

Rapid Commun. Mass Spectrom. 2014, 28, 1371–1375  
(wileyonlinelibrary.com) DOI: 10.1002/rcm.6912

## Sensitivity of whole wood stable carbon and oxygen isotope values to milling procedures

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**RATIONALE:** Milling of wood samples is a widely applied preparation method for pooling tree-rings from different trees or periods of several years for determination of  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values. In this study, whole wood samples were milled using different procedures in order to evaluate potential effects of this preparation method on  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values.

**METHODS:** Subsamples of a 5 cm<sup>3</sup> wood piece of a single tree-ring from a lowland white fir were used. The samples were milled with different setups: (i) two and three stainless-steel balls, (ii) 3, 5 and 8 min milling time, and (iii) discontinuous and continuous milling. The  $\delta^{13}\text{C}$  values were measured using an elemental analyser connected to an IsoPrime mass spectrometer and  $\delta^{18}\text{O}$  values using a Thermo Scientific MAT 253 mass spectrometer and a TC/EA connected by a ConFlo IV.

**RESULTS:** The results show that varying the milling procedure does not alter the  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values in comparison to non-milled blank samples. For shorter milling times, an increased variance of  $\delta^{18}\text{O}$  values is recorded, probably caused by isotopic gradient between early- and latewood portions of the tree-ring and thereby biasing the insufficiently homogenised samples. No overheating effects on the  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values were detected.

**CONCLUSIONS:** Milling of wood samples for carbon and oxygen isotope analyses is an appropriate preparation method. Copyright © 2014 John Wiley & Sons, Ltd.

During the last decade, an increasing number of studies have employed carbon and oxygen stable isotope ratios from tree-rings as climate proxies.<sup>[1–4]</sup> Many of these studies use ball or centrifugal mills to pulverise and homogenise the wood samples.<sup>[1,5,6]</sup> The application of these mills is particularly useful if larger sample amounts are processed. This is, for instance, the case if material of the same calendar year from several trees is pooled<sup>[1,7,8]</sup> or if material of multiple years is analysed as a single sample.<sup>[9,10]</sup>

We use this common wood preparation procedure for  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  analyses in our laboratory, and observed a warming of the wood samples during milling and a burning smell. Pure cellulose (Merck Chemicals, Darmstadt, Germany) that was continuously milled for 8 min exhibited 1.4‰ more negative  $\delta^{18}\text{O}$  values than non-milled cellulose. This may be explained by oxidation of the cellulose with atmospheric oxygen, which has an approximately 4‰ more negative  $\delta^{18}\text{O}$  value (23.5‰<sup>[11]</sup>) than cellulose ( $\delta^{18}\text{O}$  = 27.5‰), and may thus result in a lower  $\delta^{18}\text{O}$  value. The oxidation is triggered by the heat caused by the mechanical energy produced during the milling procedure. This change in  $\delta^{18}\text{O}$

values was not, however, observed after 8 min of continuous milling with a cryogenic mill. The heat may also have an influence on the  $\delta^{13}\text{C}$  values of the whole wood. Resin will be the first component of the wood sample to be converted into CO<sub>2</sub> as a result of being heated.<sup>[12]</sup> Due to the resin having a lower  $\delta^{13}\text{C}$  value than the other wood components,<sup>[13]</sup> the released CO<sub>2</sub> will be enriched in <sup>12</sup>C. This would lead to higher  $\delta^{13}\text{C}$  values for the milled whole wood samples.

These observations question the applicability of milling as a preparation method for isotopic analyses. Our working hypothesis is that the milling procedure leads to a warming of whole wood samples, resulting in decreased  $\delta^{18}\text{O}$  values due to oxidation with atmospheric oxygen and an increase in  $\delta^{13}\text{C}$  values due to CO<sub>2</sub> losses. In order to verify this hypothesis, we performed a systematic test using a homogeneous wood sample.

We tested if different milling procedures (number of stainless-steel balls, milling duration and continuity of the milling process) altered the  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values of whole wood samples. Longer milling duration might lead to an increased development of heat and alteration of the  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values. A larger number of stainless-steel balls might result in a larger release of friction heat. Finally, discontinuous milling might lead to interim cooling of the samples and prevent or reduce alteration effects. The results of this test could be of interest to the community using milling for tree-ring sample preparation.

