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Comparison between $\delta^{13}C$ of α-cellulose and bulk wood in the mangrove tree *Rhizophora mucronata*: Implications for dendrochemistry

Anouk Verheyden[a,c,*], Maarten Roggeman[a], Steven Bouillon[b], Marc Elskens[b], Hans Beeckman[c], Nico Koedam[a]

[a]*Vrije Universiteit Brussel, Laboratory of General Botany and Nature Management (APNA), Pleinlaan, 2, 1050 Brussels, Belgium*
[b]*Vrije Universiteit Brussel, Laboratory for Analytical and Environmental Chemistry (ANCH), Pleinlaan, 2, 1050 Brussels, Belgium*
[c]*Laboratory for Wood Biology and Xylarium, Royal Museum for Central Africa (RMCA), Leuvense Steenweg 13, 3080 Tervuren, Belgium*

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Abstract

Stable carbon isotope analysis of tree rings has become a widely used proxy in environmental and palaeoclimatological studies. In those studies, α-cellulose has often been the preferred material because of its singular composition and its immobility in wood. However, cellulose extraction is a time-consuming procedure and since the development of on-line isotope ratio mass spectrometers has become the time-limiting step in the isotopic analysis of wood samples for dendrochemical purposes. In this study we evaluate the necessity of cellulose extraction for isotopic analysis of tree rings in a tropical mangrove tree, *Rhizophora mucronata* Lam. Comparison between the $\delta^{13}C$ of extracted α-cellulose and bulk wood material revealed a highly significant linear relationship ($\delta^{13}C_{bulk\ wood}=0.92\ (\pm 0.08)*\delta^{13}C_{\alpha\text{-cellulose}}-2.91\ (\pm 2.04)$; $p<0.001$) for α-cellulose values between −24‰ and −27‰. However α-cellulose was on average 0.97 ± 0.03‰ enriched in ^{13}C as compared to bulk wood. The slope of the regression was not significantly different from one ($p<0.05$). Furthermore, no significant difference was found between either the $\delta^{13}C_{bulk\ wood}-\delta^{13}C_{\alpha\text{-cellulose}}$ slopes for earlywood and latewood or between the slopes for samples from trees growing in contrasting environmental conditions. These results indicate that bulk wood can be used instead of α-cellulose when measuring stable carbon isotopes in the sapwood of *R. mucronata* in the context of a dendrochronological investigation.

Keywords: Bivariate least square regression; Dendrochronology; East Africa; Jayme–Wise; Kenya; Tree rings

1. Introduction

Stable carbon isotopes in plants have become a widely used proxy in environmental (Schleser, 1990; Lin and Sternberg, 1992; Livingston and Spittlehouse,

* Corresponding author. Current address: Laboratory for Wood Biology and Xylarium, Agriculture and Forestry Section, Royal Museum for Central Africa (RMCA), Leuvense Steenweg 13, 3080 Tervuren, Belgium. Tel.: +32 2 769 56 12.
E-mail address: anouk.verheyden@africamuseum.be (A. Verheyden).

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1996) and palaeoclimatological studies (Mazany et al., 1980; Brenninkmeijer, 1983; Leavitt and Long, 1991). In mangroves, the $\delta^{13}C$ of the photosynthetic material has been found to mainly reflect the soil water salinity (Ish-Shalom-Gordon et al., 1992; Lin and Sternberg, 1992; Medina and Francisco, 1997; Kao et al., 2001). Increased soil salinity causes a reduction of the CO_2 assimilation rate, as well as a decrease of the stomatal conductance resulting in a lower ratio of the intercellular to atmospheric CO_2 and, consequently, in a higher $\delta^{13}C$ value (Farquhar et al., 1982). Therefore, when time-series based on tree rings are analyzed, the $\delta^{13}C$ should be a valuable proxy to study changes in salinity caused by drought, reduction in freshwater input or sea-level rise and provide possible insight in the forest dynamics or explain the degradation of the forests observed in many countries. The recent discovery of the presence of annual growth rings in the mangrove *Rhizophora mucronata* Lam. (Verheyden et al., 2004a) allows the inclusion of this species in dendrochronological and dendrochemical research (Verheyden, 2004; Verheyden et al., 2004b; Yu et al., 2004).

