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Technical Note: An improved guideline for rapid and precise sample preparation of tree-ring stable isotope analysis

K. Schollaen[1,2], **H. Baschek**[1], **I. Heinrich**[1], **and G. Helle**[1]

[1]German Centre for GeoSciences – GFZ –, Section 5.2 Climate Dynamics and Landscape Evolution, Potsdam, Germany
[2]Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Research Section Potsdam, Germany

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Correspondence to: K. Schollaen (karina.schollaen@gmail.com); G. Helle (ghelle@gfz-potsdam.de)

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An improved guideline for rapid and precise sample preparation

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Abstract

The procedure of wood sample preparation, including tree-ring dissection, cellulose extraction, homogenization and finally weighing and packing for stable isotope analysis is labour intensive and time consuming.

We present an elaborated methodical guideline from pre-analyses considerations, wood sample preparation through semi-automated chemical extraction of cellulose directly from tree-ring cross-sections to tree-ring dissection for high-precision isotope ratio mass spectrometry. This guideline reduces time and maximizes the tree-ring stable isotope data throughput significantly.

The method was applied to ten different tree species (coniferous and angiosperm wood) with different wood growth rates and differently shaped tree-ring boundaries. The tree-ring structures of the cellulose cross-sections largely remained well identifiable. FTIR (Fourier transform infrared) spectrometry and the comparison of stable isotope values with classical method confirm chemical purity of the resultant cellulose. Sample homogenization is no longer necessary.

Cellulose extraction is now faster, cheaper and more user friendly allowing (i) the simultaneous treatment of wood cross-sections of a total length of 180 cm (equivalent to 6 increment cores of 30 cm length) and thickness of 0.5 to 2 mm, and (ii) precise tree-ring separation at annual to high-resolution scale utilizing manual devices or UV-laser microdissection microscopes.

1 Introduction

Stable isotope records from tree rings are powerful proxies in paleoclimatic (e.g. Treydte et al., 2006; Loader et al., 2010; Brienen et al., 2012; Heinrich et al., 2013; Konter et al., 2014) and plant physiological studies (e.g. Helle and Schleser, 2004; Simard et al., 2012; Gessler et al., 2013). For stable isotope analyses of carbon (C), oxygen (O) or hydrogen (H) different wood components (wood, lignin or cellulose) can be anal-

ysed. Cellulose, as a primary carbohydrate, is often the preferred sample material because of its short synthesis pathway, singular chemical composition and its immobility (McCarroll and Loader, 2004).

Rapid advancements concerning the analytical methodology of stable isotope measurements have been achieved allowing for minute sample amounts of a few micrograms and large numbers of samples (Koziet, 1997; Saurer et al., 1998; Kornexl et al., 1999; Gori et al., 2013; Loader et al., 2014). However, these rapid analytical developments are challenging in regard to the sample preparations because they demand very high standards concerning sample purity and homogeneity. Furthermore, tree-ring dissection and chemical preparation of cellulose from wood tissue are time-consuming processes. Therefore, a variety of approaches have continuously improved the methodical and the chemical preparation processes for stable isotope analysis (Green and Whistler, 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; Laumer et al., 2009; Wieloch et al., 2011). In all of these approaches, tree rings are first subdivided, i.e. peeled manually by knife/scalpel or sledge microtome (e.g. Polycut E, Leica microsystems, Bentheim, Germany), before being passed through the cellulose extraction process. Despite novel techniques facilitating simultaneous treatments of up to several hundred micro samples (Wieloch et al., 2011) or individual pre- and post-homogenizations through grinding or ultra-sonic treatment (Laumer et al., 2009), chemical treatments of every single tree-ring sample are still required.

In order to reduce the peeling-grinding-chemical processing for each individual tree ring Loader et al. (2002) made a first attempt to extract cellulose directly from standard increment cores (5 mm Ø). More recently, Li et al. (2011) reported about a technique to extract α-cellulose directly from wood cross-sections. They conducted a high-resolution intra-annual δ^{13}C and δ^{18}O analysis on a 3 mm wide annual ring from a 3.5 to 4 mm thick α-cellulose cross-section revealing no discrepancies from the usual method. A breakthrough in terms of high sample throughput was achieved by Nakatsuka et al. (2011), with methodological improvements by Kagawa et al. (2015). Here,

a container made of teflon (PTFE, polytetrafluoroethylene) punching sheet was designed to prevent disintegration of cellulose laths (see also Xu et al., 2011). The basic principle allows cellulose extraction from wood laths in one single batch providing the same chemical conditions for all samples by using an extracting device made of teflon material. This method significantly reduces the time needed for cellulose preparation while retaining the wood cell fabric mostly intact.

Based on the approach presented by Kagawa et al. (2015), we modified and redesigned the technique and developed an improved semi-automated cellulose extraction system. We evaluated the performance of this high-throughput cellulose extraction method and modified the overall procedure of sample preparation to provide precisely dissected, homogenous tree-ring cellulose samples for high-precision IRMS analysis. To validate the methodology we tested several coniferous and broadleaf tree species with different growth rates, differently shaped tree-ring boundaries as well as with a broad range of various features of cell structures. Furthermore, we compared FTIR (Fourier transform infrared) spectra and measurements of stable isotope ratios with results from the classical tree-ring preparation procedure. We highlight similarities and dissimilarities between the cellulose extraction technique presented here and the approach by Kagawa et al. (2015). Furthermore, we present a comprehensive guideline comprising pre-analyses considerations and specific measures for tree-ring dissection for high-precision isotope ratio mass spectrometry. In particular, we tested for potential effects of contaminants, e.g. pencil marks, chalk and corn starch, on the original isotope composition (δ^{13}C, δ^{18}O) of samples. Pencil marks and chalk are commonly used in dendrochronology and -ecology to highlight the contrast between different wood cell structures for a better detection of narrow or indistinct annual growth boundaries during microscopic tree-ring measurements. In addition, it has become popular to apply a mixture of corn starch, water and glycerol (so called non-Newtonian fluid) to the wood surface in order to penetrate the cell lumens and then stabilize the cell walls during micro sectioning (Schneider and Gärtner, 2013). However, original isotope ratios may be adulterated if potential contaminants such as non-Newtonian fluid, pencil marks or

chalk cannot be removed prior to stable isotope analysis. This is becoming increasingly important as not only tree physiological and -ecological studies are combining quantitative wood anatomical measures with stable isotope analysis for improved understanding of tree structure and related function, but also dendroclimatological studies have begun combining these methods (e.g. Szymczak et al., 2014).

Hence, this article will finally give a well-elaborated guideline for modern tree-ring stable isotope research.