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## EXPERIMENTAL

A stem disc from a fast growing lowland white fir (*Abies grandis*) from Laubach (Hunsrueck, western Germany) was cut down in 2011. The stem disc included a rather wide tree-ring (approximately 2 cm) of the year 2002, which was used for the milling test. A wood sample of approximately 5 cm<sup>3</sup> was cut out from this tree-ring. The prerequisite for the milling test with different setups is the homogeneity of its stable isotope composition. Fifteen radial and ten horizontal thin slices, which were equally distributed on the 5 cm<sup>3</sup> wood sample, were taken using a sliding microtome.<sup>[14]</sup> All slices contained both early- and latewood. Since the  $\delta^{18}\text{O}$  values of wood samples typically show a higher intra-tree and inter-tree variability than the  $\delta^{13}\text{C}$  values,<sup>[15]</sup> the homogeneity was only tested for the  $\delta^{18}\text{O}$  values. The mean value of these results was also used as the blank sample for the  $\delta^{18}\text{O}$  milling test.

Subsequent to the homogeneity test, 13 thin slices were cut from the tree-ring block, and 12 different milling procedures (Table 1) were applied to these samples. One slice was not milled and used as the  $\delta^{13}\text{C}$  blank sample. For each milling procedure, approximately 40 mg of wood material was put into 2 mL micro-centrifuge tubes and freeze-dried for 24 h. Subsequently, the samples were milled using a Retsch MM 200 (Haan, Germany) swing mill with a frequency of 30 Hz.

Depending on the milling procedure, two or three stainless-steel balls (diameter 5 mm) were filled into the micro-centrifuge tubes. Furthermore, the milling duration (3, 5 or 8 min) and the milling continuity (with and without 1 min breaks after each minute of milling) were controlled (Table 1). The coarseness of the milled samples was determined using an optical microscope (Leica, Wetzlar, Germany).

For the  $\delta^{18}\text{O}$  analyses, up to three subsamples were taken for each milling procedure, while two subsamples were used for the  $\delta^{13}\text{C}$  analyses. For the  $\delta^{13}\text{C}$  analyses, 1–2 mg were weighed using a XP6 microbalance (Mettler Toledo, Gießen, Germany) ( $\pm 1 \mu\text{g}$ ) and filled into tin capsules. These samples were subsequently combusted at 1150°C in a Vario EL III elemental analyser (Elementar Analysensysteme GmbH, Hanau, Germany) connected to an IsoPrime isotope ratio mass spectrometer (GV Instruments Ltd., Manchester, UK). Standards from IAEA (Vienna, Austria), IAEA-CH-6 (sucrose) and IAEA-CH-7 (polyethylene), were each measured three times during the sample run. Due to the low number of standard measurements, the analytical error is given by the higher standard deviation of the two standards. The standard deviation of the IAEA-CH-7 measurements was 0.4%. For the  $\delta^{18}\text{O}$  analyses, approximately 250–300  $\mu\text{g}$  were weighed with a XP26 microbalance (Mettler Toledo, Gießen, Germany) ( $\pm 1 \mu\text{g}$ ) and filled into silver capsules. These were then pyrolysed at 1450°C in a high-temperature conversion elemental analyser

**Table 1.** Compilation of the parameters used for the twelve different milling procedures. Also shown are the coarseness and the standard deviations (SDs) of the  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values of the subsamples

Test number	Number of stainless-steel balls	Milling time [min]	1 min breaks after each milling minute	Coarseness	SD of the $\delta^{18}\text{O}$ subsample values [% VSMOW]	SD of the $\delta^{13}\text{C}$ subsample values [% VPDB]
1	2	3	Yes	0.5–3 mm large pieces	0.5	0.3
2	3	3	Yes	Powder (<200 $\mu\text{m}$ ) with a lot of 0.5–2 mm large pieces	0.1	0.03
3	2	3	No	0.5–3 mm large pieces	0.4	0.2
4	3	3	No	Powder (<200 $\mu\text{m}$ ) with a lot of 0.5–2 mm large pieces	0.2	0.3
5	2	5	Yes	Powder (<200 $\mu\text{m}$ ) with sporadic 0.5–2 mm large pieces	0.6	0.01
6	3	5	Yes	Powder (<200 $\mu\text{m}$ ) with sporadic 0.5–1 mm large pieces	0.3	0.2
7	2	5	No	Powder (<200 $\mu\text{m}$ ) with sporadic 0.5–2 mm large pieces	0.2	0.1
8	3	5	No	Powder (<200 $\mu\text{m}$ ) with sporadic 0.5–2 mm large pieces	/	0.04
9	2	8	Yes	Powder (<200 $\mu\text{m}$ ) with sporadic 0.5–2 mm large pieces	0.04	0.2
10	3	8	Yes	Powder (<200 $\mu\text{m}$ ) with sporadic 0.5–1 mm large pieces	/	0.04
11	2	8	No	Powder (<200 $\mu\text{m}$ )	0.1	0.3
12	3	8	No	Powder (<200 $\mu\text{m}$ )	0.1	0.1