Dendrochemical studies investigating the isotopic composition of tree rings have preferably used α-cellulose (or, alternatively, holocellulose, a combination of cellulose and hemicelluloses) as opposed to bulk wood, because of its singular composition and immobility in the wood (Mazany et al., 1980; Benner et al., 1987; Leavitt and Danzer, 1993). As a result of additional biochemical reactions leading to their formation, different wood compounds have been found to show a different isotopic signal, which in turn influences the $\delta^{13}C$ of the bulk wood material depending on their relative occurrence in the wood (Benner et al., 1987; Sheu and Chiu, 1995; Schleser et al., 1999).

Several methods have been developed for the extraction of α-cellulose from wood samples (see Green, 1963 for a summary of early references), but the method developed by Jayme and Wise (in Green, 1963), followed by an alkaline extraction of hemicelluloses, has been the most widely used. However, the extraction is time-consuming and was developed for relatively large samples (~1 g). The development of on-line isotope ratio mass spectrometers (IRMS) has lead to a significant increase in sample throughput as well as a decrease in sample size, offering new potentials for high-resolution (intra-annual) isotope research in tree rings. As a result of the technological advancement of the instrumentation, the cellulose extraction has become the time-limiting step in the study of isotopic composition of tree rings (McCarroll and Loader, 2004). Therefore, many authors have suggested adaptations to the Jayme–Wise method to allow a faster and/or more efficient cellulose extraction (Leavitt and Danzer, 1993; Loader et al., 1997; MacFarlane et al., 1999; Brendel et al., 2000). On the other hand, some authors have questioned the necessity of extracting cellulose for dendrochemical research (Borella et al., 1998; Schleser et al., 1999), since several studies have found a high correlation between α-cellulose and bulk wood as well as between α-cellulose and lignin (Mazany et al., 1980; Schleser, 1990; Leavitt and Long, 1991; Livingston and Spittlehouse, 1996; Borella et al., 1998; MacFarlane et al., 1999), allowing for the prediction of the $\delta^{13}C$ values of cellulose from the $\delta^{13}C$ values of bulk wood. In addition, bulk wood was found to be an equally good and sometimes an even better climate proxy than α-cellulose (Borella et al., 1998; Loader et al., 2003). Although Borella et al. (1998) predicted that this statement should hold for other hardwood tree species than the ones they studied (oak, birch and beech), they suggested that this might not be valid for coniferous wood, due to the large relative concentration of extractives. While considerable literature exists on the necessity of cellulose extraction for temperate trees, studies on tropical trees are limited (MacFarlane et al., 1999; West et al., 2001). However, tropical wood may have higher levels of extractives as compared to temperate tree species (Wagenführ and Scheiber, 1989). It is therefore advised to compare the isotopic signals of α-cellulose and bulk wood for each new tropical tree species investigated.

The aim of this study is to evaluate the necessity of cellulose extraction for the stable carbon isotope analysis of tree rings in *R. mucronata* wood. To achieve this aim, we compared the isotopic signal of extracted α-cellulose and bulk wood by calculating a regression line using bivariate least square statistics and testing the deviation of the slope from one, which will indicate whether the difference between the $\delta^{13}C$ of α-cellulose and the $\delta^{13}C$ of the bulk wood is independent of the isotopic signal itself. Since the chemical composition (e.g. lignin content) of the

wood may differ between seasons (Borella et al., 1998), which in turn may influence the isotopic composition of the bulk wood, the earlywood and latewood of each annual ring were processed separately. *R. mucronata* wood displays an alternation of dark brown earlywood layers, characterized by a low vessel density, and light brown latewood layers, characterized by a high vessel density (Verheyden et al., 2004a; see Fig. 1). While the color alternation is largely the result of a change in porosity of the wood (higher vessel density in the latewood, see Verheyden et al., 2004a), it is not impossible that differences in lignin content between earlywood and latewood also contribute to the difference in color. Finally, this study uses two samples from sites with contrasting environmental conditions. The two samples display a difference in color (see Materials and Methods), which again may partially or mainly be the result of a different chemical composition of the wood.