2 Preliminary consideration: wood or cellulose?

Prior to any tree-ring stable isotope study a decision has to be made whether wood or only cellulose shall be investigated. Although the extraction of cellulose from wood is very labour intensive and time consuming, cellulose is often the preferred sample material as it prevents any distortion of the isotope signal due to changing mass proportions of the different wood components with various stable isotopic signatures. In principle, carbohydrates from primary metabolism, e.g. sugars, starch or cellulose, have significantly different – normally heavier – isotopic signatures than secondary metabolites, as for example lignin or fatty acids. Accordingly, cellulose is always found enriched in ^{13}C over lignin or fatty acids by 2 to 4 ‰ (Schmidt et al., 1998 and refs. therein). Although cellulose and hemicelluloses represent by far the largest proportion of wood (on average 65–75 %), the high carbon content of lignin as compared to cellulose, which is almost 50 % higher (e.g. Kürschner and Popik, 1962; Pettersen, 1984), results in a considerable contribution of carbon from lignin. Any shift in the relative mass proportion of cellulose to lignin could indicate that wood and cellulose may not lead to the same variations in their isotopic composition, especially with regard to long isotope records.

In addition, extractives, such as fatty acids, waxes, alkaloids, proteins, phenolics, pectins, gums, resins, terpenes, starch, glycosides, saponins and essential oils may represent an additional contribution of carbon, oxygen and hydrogen which show a wide range of δ values as they are derived from the secondary plant metabolism

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(Schmidt et al., 1998, 2001). Extractives obtained from woody species growing in temperate climates constitute 4–10 % of dry weight but their portions may increase up to 20 % for tropical trees (Pettersen, 1984). Nonetheless, their contribution to the overall δ^{13}C value of wood, for example, may be negligible if these extractives make up only a small percentage of the wood and/or the isotope values of these fractions are mainly in the cellulose to lignin range. Extractives can, however, frequently be related to particular environmental incidents such as fire or drought which may induce for example resin production or may be a part of a tree‘s defence mechanism against microbial or herbivore attack (e.g. Chapman, 1980; Schweingruber, 1996; Guest and Brown, 1997). These environmentally induced changes in relative proportions of wood components may enhance or attenuate the isotope signature that can be expected according to the common models of isotope fractionation in the arboreal system (e.g. Farquhar et al., 1982; Roden et al., 2000).

Because of the considerations outlined above, several studies tested whether wood or cellulose isotope chronologies better reflect environmental and particularly climate variations, with contradictory results. Some studies suggest that cellulose extraction may not be necessary, since differences between the stable carbon and oxygen isotope data of wood and cellulose were found rather constant or revealed no difference in statistical relationship with environmental data (Barbour et al., 2001; Verheyden et al., 2005; Harlow et al., 2006; Taylor et al., 2008). On the other hand, researchers report that cellulose provides a temporally much more stable proxy of past climate and suggest that cellulose extraction is a necessary step in studies seeking to investigate climate signals in stable isotope chronologies (Ferrio and Voltas, 2005; Cullen and Grierson, 2006; Battipaglia et al., 2008; Szymczak et al., 2011). According to Loader et al. (2003), cellulose extraction may provide little additional information when the main focus lies on long-term climate trends, while it can be more critical if information on extreme events such as droughts or high-rainfall years is required.

While cellulose extraction for isotope studies has not become obsolete, the decision to just use raw wood or resin extracted wood is often taken in order to save time and money, however often at the expense of data quality.

3 Material and methods

3.1 Sample material

Sample material from 10 different tree species (*Pinus sylvestris, Quercus robur, Larix decidua, Tectona grandis, Picea abies, Cedrela lilloi, Juniperus seravschanica, Adansonia digitata, Pseudotsuga menziesii, Fagus sylvatica*) growing at low to high elevation (timberline) sites in various climatic zones (boreal, temperate, tropical and semi-arid) was chosen to represent different wood growth rates and differently shaped tree-ring boundaries, as well as a broad range of various cell structure features. Additional information can be found in the Table S1 of the Supplement.

3.2 Potential implications of contaminants

In general, possible shifts of original isotope values from contaminants do not only rely on their isotopic deviation from the sample material to be analysed, but also on their relative contribution to the sample mass. Potential effects can be determined by the following simple equation:

$$\mathrm{Impact}_{\mathrm{con}}\ [‰] = \left(\frac{(mE_{\mathrm{sample}} \times \delta nE_{\mathrm{sample}}) + (mE_{\mathrm{con}} \times \delta nE_{\mathrm{con}})}{mE_{\mathrm{sample}} + mE_{\mathrm{con}}} \right) - \delta nE_{\mathrm{sample}} \tag{1}$$

where

E Element of interest (e.g. carbon or oxygen)

$\mathrm{Impact}_{\mathrm{con}}$ Impact of contamination,

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mE_{sample}, mE_{con}	are the respective relative mass fractions of the element, e.g. carbon or oxygen in sample or contaminant,
δE_{sample}	is the original δ value of a sample, e.g. $\delta^{13}C$ or $\delta^{18}O$ value of the cellulose sample,
δE_{con}	is the δ value of a contaminant, e.g. $\delta^{13}C$ or $\delta^{18}O$ of pencil, chalk or starch

For testing potential implications of contaminating substances we repeatedly analysed and applied chalk, pencil lead and starch to different wood samples of teak (*Tectona grandis*), a ring porous to semi-ring porous wood species of the tropics. Substances were taken from maize starch flour ($(C_6H_{10}O_5)_n$) and pencil leads which consist of varying mixtures of clay minerals with graphite (C) and traces of palm oil. Chalk consists of calcium sulphate (gypsum, $CaSO_4 \times 2H_2O$). The mass fraction of oxygen contributes about 56 % of the total mass of gypsum. Starch contains mass fractions of oxygen and carbon of about 49 and 44 %, respectively (similar to cellulose $[(C_{12}H_{20}O_{10})_{[n]}]$). Wood structure analyses on Scots pine (*Pinus sylvestris*) from Sweden, Poland and Germany revealed a variable ratio of cell wall to cell lumen (Liang et al., 2013a, b; Pritzkow et al., 2014). For earlywood it was found to be around 50 : 50 on average, for latewood the cell wall portion can reach values of 80–90 %. Hence, we expected a significant contamination of wood carbon and oxygen isotope ratios, in particular from maize and chalk. We carefully prepared the surfaces of wood cross-sections with a core microtome of the Swiss Federal Institute for Forest, Snow and Landscape Research (Gärtner and Nievergelt, 2010) ensuring empty cell lumens (i.e. no remains of wood dust from sanding). Then chalk was repeatedly rubbed into the cells of 2 mm thick wood cross-sections with a surface of 15 cm × ca. 0.5 cm in size (ca. 7.5 cm^2). The potential mass fractions were determined from repeated weighing of wood cross-sections before and after the application of chalk and non-Newtonian fluid. Stable isotope ratios of

oxygen and carbon were measured from the contaminants as described in the next section.