(TC/EA; ThermoFinnigan, Bremen, Germany) equipped with a Zero Blank autosampler (Costech Analytical Technologies, Valencia, CA, USA) and connected to a MAT 253 mass spectrometer via a ConFlo IV interface (both from Thermo Scientific, Bremen, Germany). IAEA-601 and IAEA-602 (both benzoic acids) standards were used. For the sample run of the homogeneity test, both standards were analysed six times. For the milling test, the standards were each measured nine times. Due to the low number of measurements of the standards, the analytical error is given by the higher standard deviation obtained for the two standards for each sample run, which in both cases was IAEA-602. The analytical error was  $\pm 0.5\%$  for the homogeneity test and  $\pm 0.3\%$  for the milling test. The  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values are expressed as deviations (in ‰) from VPDB and VSMOW, respectively.

## RESULTS AND DISCUSSION

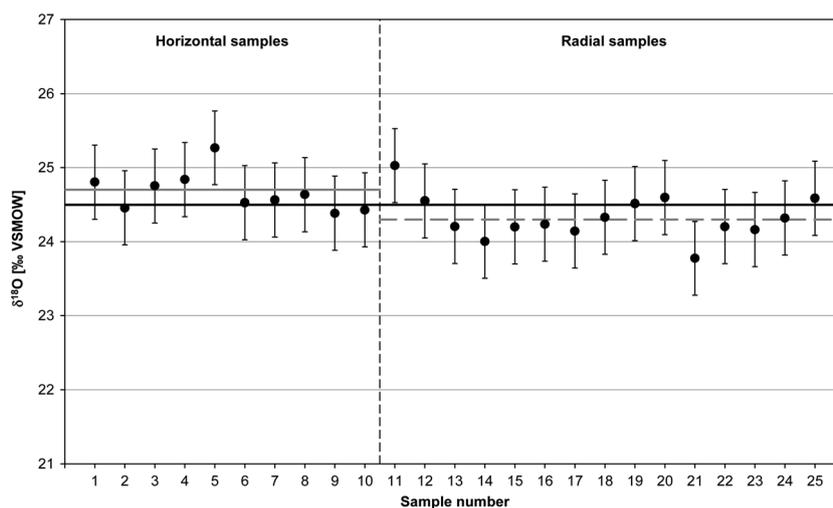
The homogeneity test gave mean  $\delta^{18}\text{O}$  values of  $24.3 \pm 0.3\%$  for the 15 radially cut samples and  $24.7 \pm 0.3\%$  for the 10 horizontally cut samples. The overall mean is  $24.5 \pm 0.3\%$  (Fig. 1) indicating that, within analytical error, the 5 cm<sup>3</sup> tree-ring piece has a homogenous  $\delta^{18}\text{O}$  value and is suitable for the milling test.

The coarseness of the milled samples (Table 1) is reflected in the  $\delta^{18}\text{O}$  values (Fig. 2). For tests #1 and #3, we observed 0.5–3 mm large pieces. For tests #2 and #4 to #10, we observed powder (<200  $\mu\text{m}$ ) with only sporadic 0.5–2 mm pieces. For tests #11 and #12, we observed powder (<200  $\mu\text{m}$ ) with no visible larger pieces (Table 1). The standard deviation of the  $\delta^{18}\text{O}$  values of the subsamples from the first six milling procedures was generally higher than the analytical error (Table 1). This is probably due to an insufficiently milled and therefore inhomogeneous sample. Tests #2 and #4 represent an exception in this context. For tests #7 to #12, the standard deviations were lower than the analytical