2. Materials and methods

2.1. Samples and sample preparation

Stem discs of two *R. mucronata* trees were collected in 1999 from two sites with contrasting environmental conditions in Gazi Bay, Kenya (39°30′E, 4°25′S). The stem discs are now part of the Tervuren wood collection of the Royal Museum for Central Africa, Tervuren, Belgium (accession numbers: Tw55891 and Tw55943) (Fig. 1). Stem disc Tw55891 was collected from a basin type forest (sensu Lugo and Snedaker, 1974), which is inundated by the high tides about 50 times a month. Stem disc Tw55943 was collected from a riverine forest type (sensu Lugo and Snedaker, 1974), which is inundated by the high tides about 20 times a month. The two samples show different wood colors. Tw55891 exhibits a light orange-brown color, while Tw55943 displays a dark chocolate-brown color. Earlywood and latewood of each annual ring (see Fig. 1) were carefully separated over the entire circumference of the stem disc. A total of 27 wood samples were obtained from the two stem discs (19 samples from Tw55891 and eight samples from Tw55943). Each wood sample was ground with a slightly modified coffee mill (KENWOOD CG100). The modification consisted of fixing sharper knives on the blades of the coffee mill and reducing the height of the lid, so that wood particles would be held down and grinding would be more efficient. The standard deviation of six repeated measurements of $\delta^{13}C$ on three samples was equal to 0.05‰ (1σ), which is of the same order of magnitude as the analytical precision of the instrument (see further) and certifies that the ground samples were homogenous. Sub-samples of the ground material were taken for cellulose extraction and bulk analysis.

2.2. Cellulose extraction

The method follows the description of Green (1963), however longer reaction times were used for each step (see further), comparable to those reported in Leavitt and Danzer (1993) and MacFarlane et al. (1999), to assure a complete cellulose extraction.

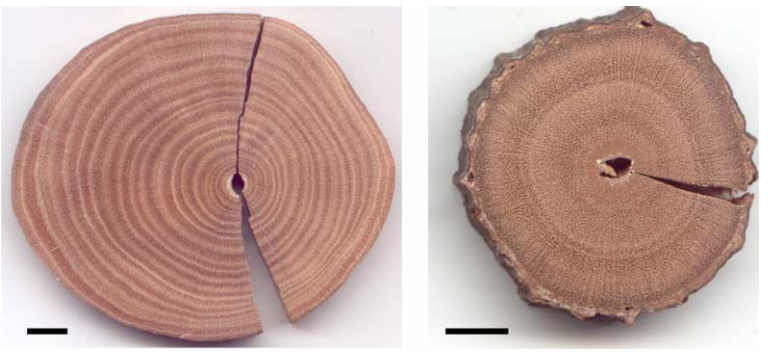

Fig. 1. Stem disc Tw55891 (left) and Tw55943 (right) collected from Gazi Bay, Kenya displaying the indistinct annual growth rings, which consist of dark-colored earlywood layers, characterized by a low vessel density, and light-colored latewood layers, characterized by a high vessel density (see Verheyden et al., 2004a for more details). Scale bar represents 1 cm.

Between 0.15 g and 1.24 g wood material from each earlywood and latewood layer were processed for cellulose extraction. The first step consists of washing out extractives, such as oils and resins using a Soxhlet extraction line with a 2:1 (by volume) benzene–ethanol mixture for 16 h, followed by ethanol, during 16 h. The second step in the cellulose extraction consists of removing the lignin. The wood material was transferred into an Erlenmeyer and 200 ml of deionized water, 0.3 ml glacial acetic acid (CH_3COOH, analytical grade) and 1.25 g sodium chlorite ($NaClO_2$, technical grade) were added. The solution was brought to 70 °C in a water bath and left overnight. An additional 0.3 ml glacial acetic acid and 1.25 g sodium chlorite was added in the morning and again 5 h later. Samples were then rinsed thoroughly with deionized water and collected on a glass fiber filter (Whatmann GF/C, 47 mm). The last step in the procedure consists of the removal of hemicelluloses, which was achieved using a 4% solution of NaOH at 70 °C, for 24 h (Brenninkmeijer, 1983). The extracted α-cellulose was again rinsed thoroughly with deionized water and collected on a GF/C filter. Fifteen of the twenty-seven samples were oven dried (60 °C) after each step and weighed, from which the yield was calculated. The remaining samples were dried at the end of the procedure only.