3.3 Stable isotope analysis

Measurements of stable carbon isotopes were undertaken by combustion using an elemental analyser (Model NA 1500; Carlo Erba, Milan, Italy) coupled online via open split to an Isoprime IRMS (Isoprime Ltd, Cheadle Hulme, UK). The operating temperature of the combustion oven set to 1080 °C. The masses of the cellulose samples and reference materials for stable carbon isotope analyses lay between 180–220 µg. The analytical error (better than ±0.1 ‰) is being monitored constantly by measurements of reference material after every 10th sample and replicated (3–6 times) analyses of individual cellulose samples. For isotope analyses, we used Helium 5.0 as carrier gas. The isotope ratios are reported as per mille (‰) deviations and given in the delta (δ) notation, relative to the standards VPDB (Vienna-PDB) for δ^{13}C (Craig, 1957; Hut, 1987).

3.4 Fourier Transform Infrared (FTIR) spectroscopy

The chemical purity of the cellulose gained from wood cross-sections was evaluated by applying FTIR spectrometry, identifying the functional groups for resin, lignin, cellulose and hemicellulose. We compared the spectra of (holo)-cellulose cross-sections from teak wood samples with the corresponding wood samples (completely untreated and resin extracted) and laboratory cellulose standards. The FTIR spectra were measured by direct absorbance using the potassium bromide (KBr) pellet technique. 1 mg of different cellulose cross-sections and reference samples of α-cellulose powder (Fluka and Merck-α−cellulose) were mixed with 450 mg of KBr-powder and pressed into thin pellets. The resultant pellets were vacuum dried at 50 °C for 24 h before placing them on a Vertex 80v FTIR-spectrometer (Bruker Optics, Germany). Each spectrum was

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measured at a spectral resolution of $0.25\,cm^{-1}$ and 128 scans were conducted per sample.

3.5 Classical cellulose extraction method vs. cross-section cellulose method

Besides the evaluation of the chemical purity by FTIR we compared the isotope ratios of cellulose produced by the cross-section extraction method with the ratios of cellulose derived from the classical extraction method. We wanted to test for discrepancies in the stable isotope ratios. Therefore, we measured the stable isotope values of carbon in two different teak trees by analyzing a time period at the beginning of a tree's life (heartwood part) and a time period covering the most recent tree rings (sapwood part). The strength of the linear relationships between the tree-ring series were assessed by Pearson's correlation coefficients (r). High-frequency coherence was measured using the Gleichläufigkeit (GLK) (Eckstein and Bauch, 1969; Schweingruber, 1983; Esper et al., 2001), that is, a simple sign test of synchronous year-to-year changes amongst single series. A value of 1 implies parallel series.

4 Experimental design

4.1 Guideline for cellulose extraction and tree-ring dissection at a glance

We followed a preparation scheme that requires ten steps. The procedure finally provides specimens that are ready for IRMS analysis, but also allows other dendrochronological methods to be applied, as large parts of the original samples (wood cores/segments) remain intact due to the use of thin cross-sections (step 1–5 see Fig. 1, step 6–8 see Fig. 2, step 9–10 see Fig. 3):

1. *preparation of a plane cross-sectional wood surface* by sanding or cutting with a microtome or a razor blade (Fig. 1a),

2. *measurement of tree-ring width (TRW)* with analog or digital systems followed by chronology building; recommendation: scanning of the wood core and using digital systems for tree-ring width analysis (Fig. 1b),
3. *preparation of thin wood cross-sections* from stem disks or increment cores (0.5–2 mm thickness and up to 30 cm in length) with a high-precision diamond wafering saw, a core microtome or razor blade (Fig. 1c),
4. *subdivision of cross-sections into segments* (max. 6 cm long and 1.5 cm width) along tree-ring boundaries by using a razor blade or scalpel (Fig. 1d),
5. *cleaning wood cross-sections in an ultrasonic bath* to at least partly remove contaminants (e.g. chalk, safranin and starch remains) from dendrochronological and wood anatomical analysis or wood swarf (Fig. 1e),
6. *enclosing wood cross-sections in the extraction system (Fig. 2)* that contains 5 individual extraction units (Fig. 2b) with max. 6 single punching sheet holders (Fig. 2a), allowing the treatment of wood cross-section with a total length of up to 180 cm,
7. *semi-automated cellulose extraction* with 5 % sodium hydroxide (2 × 2 h, 60 °C) and 7 % sodium chlorite (pH 4–4.5 (acetic acid), 36 h, 60 °C) using a peristaltic pump with two silicon tubes (optional) for continuous circulation and easy renewal of chemical solutions and gentle washing with de-ionized water (Fig. 2c),
8. *freeze-drying of cellulose cross-sections* within their extraction units (Fig. 2d),
9. *dissection of tree rings* with a scalpel under a binocular microscope or with an UV-laser microdissection microscope and specimen collection in tin or silver capsules avoiding the extra step of sample homogenization (Fig. 3) and
10. *stable carbon (C) and oxygen (O) isotope analysis via conventional IRMS* coupled online to a combustion or pyrolysis furnace.

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The experimental design is explained in more detail in the following subsections.

4.2 Preparation of wood cross-sections

The procedure starts with wood surface preparation by razor blade, microtome or sanding machine (Fig. 1a). For better handling we recommend to fix wood cores on a carrier material. The prepared wood segments are scanned and tree-ring widths (TRW) are measured (Fig. 1b) following dendrochronological procedures described by Cook and Kairiukstis (1990). We recommend using digital systems for tree-ring width analysis (e.g. WinDendro™, Regent Instruments Inc., Canada) in combination with direct visual control of very narrow tree-ring sequences using a binocular. Thereafter, wood cross-sections of varying thicknesses, ranging from 0.5 to 2 mm are produced (Fig. 1c). This is done by using a special high-precision, water cooled, diamond saw (IsoMet 5000 with a 15HC precision sectioning blade, ITW Test & Measurement GmbH, Düsseldorf, Germany), that was modified at the GFZ for handling samples of up to 30 cm in length. Alternatively and depending on the properties of sample material a microtome (Gärtner and Nievergelt, 2010) or any other appropriate saw (e.g. a double bladed saw, Dendrocut from Walesch Electronics GmbH, Illnau-Effretikon, Switzerland) or even razor blades (e.g. Li et al., 2011) can be used. Very soft woods, e.g. from African baobab (*Adansonia digitata*), with high water content should be frozen with liquid nitrogen and kept in dry ice during preparation of cross-sections. This provides sufficient hardiness for the cutting process ensuring rather accurate cross-sections (F. Slotta, pers. obs.).

Wood cross-sections are separated into segments of max. 6 cm length (Fig. 1d) for easy handling before and after the cellulose extraction process. Especially for manual tree-ring dissection using a binocular or for UV-laser-based microdissection as well as confocal laser scanning microscopy for quantitative wood anatomy, samples with a length of 6 cm are preferred according to the size of the respective object holders.