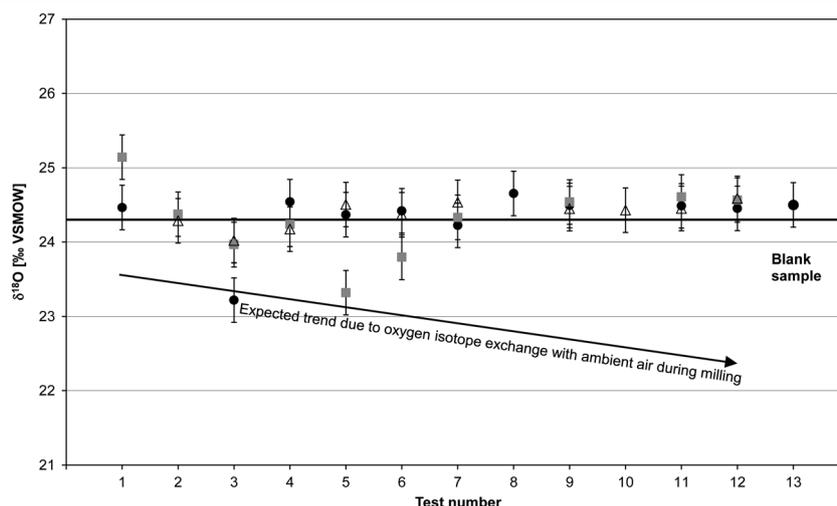
error, and this was also reflected by the predominantly homogeneous powder of the samples (Table 1 and Fig. 2). The lower  $\delta^{18}\text{O}$  values of some subsamples of tests #3, #5 and #6 were probably influenced by the presence of higher amounts of earlywood, which typically shows lower  $\delta^{18}\text{O}$  values than the latewood of a tree-ring.<sup>[16]</sup> Due to insufficient milling, 0.5–3 mm large pieces are visible in the sample mass of these milling tests. For the measurement, 250–300  $\mu\text{g}$  of sample was used, and the sample possibly contained of one or two of these pieces, probably consisting only of earlywood. Therefore, insufficient milling together with low sample amounts probably led to the lower  $\delta^{18}\text{O}$  values of these subsamples. Tests #7 to #12 show  $\delta^{18}\text{O}$  values within the analytical error of 0.3‰ (Table 1), which is due to sufficient homogenisation through longer milling. Therefore, a milling duration of 8 min is recommended to obtain a homogeneous sample mass.

The mean  $\delta^{18}\text{O}$  value of all the analysed samples is  $24.3 \pm 0.4\%$ , in agreement with the value for the blank sample (mean of the homogeneity test,  $24.5 \pm 0.3\%$ , Fig. 1). The expected trend of decreasing  $\delta^{18}\text{O}$  values, due to potential oxidation and exchange with atmospheric oxygen ( $\delta^{18}\text{O} = 23.5\%$ <sup>[11]</sup>) during milling, is not observed (Fig. 2). Furthermore, there is no difference in the  $\delta^{18}\text{O}$  values of tests #9 to #12 (Table 1), where we systematically varied the number of stainless-steel balls (Fig. 2). In addition, potential cooling of the samples due to discontinuous milling has obviously no effect on the  $\delta^{18}\text{O}$  values (Fig. 2, tests #9/#10 compared with tests #11/#12).

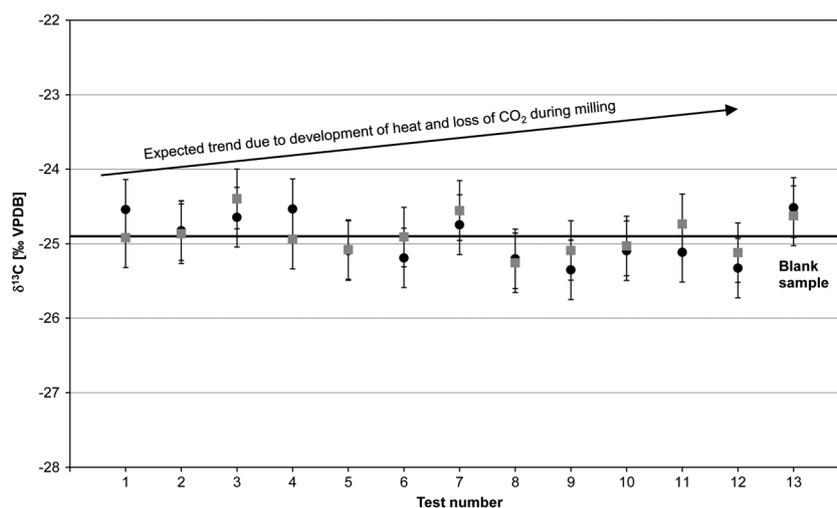
The results of the  $\delta^{13}\text{C}$  analyses show a similar pattern (Fig. 3). The mean  $\delta^{13}\text{C}$  values for the different milling procedures ( $-24.9 \pm 0.3\%$ ) and of the non-milled blank sample ( $-24.6 \pm 0.1\%$ ) are identical within the analytical error of 0.4‰. The number of stainless-steel balls (two or three), the milling duration (3, 5 or 8 min) as well as continuous or discontinuous milling do not influence the  $\delta^{13}\text{C}$  value. In addition, the expected trend of increasing  $\delta^{13}\text{C}$  values due to loss of CO<sub>2</sub>, which preferentially contains <sup>12</sup>C from the conversion of resin, is not observed (Fig. 3). In comparison with



