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Validation of a coupled $\delta^2H_{n\text{-alkane}}$-$\delta^{18}O_{sugar}$ paleohygrometer approach based on a climate chamber experiment

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Keywords

hydrogen stable isotopes, oxygen stable isotopes, hemicellulose sugars, leaf waxes, leaf water enrichment, deuterium-excess, relative humidity

Abstract

The hydrogen isotopic composition of leaf wax-derived biomarkers, e.g. long chain *n*-alkanes ($\delta^2H_{n\text{-alkane}}$), is widely applied in paleoclimatology research. However, a direct reconstruction of the isotopic composition of source water based on $\delta^2H_{n\text{-alkane}}$ alone can be challenging due to the alteration of the soil water isotopic signal by leaf-water heavy-isotope enrichment. The coupling of $\delta^2H_{n\text{-alkane}}$ with $\delta^{18}O$ of hemicellulose-derived sugars ($\delta^{18}O_{sugar}$) has the potential to disentangle this effect and additionally to allow relative humidity reconstructions. Here, we present $\delta^2H_{n\text{-alkane}}$ as well as $\delta^{18}O_{sugar}$ results obtained from leaves of the plant species *Eucalyptus globulus*, *Vicia faba* var. *minor* and *Brassica oleracea* var. *medullosa*, which grew under controlled conditions. We addressed the questions (i) do $\delta^2H_{n\text{-alkane}}$ and $\delta^{18}O_{sugar}$ values allow precise reconstructions of leaf water isotope composition, (ii) how accurately does the reconstructed leaf-water-isotope composition enables relative humidity (RH) reconstruction in which the plants grew, and (iii) does the coupling of $\delta^2H_{n\text{-alkane}}$ and $\delta^{18}O_{sugar}$ enable a robust source water calculation?

For all investigated species, the alkane *n*-C_{29} was most abundant and therefore used for compound-specific δ^2H measurements. For *Vicia faba*, additionally the δ^2H values of *n*-C_{31} could be evaluated robustly. With regard to hemicellulose-derived monosaccharides, arabinose and xylose were most abundant and their $\delta^{18}O$ values were therefore used to calculate weighted mean leaf $\delta^{18}O_{sugar}$ values. Both $\delta^2H_{n\text{-alkane}}$ and $\delta^{18}O_{sugar}$ yielded significant correlations with $\delta^2H_{leaf\text{-}water}$ and $\delta^{18}O_{leaf\text{-}water}$, respectively ($r^2$ = 0.45 and 0.85, respectively; $p < 0.001$, n = 24). Mean fractionation factors between biomarkers and leaf water were found to be -156‰ (ranging from -133 to -192‰) for $\varepsilon_{n\text{-alkane/leaf-water}}$ and +27.3‰ (ranging from +23.0 to 32.3‰) for $\varepsilon_{sugar/leaf\text{-}water}$, respectively. Modelled RH_{air} values from a Craig-Gordon model using measured T_{air}, $\delta^2H_{leaf\text{-}water}$ and $\delta^{18}O_{leaf\text{-}water}$ as input correlate highly significantly with measured RH_{air} values (R^2 = 0.84, $p < 0.001$, RMSE = 6%). When coupling $\delta^2H_{n\text{-alkane}}$ and $\delta^{18}O_{sugar}$ values the correlation of modelled RH_{air} values with measured RH_{air} values is weaker but still highly significant with R^2 = 0.54 ($p < 0.001$, RMSE = 10%). Finally, the reconstructed source water isotope composition (δ^2H_s and $\delta^{18}O_s$) as calculated from the coupled approach matches the source water in the climate chamber experiment ($\delta^2H_{tank\text{-}water}$ and $\delta^{18}O_{tank\text{-}water}$). This highlights the great potential of the coupled $\delta^2H_{n\text{-alkane}}$-$\delta^{18}O_{sugar}$ paleohygrometer approach for paleoclimate and relative humidity reconstructions.

