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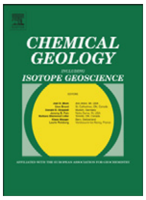

# An optimized method for stable isotope analysis of tree rings by extracting cellulose directly from cross-sectional laths

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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