2.3. Stable carbon isotope measurements

Bulk wood material and extracted α-cellulose were sub-sampled (1 mg) for isotopic analysis and wrapped into tin cups. Stable carbon isotopic composition of the samples was measured on-line using an Element Analyzer (Flash 1112 Series EA Thermo Finnigan) coupled via a CONFLO III to an IRMS (DELTA^{plus}XL, Thermo Finnigan). Sucrose was used as a standard (IAEA-CH-6, $\delta^{13}C = -10.4 \pm 0.1‰$). Results are reported using the conventional δ notation relative to the VPDB standard. The reproducibility of the method, including the cellulose extraction, is 0.05‰ (1σ; $n=9$), which is of the same order of magnitude of the analytical precision of the instrument ($1\sigma = 0.08‰$, $n=214$).

2.4. Statistics

The relation between the $\delta^{13}C$ of α-cellulose and bulk wood was assessed using least square regression. When both the dependent and independent variables are measured with errors (as is the case in this study), the reduced major axis regression (RMA) and the bivariate least squares regression (BLS) are more appropriate than the ordinary least square regression (OLS). While RMA allows correcting for the estimation of the slope, BLS allows correcting for estimations of both the slope and intercept (Riu and Rius, 1996; Martinez et al., 1999). Since the estimates of slope and intercept are not independent, and a value for one of the parameters automatically influences that of the other, BLS was used in this study. Significance tests for the slope and intercept of the regression and correlation coefficients were based on the joint confidence interval, which was calculated taking the analytical uncertainties on the measurements of both extracted cellulose and bulk wood into account (Martinez et al., 1999). Further investigation of the slope was by the Student's t-test. Data are given as means ± standard error.

3. Results and discussion

The yield after the Soxhlet extraction was 93.7 ± 0.4% ($n=15$), 65.9 ± 0.3% ($n=15$) after lignin extraction and 35.7 ± 0.4% ($n=15$) after the alkaline extraction. These values are comparable to those reported by Loader et al. (1997) and MacFarlane et al. (1999) and indicate that only little material was lost during the cellulose extraction. However, it should be noted that Brendel et al. (2000) were critical of the yields reported by Loader et al. (1997). Our yield data suggest that *R. mucronata* wood is composed approximately of 6% extractives, 28% lignin, 30% hemicelluloses and 36% α-cellulose.

The $\delta^{13}C$ of the α-cellulose and of the bulk wood material showed a highly significant linear relationship (BLS: $\delta^{13}C_{bulk\ wood} = 0.92\ (\pm 0.08) * \delta^{13}C_{\alpha\text{-cellulose}} - 2.91\ (\pm 2.04)$; $p<0.001$; $n=27$; $r^2=0.96$; Fig. 2), as has been found by other authors for different tree species (e.g. Borella et al., 1998; MacFarlane et al., 1999). On average, the bulk wood of *R. mucronata* are 0.97 ± 0.03‰ (range = 0.64–1.29‰; $n=27$) isotopically lighter than the corresponding α-cellulose samples (for samples with $\delta^{13}C_{\alpha\text{-cellulose}}$ ranging from −26.9‰ to −24.1‰). Similar differences have been reported for

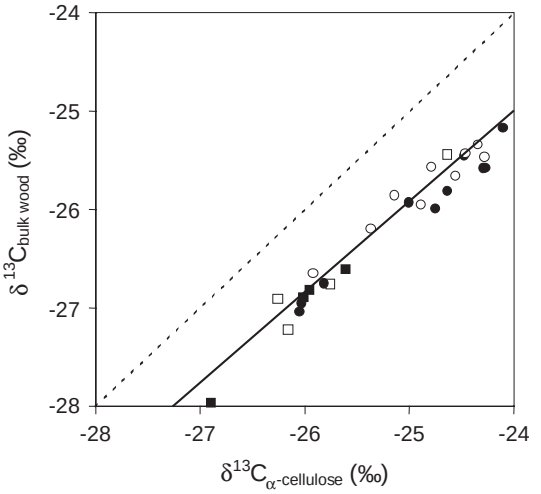

Fig. 2. Values of the $\delta^{13}C$ (‰) of α-cellulose versus $\delta^{13}C$ (‰) of the corresponding bulk wood for the two sampled stem discs (Tw55891: circles, Tw55943: squares). Solid symbols represent earlywood samples, open symbols represent latewood samples. Each value represents a single measurement. The BLS (bivariate least square) regression (solid line, $\delta^{13}C_{bulk\ wood} = 0.92\ (\pm 0.08) * \delta^{13}C_{\alpha\text{-cellulose}} - 2.91\ (\pm 2.04)$, $p < 0.001$, $n = 27$) and the one to one line (dashed line) are also shown.