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1 Introduction

Leaf-wax-derived biomarkers, such as long chain *n*-alkanes, and their stable hydrogen isotopic composition ($\delta^2H_{n\text{-alkane}}$) are widely applied in paleoclimatology research. Sedimentary $\delta^2H_{n\text{-alkane}}$ values correlate with δ^2H of precipitation (Huang et al., 2004; Mügler et al., 2008; Sachse et al., 2004; Sauer et al., 2001), confirming the high potential of $\delta^2H_{n\text{-alkane}}$ to establish δ^2H records of past precipitation (Hou et al., 2008; Rao et al., 2009; Sachse et al., 2012). However, the alteration of the isotopic signal because of leaf water heavy-isotope enrichment caused by evapotranspiration can be several tens of per mil. This poses a challenge for accurate data interpretation (e.g. Zech et al., 2015), especially in respect of single proxy ($\delta^2H_{n\text{-alkane}}$)-based climate records. Apart from studies of sedimentary cellulose (Heyng et al., 2014; Wissel et al., 2008), the oxygen stable isotope composition of sugar biomarkers ($\delta^{18}O_{sugar}$) emerged as complementary paleoclimate proxy during the last decade (Hepp et al., 2015, 2017, Zech et al., 2013a, 2014a). The interpretation of the $\delta^{18}O_{sugar}$ values is comparable to those of $\delta^2H_{n\text{-alkane}}$. When sugars originate primarily from leaf biomass of higher terrestrial plants, they reflect the plant source water (which is often directly linked to the local precipitation) modified by evapotranspirative enrichment of the leaf water (Tuthorn et al., 2014; Zech et al., 2014a). The coupling of $\delta^2H_{n\text{-alkane}}$ with $\delta^{18}O_{sugar}$ values allows quantifying the leaf-water isotopic enrichment and relative air humidity (Zech et al., 2013a). This approach was validated by Tuthorn et al. (2015) by applying it to topsoil samples along a climate transect in Argentina. Accordingly, the biomarker-derived relative air humidity values correlate significantly with actual air relative humidity from the respective study sites, highlighting the potential of the $\delta^2H_{n\text{-alkane}}$-$\delta^{18}O_{sugar}$ paleohygrometer approach.

The coupled approach is based on the observation that the isotope signature of precipitation ($\delta^2H_{precpitation}$ and $\delta^{18}O_{precpitation}$) typically plots on or adjacent to the global meteoric water line (GMWL), in a δ^2H-$\delta^{18}O$ diagram. The GMWL is characterized by the equation $\delta^2H_{precipitation} = 8 \cdot \delta^{18}O_{precipitation} + 10$ (Craig, 1961). In many cases, the local precipitation is directly linked to the source water of plants, which is indeed soil water and eventually shallow groundwater. The isotopic composition of xylem water of plants readily reflects these sources (e.g. Dawson, 1993). However, leaf-derived biomarkers reflect the leaf water isotope composition, which is, unlike xylem water, prone to evapotranspiration (e.g. Barbour and Farquhar, 2000; Helliker and Ehleringer, 2002; Cernusak et al., 2003; Barbour et al., 2004; Cernusak et al., 2005; Feakins and Sessions, 2010; Kahmen et al., 2011; Sachse et al., 2012; Kahmen, et al., 2013; Tipple et al., 2013; Lehmann et al., 2017; Liu et al., 2017). During daytime, the leaf water is typically enriched in the heavy isotope compared to the source water because of the evapotranspiration through the stomata. Thereby, lighter water isotopologues evaporate preferentially, which leads to gradual reduction of a deuterium-excess parameter of the remaining water compared to the precipitation water ($d = \delta^2H_{precitation} - 8 \cdot \delta^{18}O_{precitation}$; according to Dansgaard, 1964). The degree of enrichment by evapotranspiration is mainly controlled by the relative air humidity (RH_{air}) in the direct surrounding of the plant leaves (e.g. Cernusak et al., 2016). Although the biomarkers reflect the isotopic composition of leaf water, there is still a modification by biosynthetic fractionation during the synthesis, leading to an offset between leaf water and biomarker isotope composition. In case the biosynthetic fractionation is known and constant, there is a great potential to derive RH_{air} from coupling $\delta^2H_{n\text{-alkane}}$ with $\delta^{18}O_{sugar}$ values.