Additional removal of swarf, chalk or starch remains is achieved by means of an ultrasonic bath (Fig. 1e). The cleaned and dried wood cross-sections are stored be-

tween well labeled microscopic slides (26 mm × 76 mm, Thermo Fisher Scientific; Menzel GmbH, Braunschweig, Germany).

4.3 The semi-automated cellulose extraction system

The basic principle of this cellulose extraction method has been described by Li et al. (2011) and Kagawa et al. (2015). In the following we describe a modified version using a special extraction system. Technical drawings can be found in the in the Fig. S1 of the Supplement.

The extraction system contains a modular structure and is basically made of two components. The first component is the sample holder made of teflon punching sheets. During the extraction process several sample holders accommodate, in combination with spacer slides, wood cross-sections of up to 2 mm thickness and maximum widths of 15 mm (e.g. from 5 or 10 mm increment cores) (Fig. 2a). The second component is a casing (Fig. 2b) consisting of a lower and upper mount enclosing six rectangular wells, which hold the punching sheet sample holders. Both mounts are fitted together and fixed hand-tight with screws. Six punching sheet holders placed in the casing built up an extraction unit (Fig. 2b). Several of these individual extraction units (max. 5 units) can be placed on top of each (Fig. 2c). The extraction device is placed into a glass container (Fig. 2c) accommodating the relevant chemical solutions and completing the extraction system which is then positioned in a bath with heated water of a constant temperature of 60 °C (Julabo SW-21C, JULABO GmbH, Seelbach, Germany). We are following a chemical treatment protocol that is outlined in detail by Green and Whistler (1963) with modifications described by Wieloch et al. (2011).

The cellulose extraction process works semi-automatically due to the use of a peristaltic pump system with two silicon tubes (e.g. Roth Cyclo II pericyclic pumps, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Such a pump system facilitates adequate temperature distribution and continuous circulation of the chemical solution (flow rate: 0.1 L min^{-1}) within the extraction system (Fig. 2c). For renewal of chemical solutions silicone tubes can be connected to a waste reservoir or flask with new chemical bleach.

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Likewise, for washing and neutralizing, the extraction system can be connected via tubes to a waste container and reservoir filled with de-ionized water. Manual exchange of chemical solutions and washing/neutralizing of the samples can also be applied, however special care needs to be taken, particularly when processing very thin cross-sections or partly decomposed wood material.

After cellulose extraction the extraction device or its individual extraction units with their thin section cellulose samples are taken out of the glass container and stored in a freezer at −20 °C. Thereafter, the frozen cellulose cross-sections are being freeze-dried within their extraction units for two days before they can be taken out carefully with a forceps. Dry cellulose cross-sections are kept between two labeled microscope slides that are fixed with a clamp or adhesive tape (Fig. 2d).

This study focused on holocellulose, because isotopic differences between holo- and α-cellulose were found to be relatively small (ca. 0.25–0.3 ‰ for $\delta^{13}C$ and $\delta^{18}O$, Boettger et al., 2007) and rather constant in time (Wieloch et al., 2011 and refs. therein). However, the additional step of α-cellulose extraction with 17 % NaOH at room temperature can easily be performed as shown in earlier approaches by Nakatsuka et al. (2011). Additional information regarding the extraction device can be found in the Supplement Notes S1.

4.4 Tree-ring dissection from cellulose cross-sections

For stable isotope analysis tree-rings, or parts thereof (e.g. earlywood/latewood), can be dissected from cellulose cross-sections by manual dissection with a scalpel under a binocular microscope or by UV-laser microscopic dissection (Fig. 3). In particular when ring widths are very narrow and/or growth ring boundaries are curved or asymmetric the dissection with an UV-laser is of advantage (Schollaen et al., 2014a). Apart from UV-laser dissection, the use of UV-light can facilitate the visualization of tree-ring structures (see Fig. 6, Juniper). Since the density of the cellulose is changing throughout the tree ring (e.g., latewood cellulose is more dense than earlywood cellulose), denser parts of a tree ring have a higher fluorescence.

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Tree-ring samples need to be cut along the tree-ring boundaries with great caution ensuring that the entire ring width is represented by the isotope sample (Fig. 3). The exact sample masses required for IRMS analysis can be achieved by performing variable radial cuttings. They always have to comprise the entire tree-ring width but vary in width perpendicular to the tree-ring boundaries (blue arrows in Fig. 3). This results in specimens of different sizes/volumes but similar mass. In doing so, it is ensured that all samples have the appropriate weight and are homogenous representatives of the individual tree rings. The sample weight is controlled with a microbalance (AX26 DeltaRange; Mettler Toledo GmbH, Greifensee, Switzerland). On the condition that the relative proportions of earlywood and latewood width are kept, homogenization is no longer required and cellulose can be loaded directly into tin or silver capsules for carbon and oxygen isotope analysis, respectively. When using a UV-laser microdissection microscope the proper size of the cutting area can be calculated before the laser dissection, provided that the thickness of the cellulose cross-section is known and the earlywood-to-latewood proportions do not vary significantly.

5 Results

5.1 Possible influence of contaminants on stable isotope values

The impact of potential contamination [δ ‰] from chalk, pencil marks and maize starch on the tree-ring stable isotope values of wood is shown in Table 1. From repeated weighing we determined a relative contribution of oxygen and carbon deriving from chalk and maize starch of less than 1 % per tree ring. This indicates that the substances do not penetrate deeply into the wood cross-sections, despite partly high ratios of cell lumen vs. cell wall. For pencil marks the relative mass contributions could not even be measured as only some rings were marked and material from pencil lead was not rubbed into the cell lumens. However, other woody tree species than teak show higher ratios of cell lumen vs. cell wall. Hence, we calculated the potential effects of contam-

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inants with mass fractions of 10 % demonstrating possible isotopic shifts of more than 2 ‰ deviation from the original value (Table 1, bold numbers). But even remnants (up to 1 %) of chalk and maize starch can slightly shift the original $\delta^{18}O$ and $\delta^{13}C$ values of wood exceeding the margins of analytical error (Table 1, italic numbers).

5 With respect to cellulose cross-sections it became clear, that most of the contaminants are dispersed and removed by the ultra-sonic treatment (Fig. 1e). But pencil marks still remain visible on the cellulose cross-sections after the extraction. Most of the chalk and starch are completely dissolved during cellulose extraction at 60 °C. Even the hardly soluble gypsum remains from the chalk (solubility in water: $2\,g\,L^{-1}$) is being
10 dissolved and removed by the several liters of water applied during the extraction procedure.

5.2 Classical vs. cross-section cellulose extraction method

We found highly significant correlations and high levels of common variance between the stable carbon isotope datasets of both methods (classical and cross-section cel-
15 lulose extraction method) with correlation coefficients of $r = 0.99/0.96$ ($R^2 = 0.98/0.92$) and GLK of 0.86/0.91 ($n = 36$) (Fig. 4). The average difference between tree-ring isotope ratios of both methods is 0.13 ‰. This comparison shows that the extracted cellulose following the cross-section extraction method results in similar isotope values as in cellulose obtained by the classical extraction method.