Eucalyptus globulus Labill. as well as for *Pinus* spp. and *Quercus* spp. (Borella et al., 1998; MacFarlane et al., 1999). However, the difference in $\delta^{13}C$ between α-cellulose and bulk wood is dependent on the relative concentration of other wood compounds and has been reported to be as high as 2.5‰ (*Juniperus* spp.; Leavitt and Long, 1991). These differences are caused mainly by the presence of lignin, which is on average 2–4‰ lighter than α-cellulose (Benner et al., 1987). Nevertheless, lignin does not affect the correlation between $\delta^{13}C$ of bulk wood and α-cellulose, as the isotopic signal of lignin correlates well with the isotopic signal of α-cellulose (Mazany et al., 1980). In contrast, the isotopic composition of extractives is usually uncorrelated to the signal of α-cellulose and can dampen the isotopic signal, especially at more positive $\delta^{13}C$ values (Borella et al., 1998). Therefore, an important prerequisite in using bulk wood instead of α-cellulose is that the slope of the regression is not significantly different from one. In theory, the presence of a linear regression already allows the use of bulk wood instead of extracted α-cellulose; however, due to a possible dampening of the isotopic signal, α-cellulose values will have to be predicted from the bulk wood using the linear regression. If the slope of the regression is not significantly different from one, this procedure becomes unnecessary, allowing the use of bulk wood for dendrochemical applications.

Unlike Borella et al. (1998), we found that the slope of our regression was not significantly different from one (*t*-test; 95% confidence interval: 0.84, 1.004). However, care should be taken when using the regressions of older publications for comparison, as these studies have mostly (incorrectly) used the ordinary least square regression (see Materials and

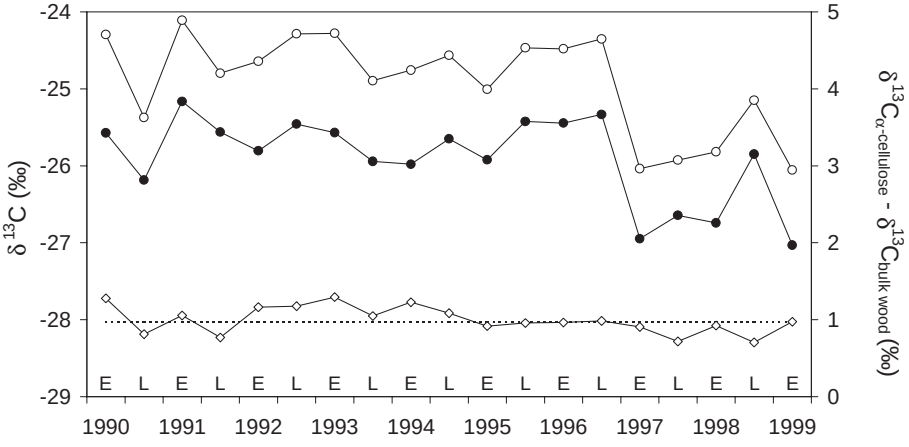

Fig. 3. Stable carbon isotope chronology obtained from consecutive earlywood (E) and latewood (L) layers from stem disc Tw55891 illustrating the relatively constant difference (open diamonds) between the bulk wood (solid circles) and extracted α-cellulose (open circles). The average difference is shown as the dashed line.

