves with acetone-soluble adhesive (Cemedine C, Cemedine Co., Ltd., Tokyo, Japan) before being sliced into laths and soaked in acetone for a few hours to remove the adhesive. Also, compared to laths prepared by circular (twin) saw, laths prepared by diamond saw have the advantage of distinct anatomical structure at the cutting surface. However, a diamond saw requires longer cutting time (more than 5 min per lath) and produces laths with greater variation in thickness. When working with 5 mm cores, two or three laths of 1-mm thickness can be prepared by diamond saw (Xu et al., 2011), and α-cellulose was successfully extracted from laths thus prepared in our previous studies (Sano et al., 2012, 2013; Xu et al., 2013).

The cross-section method can be applied not only to isotope analysis of tree rings at annual resolution, but also to intra-annual isotope analysis. When a cellulose lath is manually separated with knife under stereomicroscope, the resolution (or minimum ring width) can be as fine as 0.1–0.2 mm, depending on tree species. Working with thinner laths may even achieve resolutions finer than 0.1–0.2 mm, which is important when working with narrow tree rings. And we had no problem separating tree rings with high curvature, such as those of oak, because small tree-ring pieces with tangential length of 0.5–2.5 mm were sufficient for isotope analysis.

Another application of the cross-section method is radiocarbon analysis of tree rings (Shinta Ohashi, personal communication). Normally, we use cotton thread for sealing the tree-ring lath into the PTFE case (Fig. 1b). However, by using PTFE thread, contamination of modern carbon can be avoided, and the cross-section method can be used for preparing α-cellulose lath for tree-ring ^{14}C analysis.

Another modification we suggest relates to the frame material: When used repeatedly, the PTFE cases tend to warp slightly, but a frame made of rigid material such as ceramic would prevent warping.

For analysis of narrow tree rings with widths less than 1.5–2.0 mm, rectangular pieces weighing about 100–400 μg (about 0.4–1.6 mm^3 volume of α-cellulose) were cut out from α-cellulose laths and weighed directly into silver capsules for oxygen measurement. Dissecting, weighing and wrapping the rectangular pieces into capsules required about 3 min per sample. However, when tree rings were wide, cutting the rings into rectangles without exceeding the weight limit became difficult. It became more difficult to keep the tangential width of the dissected piece constant throughout the earlywood and latewood zones and prevent the dissected piece from becoming trapezoidal with different lengths on the two ring boundaries. As there is significant isotopic variation across a single ring (Ogle and McCormac, 1994; Loader et al., 1995; Kagawa et al., 2003; Helle and Schleser, 2004; Nakatsuka et al., 2004; Roden et al., 2009), the isotope ratios of trapezoidal samples may not be representative of whole tree ring. To avoid this problem, when tree rings were wide, we ultrasonically homogenized a larger rectangular sample. Then, after freeze-drying, aliquots were weighed into capsules (Laumer et al., 2009).

Reaction times should be adjusted according to species and lath thickness. Extraction from teak laths of 2.5 mm thickness with reaction time described in Section 2.3. resulted in α-cellulose laths with interiors that remained yellowish. However, we believe this could be easily corrected by extending the reaction time, because tree-ring laths of up to 4-mm thickness have been successfully processed with two days of bleaching reaction by Li et al. (2011). The minimum thickness of tree-ring laths that can be processed without serious disintegration was 0.5 mm for oak and teak. Therefore, we recommend preparing tree-ring laths with thickness between 0.5 and 4.0 mm for α-cellulose extraction.

We were able to avoid serious disintegration when extracting α-cellulose laths from rigid archeological wood of the seven tree species without significant decomposition. However, when the wood was significantly decomposed to the point where it was easily deformable by pressing on it with our fingers, α-cellulose extraction was difficult.

In our laboratory, the standard method can process about a hundred tree rings in a week. The cross-section method can process thousands of tree rings in a week at the same cost and labor requirements, making cellulose extraction more than 10-fold efficient. In the case of narrow tree rings, efficiency increases even further, because separated rings can be directly loaded into capsules without homogenizing.

4. Conclusions

The perforated PTFE case we designed (Fig. 1a and b) enables extraction of α-cellulose laths from thin cross-sectional laths (thickness of 0.5–1.0 mm) without disintegration. Our PTFE case broadens the applicability of the "cross-section" method to fragile tree-ring samples, such as teak or rigid archaeological wood without significant decomposition. For drying α-cellulose laths, we found freeze-drying to be the most practical method, causing the least shrinkage and best preserving anatomical structure. Thanks to the well-preserved anatomical structure of freeze-dried α-cellulose laths, ring boundaries were clearly recognizable (Fig. 1g). Tree rings were dissectible precisely at ring boundaries under stereomicroscope, especially with transmitted light.

By adhering the α-cellulose laths to fixation sheets, we successfully prevented the fragile freeze-dried laths from disintegrating (Fig. 1d and e). There was no sign of significant contamination from adhesives, and any possible contamination had negligible effect on tree-ring oxygen and carbon isotope ratios. Both oxygen and carbon isotope ratios of tree rings obtained with the cross-section method closely matched those obtained with the standard method (Leavitt and Danzer, 1993; Loader et al., 1997; Harada et al., 2014) in all five tree species tested (Fig. 3). Because of the similarity in the chemical purities between the

two methods, we believe the effect of residual hemicellulose and lignin on the oxygen and carbon isotope ratios of α-cellulose laths to be negligible. Purity of α-cellulose significantly changes depending on the period of chemical treatment (Harada et al., 2014) and Loader et al. (1997) recommends modification of the period depending on species and physical nature of the sample (powder or slivers). However, for 1-mm thick tree-ring laths, 4-h bleaching at 70 °C and 3-h alkali treatment at 80 °C were sufficient to obtain α-cellulose with high chemical purity.

Both the purity and tree-ring isotope ratios of α-cellulose laths closely matched those of the standard method. We therefore confidently encourage adoption of the cross-section method as an attractive alternative to the standard method for extracting tree-ring cellulose.

Tree-ring samples were cut from the α-cellulose lath and weighed directly into silver or tin capsules, or, in case of wide rings, individual rings were homogenized before aliquots are weighed into the capsules. The simple procedures involved in the cross-section method resulted in more than ten-fold reduction in both cost and time associated with tree-ring oxygen and carbon isotope analysis of α-cellulose.

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For the latest protocol of our cellulose extraction method, please contact the corresponding author (Akira Kagawa and Takeshi Nakatsuka). This study was funded by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (25292111), and the Global Environmental Research Fund RF-1011 of the Japan Environment Agency. We thank Jennifer Lue for reviewing early drafts of this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.chemgeo.2014.11.019.

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