**Figure 1.**  $\delta^{18}\text{O}$  values of the 25 samples used for the homogeneity test. Also shown is the analytical error of 0.5‰, given by the standard deviation of the standard of the corresponding sample run. The solid black line is the mean  $\delta^{18}\text{O}$  value of all samples ( $24.5 \pm 0.3\%$ ). The first 10 samples were cut horizontally, and their mean  $\delta^{18}\text{O}$  value is  $24.7 \pm 0.3\%$  (solid grey line). Samples 11 to 25 were cut radially, and their mean  $\delta^{18}\text{O}$  value is  $24.3 \pm 0.3\%$  (dashed grey line).



**Figure 2.** Compilation of the  $\delta^{18}\text{O}$  values of the samples milled using different procedures. Also shown is the mean  $\delta^{18}\text{O}$  value of the homogeneity test (blank sample). The solid line indicates the mean  $\delta^{18}\text{O}$  value of all milled samples. Grey squares, black circles and open triangles indicate three subsamples of the same milling procedure. For the corresponding sample numbers, see Table 1.



**Figure 3.** Compilation of the  $\delta^{13}\text{C}$  values of the samples milled using different procedures. Also shown is the non-milled blank sample. The solid black line shows the mean  $\delta^{13}\text{C}$  value of all milled samples. Grey squares and black circles indicate different subsamples of different milling procedure. For the corresponding sample numbers, see Table 1.

the results from the  $\delta^{18}\text{O}$  analyses, an increased standard deviation between the subsamples for shorter milling times is not observed (Table 1). This is probably due to the three to six times larger sample amount of 1–2 mg (compared with 250–300  $\mu\text{g}$  for the  $\delta^{18}\text{O}$  samples) and the consequently much lower probability of having only earlywood in the samples.

## CONCLUSIONS

The coarseness of the material after the different milling tests is reflected in the standard deviation of the  $\delta^{18}\text{O}$  values. Samples including pieces that are 0.5–2 mm also reveal higher standard deviations between the  $\delta^{18}\text{O}$  values of the

subsamples, exceeding the analytical error. These samples were probably not milled long enough to guarantee a homogenous sample powder. Thus, wood samples should be milled for at least 8 min to obtain a homogeneous sample.

Within analytical error (0.3‰), the mean  $\delta^{18}\text{O}$  value of all milled samples ( $24.3 \pm 0.4\text{‰}$ ) was identical to that of the blank sample ( $24.5 \pm 0.3\text{‰}$ ). Likewise, the mean  $\delta^{13}\text{C}$  value of all milled samples ( $-24.9 \pm 0.3\text{‰}$ ) was in agreement with that of the blank sample ( $-24.6 \pm 0.1\text{‰}$ ). These results clearly demonstrate that heat development during milling has no effect on the wood  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values. A systematic bias resulting from the number of stainless-steel balls, milling duration as well as interruptions during milling was not observed. Milling is, thus, an appropriate preparation method for wood samples.

## Acknowledgements

We are grateful to B. Müller for the  $\delta^{13}\text{C}$  analyses and thank M. Kochbeck, L. Klippel, and P. Römer for sample preparation and laboratory work. We also thank K.-H. Bohn for providing the stem disc. Two anonymous reviews helped to improve a former version of the manuscript. We are also grateful to the editor, John Monaghan, for his constructive comments.

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