methods). Our results indicate that the difference in $\delta^{13}C$ between α-cellulose and bulk wood can be considered constant in *R. mucronata* wood and is independent from the isotopic signal itself. This is further illustrated in Fig. 3, which shows a relatively constant difference between α-cellulose and bulk wood in the stable carbon isotope chronology obtained from stem disc Tw55891. Furthermore, no significant difference (*t*-test, $p=0.91$) was found between the slopes obtained for earlywood ($\delta^{13}C_{bulk\ wood}=0.90\ (\pm 0.09)*\delta^{13}C_{\alpha\text{-cellulose}}-3.47\ (\pm 2.17)$; $n=14$; $r^2=0.98$) and latewood ($\delta^{13}C_{bulk\ wood}=0.92\ (\pm 0.15)*\delta^{13}C_{\alpha\text{-cellulose}}-2.98\ (\pm 3.65)$; $n=13$; $r^2=0.94$). High-resolution bulk wood $\delta^{13}C$ values measured in eight *R. mucronata* trees range from −23‰ to −29‰ (Verheyden et al., 2004b). Calculating α-cellulose $\delta^{13}C$ using these values for both the earlywood and latewood equations results in differences which are smaller than the analytical precision of this method. This suggests that it is not necessary to use separate equations to calculate α-cellulose from bulk earlywood and latewood (within the natural range of $\delta^{13}C$ encountered in *R. mucronata* wood).

In this study, the earlywood and latewood were processed separately, based on the hypothesis that the difference in color between earlywood and latewood might partially be the result of a difference in lignin content (see Introduction). Our results show that if variation in chemical composition does exist, the influence on the carbon isotopic composition is minimal. However, it is important to note here that the use of earlywood and latewood isotopic signals for dendrochemical applications should be re-evaluated. Recent studies on high-resolution (intra-seasonal) stable carbon isotope measurements in tree rings have demonstrated that the variations in the isotopic signal do not correspond to changes in wood anatomical features, indicating that the isotopic signal has a certain independence from the wood anatomical boundaries used to define earlywood and latewood. This has been found in temperate trees (Helle and Schleser, 2004) and has also been shown to occur in *R. mucronata* (Verheyden et al., 2004b). Verheyden et al. (2004b) found that low resolution $\delta^{13}C$ measurements resulted in an artificial dampening of the signal (averaging effects) as well as in an artificial between-year and within-year variation (see Verheyden et al. (2004b)).

Similar to the comparison between earlywood and latewood, the slopes obtained from two samples displaying a different wood color and collected from contrasting environmental conditions were also compared. No significant difference (*t*-test, $p=0.33$) was found between the slopes obtained for sample Tw55891 (bulk wood = 0.86 (±0.12) * α-cellulose − 4.39 (±2.87); $n=19$; $r^2=0.93$) and sample Tw55943 (bulk wood = 1.09 (±0.21) * α-cellulose + 1.36 (±5.53); $n=8$; $r^2=0.96$), indicating again that if variation in chemical composition does exist between the two samples, the influence on the carbon isotopic composition is minimal.

4. Conclusions

In conclusion, the high correlation between $\delta^{13}C$ of α-cellulose and bulk wood in *R. mucronata* together with a slope which is not significantly different from one, allows the use of bulk wood instead of α-cellulose for dendrochemical applications. However, it should be noted here that these results cannot be applied when using fossil wood or decayed wood pieces, since the degradation of cellulose and hemicelluloses leads to an increased proportion of lignin (see, for example, Schleser et al., 1999; Robertson et al., 2004a). Furthermore, care should be taken when analyzing longer time records, since heartwood samples may have a higher amount of extractives than sapwood (see, for example, Borella et al., 1998). This study did not include the analysis of heartwood samples due to the overall young age of the mangrove trees in Kenya (see Verheyden et al., 2004a).

The possibility of using bulk wood, allows for the analysis of small (down to 20 μg or even less, using conventional online IRMS instrumentation) and many samples with relatively little sample preparation. We estimated that the preparation time for 100 samples amounts to 3 h (including cutting slivers using a sliding microtome and wrapping of the samples in tin cups as a preparation for $\delta^{13}C$ analysis), without cellulose extraction, as compared to 10 days using the extraction method described in this paper. A number of recent studies have demonstrated the importance of stable isotope analysis at annual resolution (Robertson et al., 2004b), but in particular at high resolution for tropical dendrochronology (Evans and Schrag, 2004; Poussart

et al., 2004; Verheyden et al., 2004b), further illustrating the importance of the results presented here.

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