20 ## 5.3 Purity of cellulose cross-sections

The FTIR method is a well-known method to analyze the major wood components α-cellulose, hemicellulose, lignin as well as extractives. Here we compared the spectra of holocellulose cross-sections from teak wood samples with the corresponding wood samples (completely untreated and resin extracted) and laboratory cellulose standards
25 (Fig. 5). Many well-defined peaks exist (Fig. 5 grey shaded bars) in wood samples which are caused by functional groups of lignin (Harrington et al., 1964; Pandey and

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Theagarajan, 1997; Pandey and Pitman, 2003): 1598 cm^{-1}/1511 cm^{-1} for aromatic skeleton in lignin, 1460 cm^{-1} C-H resulting from deformation and benzene vibration in lignin and 1270 cm^{-1}/1226 cm^{-1} from C-O stretching vibration in lignin. The particular absorption of hemicellulose components is around 1732 cm^{-1}. The spectra of the cellulose cross-sections reveal none of these peaks and the positions of bands are approximately the same as for the reference cellulose samples of Fluka and Merck α-cellulose.

5.4 Cellulose cross-sections from different tree species

We found that the tree-ring structures largely remain well identifiable for a number of different coniferous and angiosperm tree species from various climate zones (tropical, temperate, boreal), as well as low elevation and alpine timberline sites (Fig. 6). Not only the tree-ring boundaries, but also the basic tree-ring structures largely remained well identifiable. Characteristic features as tracheids (see coniferous wood samples), vessels (see angiosperm wood samples), parenchyma bands (e.g. teak and baobab sample) or woody rays (e.g. oak sample) are equally visible in the wood and cellulose cross-sections. Even rings which are very narrow or with irregular shapes, remain visible, as demonstrated here for larch and teak. Furthermore, the extraction method works properly for subfossil wood as was demonstrated for a partly decomposed subfossil pine sample from the vicinity of Zurich, Switzerland (year of sample: 12 910–12 906 kyrs cal BP, Pieper et al., 2014).

6 Discussion

6.1 Benefits of using cellulose instead of wood

Testing the potential influences of contaminating substances (chalk, pencil marks, maize starch) on the original stable isotope values of wood revealed no significant influ-

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ences as was also shown for chalk and pencil marks in a previous experiment (Schollaen and Helle, 2013). However, especially chalk and maize starch may slightly shift the original $\delta^{18}O$ and $\delta^{13}C$ values of wood samples. In order to prevent possible contamination of the original stable isotope ratios when using these auxiliary agents that often simplify tree-ring width measurements as well as quantitative wood anatomical analysis, we recommend the extraction of cellulose instead of using wood samples for dendro-isotopic studies. Through mechanical (Fig. 1e) and chemical treatments (Fig. 2) the contaminants applied are completely dissolved, except for pencil marks. Nonetheless, their contribution to the overall $\delta^{13}C$ value of wood or cellulose, respectively, is negligible, because our calculations, weighing experiments and isotope measurements have demonstrated that pencil marks make up no weighable percentage of the wood or cellulose and their $\delta^{13}C$ values were mostly found close to the ranges found in cellulose and wood. Besides, prior to cellulose extraction pencil marks can easily be removed mechanically with a razor blade or scalpel. However, they help distinguishing wood anatomical features after cellulose extraction, particularly when studying cross-sections of diffuse porous tropical wood species without distinct parenchyma bands.

The decision of whether wood or cellulose samples should be used for reasons of cost and time at the expense of data quality has become superfluous, when applying the cross-section cellulose extraction and tree-ring dissection procedure described here. We recommend the use of cellulose for stable isotope analysis, in particular, when emphasis is given to the importance of identical treatment of sample material ("IT principle", Werner and Brand, 2001; Carter and Fry, 2013; Brand et al., 2014), that should guide all isotope ratio determinations and evaluations.

6.2 Evaluation of the cross-section cellulose extraction system

The chemical purity tests of the holocellulose obtained from the cross-section extraction method confirm the findings by previous studies (e.g. Li et al., 2011; Kagawa et al., 2015). Despite the expected FTIR peak around 1732 cm^{-1} that is supposed to be significant for hemicellulose components such as mannose and xylose of wood samples

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(Fig. 5a and b), we found no significant peaks in this area for any cellulose sample (Fig. 5c–e). Cellulose is hygroscopic by nature and water adsorbs very fast to oven-dry cellulose. This might explain the relative intense peak around 1616 cm^{-1} derived from the cellulose cross-section samples.

The cellulose extraction procedure on wood cross-sections is working properly which is, additionally to the FTIR spectra, supported by the fact that the comparisons with stable carbon isotope data obtained from the classical extraction method showed a very high grade of synchronicity (Fig. 4). The studies by Li et al. (2011) and Kagawa et al. (2015) also reported only insignificant differences between the classical and the cross-section method obtaining R^2 of 0.977 and an average difference between tree-ring isotope ratios of both methods of 0.06–0.12 and 0.001 ‰, respectively.

The method has several benefits compared to the classical extraction method. The use of cross-sections permits the simultaneous extraction of wood from numerous tree-ring samples. Cross-sections of up to 180 cm in length (corresponding to 6 wood cores of 30 cm length) can be extracted in one step. A disadvantage, like an evaporation of water resulting in enrichment of chemical concentration due to long extraction intervals when using filter funnels for the cellulose extraction (Wieloch et al., 2011), are becoming negligible. The chemical treatment is consistent for all samples and renewal of NaOH- or $NaClO_2$-solutions is easy and fast and works semi-automatic by using a peristaltic pump system. The procedure saves time and causes minimum strain to particularly fragile thin sections from species with very low wood density, hence avoiding any damage to the cellulose cross-sections. Not only tree-ring boundaries, but also the cell structures largely remain visible, earlywood and latewood can easily be distinguished under a binocular with transmitted light. The flexible usage of custom designed teflon sheets within teflon casings allows cellulose extraction from wood cross-sections of variable size and thickness. Further, less space for the extraction apparatus is needed in the laboratory compared to the classical extraction method, i.e. only a part of a fume hood is required for processing up to six wood increment cores.

The basic principle of this cellulose extraction method is similar to the method described by Li et al. (2011) and Kagawa et al. (2015). The following dissimilarities and improvements to the published methods can be highlighted as follows: the cross-section cellulose extraction system described facilitates the horizontal position of cross-sections and together with the use of a peristaltic pump ensures a consistent chemical treatment of all samples. Due to the use of such a pump system the renewal of solutions works semi-automatically. No time consuming sewing of punching sheets with potentially contaminating cotton threads (Kagawa et al., 2015) is necessary to enclose the wood samples properly. The cellulose extraction of wood samples with a thickness of up to 3.5 mm is possible.

For convenient handling we recommend the use of wood samples with a length no longer than 6 cm, a width between 3 to 4 mm up to 15 mm and a thickness ranging from 0.5 to 2 mm. Caution should be exercised if the cross-sections are outside the suggested range of thickness. Cellulose cross-sections thinner than 0.5 mm are very fragile and difficult to handle subsequently. Cross-sections thicker than 2 mm may need a longer extraction procedure. Furthermore, shrinkage during cellulose extraction or drying may cause distortions in vertical direction (parallel to wood fibres or vessels) that may prevent from precise and unambiguous dissection of defined tree rings.

6.3 Application of the cellulose extraction system to different tree species

To evaluate the practical application of the method we tested the cross-section extraction and dissection procedure on several tree species with different types of cell structures. In general the cross-section extraction method works properly for both, coniferous and angiosperm wood (Fig. 6). Also subfossil timber can be treated properly with this method if not decomposed severely (example of *Pinus sylvestris*, see Table S1 for site details). The use of UV-light can facilitate the visualization of the tree-ring structure, as demonstrated for Juniper.

A special focus of the study presented here lies on tropical tree species for which the application of stable isotope analysis to dendroclimatology is increasingly used. Stable

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isotope records from tropical tree species, such as *Cedrella odorata* (e.g. Brienen et al., 2012) or *Tectona grandis* (Schollaen et al., 2013, 2014b), can significantly improve the knowledge of tropical climate in the past. The various tree samples presented here, demonstrate that the cross-section extraction and tree-ring dissection method can be applied very well to tropical tree species where alternative methods are often the only choice for successful tree-ring research (Heinrich and Allen, 2013). This opens new possibilities for studies on tropical species with more complicated wood anatomical structures, e.g. the African baobab (*Adansonia digitata*), a wide-spread and long-living species with high potential for providing stable isotope records of millennial length for climate reconstructions (Robertson et al., 2006; Slotta et al., 2014).

6.4 Evaluation of tree-ring dissection, homogenization and preparation for IRMS

Laumer et al. (2009) tested different homogenization approaches like grinding the wood sample before extraction, homogenization of cellulose by freeze milling or ultrasonic treatment and came to the conclusion that all methods are applicable for stable isotope analysis. The enormous sample loss and time intensive cleaning procedure by grinding or freeze milling makes these approaches unsuitable. In comparison, the loss of sample material by means of ultrasonic homogenization of water-dispersed cellulose is rather small (Laumer et al., 2009). However, oxygen isotopic exchange between sample and water in consequence of increased temperatures resulting from extensively long ultrasonic treatments is a known but frequently underestimated risk. Ultrasonic cavitation releases an extremely high energy per unit of volume which greatly surpasses the break tension of the cellulose fibres. The mean bond dissociation enthalpy, namely EH_m (HO-O) for water is 492 kJ mol^{-1} and the single bond enthalpy EH_m (O-H) is 463 kJ mol^{-1} (Kaye and Laby, 1973). Though these values may not be reached during the ultrasound treatment an activation could be induced such that an exchange of isotopes could be facilitated by higher temperatures, potentially resulting in depleted δ^{18}O values relative to the original cellulose samples, since δ^{18}O values of cellulose

from wood are in the range of 25 to 35 ‰ and oxygen isotopes from de-ionized water normally show values around −10 to −5 ‰ (Laumer et al., 2009). One may use larger amounts of sample material (> 1 mg) in order to reduce inhomogeneity effects. However, many tree-ring records derived from long-lived trees at timberline sites rarely provide enough material because of their narrow and frequently wedging rings. This complicates the strategy of using larger amounts of sample material.

The sample mass required for IRMS analysis can be achieved without extra homogenization by dissecting the tree rings precisely along the tree-ring boundaries (tangential direction), but with variable cuttings in radial direction that result in consistent specimens of rather similar weight despite variable ring widths. The specimens comprise the whole tree-ring width but vary in size, i.e. in tangential length parallel to the tree-ring boundaries (Fig. 3). In doing so, the cross-section dissection method may make (ultrasonic) homogenization obsolete (except for tree-ring widths > approx. 10 mm). Furthermore, provided that the IRMS holds an excellent linearity and the person who is performing the tree-ring cellulose dissection is well experienced, weighing may no longer be necessary for each individual sample when following this procedure. The method takes advantage of modern online IRMS systems that are capable of analyzing very small samples. The suggested way of separating tree rings from cellulose cross-sections which might be as thin as 0.5 mm, usually results in very small amounts of samples, which are still representative for an entire tree ring.

In general, cellulose cross-sections are more difficult to handle and more sensitive to breakage than wood cross-sections, thus gentle handling during the extraction process and especially during manual tree-ring dissection is required.

7 Conclusions

We presented a detailed methodical guideline for a semi-automated method of cellulose extraction from wood cross-sections and subsequent dissection of tree-rings or parts thereof. The procedure was applied to different tree species with a broad range

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of various cell structures, wood growth rates as well as potentially different amounts of lignin and resin.

The new guideline also describes the dissection of very narrow tree rings and furthermore offers potential for applying UV-laser-based microscopic dissection for highly resolved and very precise intra-annual tree-ring stable isotope analysis (Schollaen et al., 2014a). Due to the use of cross-sections, multidisciplinary analyses on the same wood samples (tree-ring width, stable isotope, quantitative wood anatomy, wood density) are possible, since the isotope analysis no longer consumes entire wood cores. Furthermore, the question of whether or not cellulose extraction is required for stable isotope analysis on tree-ring chronologies may become superfluous, as cellulose extraction and sample homogenization are no longer a time limiting step.

We conclude that following our guideline of semi-automated method of extracting cellulose and dissecting tree rings directly from cross-sections is an important approach to optimize stable isotope analysis in two ways: faster, cheaper and user friendly cellulose extraction, allowing the simultaneous treatment of wood cross-sections of a total length of 180 cm (equivalent to 6 increment cores of 30 cm length) and precise tree-ring dissection at annual to high-resolution scale utilizing manual devices or UV-laser microdissection microscopes. The procedure may even allow the analysis of individual tree-ring samples, where otherwise pooling (e.g. Dorado Liñán et al., 2011; Szymczak et al., 2012) would be required due to time and cost constraints.

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Table 1. Impact of potential contamination [δ ‰] from chalk ($CaSO_4 \times 2H_2O$), pencil lead (C) and maize starch ($(C_6H_{10}O_5)_n$) on the values of tree-ring stable isotope analysis of teak wood. Bold: Isotopic contamination exceeds the analytical error. Mass fractions of contaminants of 10 % (grey shaded boxes) are theoretical, but could have strong effects. In practice, mass fractions of considerably less than 1 % were constantly observed. This implies that pencil marks have no notable effect, although they remain visible on cellulose cross-sections. Remnants (up to 1 %) of chalk and maize starch can slightly shift the original $\delta^{18}O$ and $\delta^{13}C$ values of wood to more negative and more positive values, respectively (italic numbers). Note, chalk and starch are being removed during cellulose extraction (cf. text for more details).

				$\delta^{13}C_{wood}$				$\delta^{18}O_{wood}$			
				min	max	min	max	min	max	min	max
mass fraction of contaminant [%]				1	1	10	10	1	1	10	10
original value [‰]				−25.9	−23.6	−25.9	−23.6	20.1	22.9	20.1	22.9
chalk	$\delta^{18}O$	min	5					*−0.15*	*−0.18*	**−1.51**	**−1.79**
		max	8					*−0.12*	*−0.15*	**−1.21**	**−1.49**
pencil	$\delta^{13}C$	min	−28	−0.02	−0.04	**−0.21**	**−0.44**				
		max	−16	0.10	0.08	**0.99**	**0.76**				
maize starch	$\delta^{13}C$	min	−14	***0.12***	*0.10*	**1.19**	**0.96**				
		max	−11	***0.15***	***0.13***	**1.49**	**1.26**				
	$\delta^{18}O$	min	26					0.06	0.03	**0.59**	**0.31**
		max	36*					0.16	0.13	**1.59**	**1.31**
				Potential isotopic shift due to contamination							

* measured by Williams et al. (2005).

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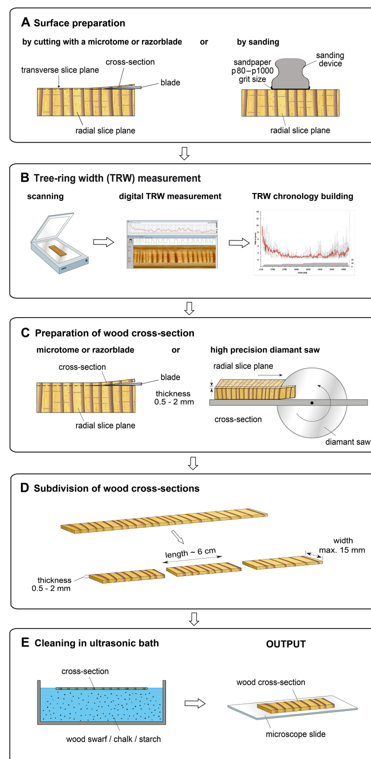

Figure 1. Preparation scheme of wood cross-sections for cellulose extraction procedure. **(a)** Preparation of a plane cross-sectional wood surface by sanding, cutting with a core-microtome or razorblade, **(b)** scanning of the wood core, TRW measurement and TRW chronology building, **(c)** preparation of thin wood cross-sections with a high-precision diamond wafering saw, a core microtome or razorblade, **(d)** subdivision of cross-sections into max. 6 cm long segments of max. 1.5 cm width, **(e)** cleaning in an ultrasonic bath to remove wood swarf and possible contaminants. For details cf. text (Sect. 2.1).

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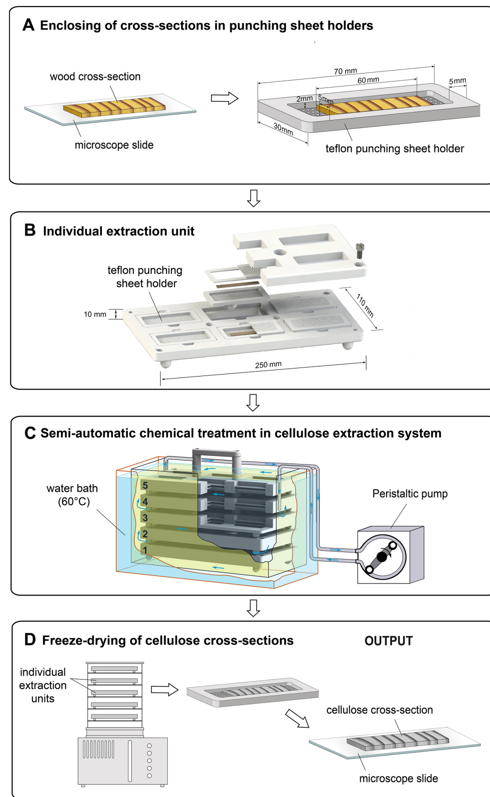

Figure 2. Scheme of the cellulose extraction method from wood cross-sections using a customized extraction device. **(a)** Enclosing of wood cross-sections in customized punching sheet holders, **(b)** up to 6 wood cross-sections can be placed in individual extraction units, **(c)** semi-automated cellulose extraction in a customized extraction system using a two silicon tubes peristaltic pump system for chemical treatment, **(d)** freeze-drying of cellulose cross-sections within their extraction units. For details cf. text (Sect. 2.2).

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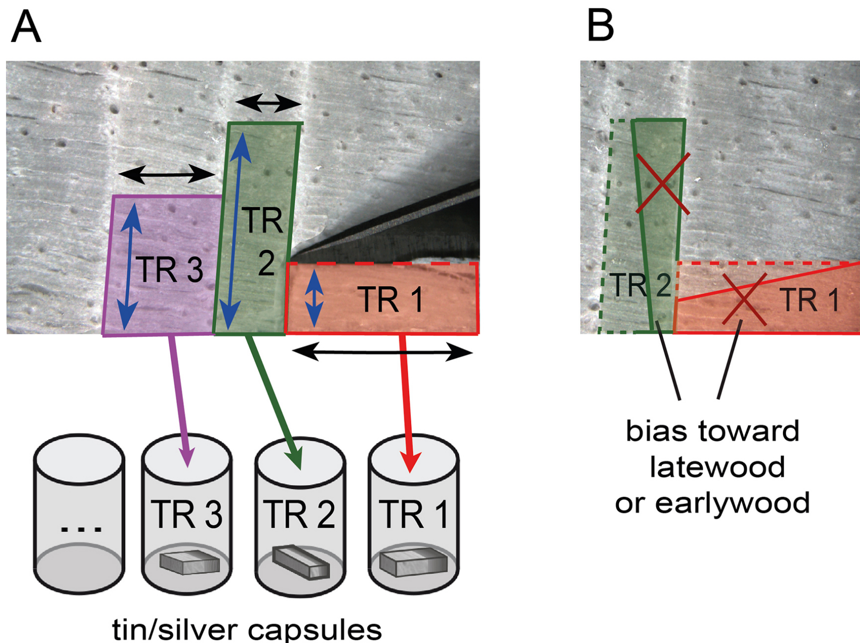

Figure 3. Tree-ring dissection using cellulose cross-sections. **(a)** The mass of tree-ring cellulose required is adjusted by variable positions (blue arrows) of radial cuttings perpendicular (black arrows) to the tree-ring boundaries. Dissection can be done manually with a scalpel under a binocular microscope or automatically with an UV-laser microdissection microscope (Schollaen et al., 2014a). After checking for appropriate mass ranges (weighing) specimen are loaded into tin or silver capsules for IRMS analysis. The variable radial cuttings result in specimens of different sizes (purple, green, orange colored areas) but similar weight that comprise the whole tree-ring width. In principle, no homogenization is required. **(b)** For avoiding biases toward late or earlywood, care must be taken, that the dissected specimen comprises the whole tree-ring width (unless intra-annual sampling is desired) and radial cuttings are performed perpendicular to the tree-ring boundaries. For details cf. text (Sect. 2.3).

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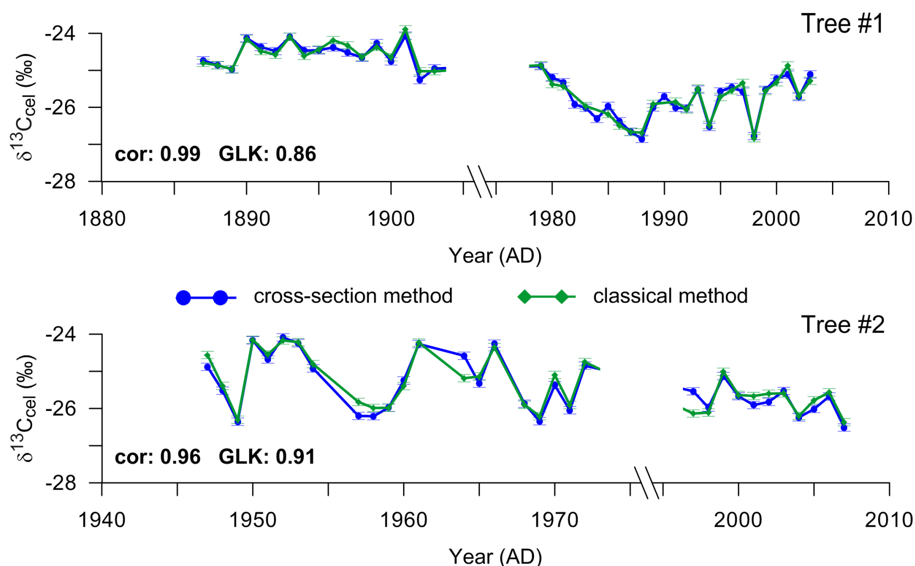

Figure 4. Comparison between $\delta^{13}C$ values from cellulose of two teak trees (*Tectona grandis*) using the classical method of cellulose extraction (green lines) and the cross-section method (blue lines). The analytical precision is shown with error bars. GLK: Gleichläufigkeit; cor: Pearson correlation coefficient. The stable isotope records of the two different methods are well correlated and show a high degree of synchronization suggesting that the cellulose extraction directly from tree-ring cross-sections is working properly.

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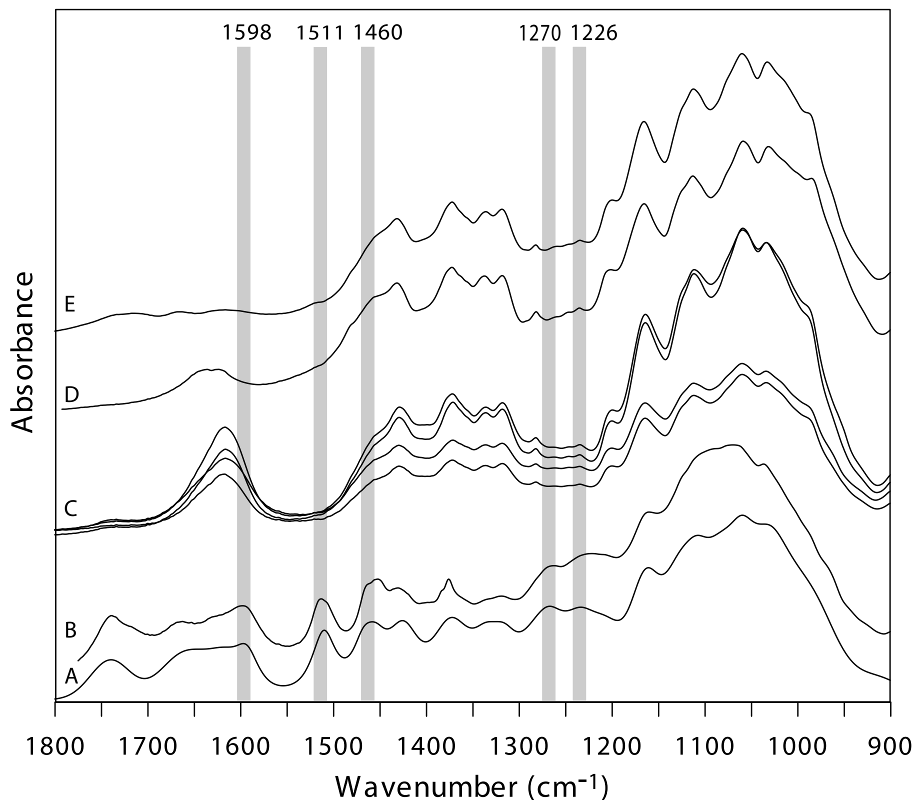

Figure 5. FTIR (Fourier transform infrared) Spectra of **(a)** untreated wood, **(b)** resin extracted wood, **(c)** holocellulose of wood samples, **(d)** α-cellulose standards (Merck) and **(e)** α-cellulose standards (Fluka). The wood samples are all from *Tectona grandis*. Grey shaded bars mark lignin-specific bands. FTIR spectra of the cellulose cross-sections show no sign of residual lignin.

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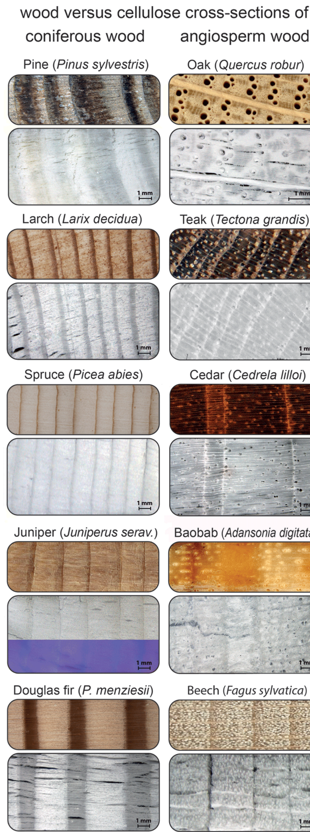

Figure 6. Tree species that have been tested for cellulose extraction and tree-ring dissection on cross-sections. The upper pictures of each species show wood cross-sections while the lower pictures display the cellulose cross-sections obtained. Tree-ring structures remained clearly visible after the cellulose extraction process. The use of UV-light can help to detect tree-ring boundaries as shown for Juniper.

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