The overall aim of this study is to evaluate the $\delta^2H_{n\text{-alkane}}$-$\delta^{18}O_{sugar}$ paleohygrometer approach by applying it to plant leaf material from three different plants grown in a climate chamber experiment under well controlled conditions. More specifically, we address the following questions:

(i) which homologues and specific monosaccharides can be used to obtain $\delta^2H_{n\text{-alkane}}$ and $\delta^{18}O_{sugar}$ results for the plant leaf material grown in our climate chamber experiment, respectively,

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(ii) how precisely do $\delta^2H_{n\text{-alkane}}$ and $\delta^{18}O_{sugar}$ values allow reconstructing $\delta^2H_{leaf\text{-}water}$ and $\delta^{18}O_{leaf\text{-}water}$, respectively,

(iii) how accurately does the leaf-water-isotope composition reflect RH_{air},

(iv) and does the coupling of $\delta^2H_{n\text{-alkane}}$ and $\delta^{18}O_{sugar}$ enable a RH_{air} reconstruction and how robust are source water calculations?

2 Material and Methods

2.1 Climate chamber experiment

A phytotron experiment was conducted at the Helmholtz Zentrum München in Neuherberg during winter 2000/2001 (Mayr, 2002). Three different dicotyledon plant species (*Eucalyptus globulus*, *Vicia faba* var. *minor* and *Brassica oleracea* var. *medullosa*) were grown in eight chambers for 56 days under seven distinct climatic conditions (same conditions in chambers 4 and 8). Air temperature (T_{air}) were set to 14, 18, 24 and 30°C and RH_{air} to around 20, 30, 50, and 70% between 11 a.m. and 4 p.m. (Fig. 1A). During the rest of the day typical natural diurnal variations were aimed for (details in Mayr, 2002). Furthermore, uniform irrigation conditions were guaranteed via an automatic irrigation system, which was controlled by tensiometers installed in 9 cm substrate depth. The tank water used for irrigation was sampled periodically (intervals of one to three days) over the whole experiment and revealed only minor variability in its isotope composition ($\delta^{18}O_{tank\text{-}water}$ = -10.7 ± 0.3‰ standard deviation (σ); $\delta^2H_{tank\text{-}water}$ = -7 ± 1‰ σ). Once a week, soil water (via ceramic cups in 13 cm soil depth) and atmospheric water vapor (via dry ice condensation traps) was sampled ($\delta^2H_{soil\text{-}water}$, $\delta^{18}O_{soil\text{-}water}$ and $\delta^2H_{atmospheric\text{-}water\text{-}vapor}$, $\delta^{18}O_{atmospheric\text{-}water\text{-}vapor}$). Additionally, leaf temperatures (T_{leaf}) were derived from gas exchange measurements, at least once a week (Mayr, 2002).

In order to analyze stable hydrogen and oxygen isotopic composition of leaf ($\delta^2H_{leaf\text{-}water}$, $\delta^{18}O_{leaf\text{-}water}$) and stem water, the plants were harvested at the end of the experiment. The vacuum distillation method was used for the extraction of the plant water. It should be noted that stem water is a mixture between phloem and xylem water. Only the latter reflects the isotopic composition of soil water. For simplification, stem water is referred to as xylem water in the following ($\delta^2H_{xylem\text{-}water}$, $\delta^{18}O_{xylem\text{-}water}$). For more details about the experiment, we refer to the original publication (Mayr, 2002).

2.2 Leaf biomarker extraction and compound-specific stable isotope analysis

A total of 24 leaf samples were prepared according to Schäfer et al. (2016) for compound specific δ^2H measurements of *n*-alkanes, at the Institute of Geography, Group of Biogeochemistry and Paleoclimate, University of Bern. Microwave extraction with 15 ml dichloromethane (DCM)/methanol (MeOH) 9:1 (v:v) at 100°C for 1 h was conducted. The resulting total lipid extracts were purified and separated using aminopropyl-silica-gel (Supelco, 45 µm) pipette columns. The hydrocarbon fractions (containing *n*-alkanes) were eluted with *n*-hexane and cleaned via silver nitrate-coated silica gel pipettes (Supelco, 60-200 mesh) and zeolite (Geokleen Ltd.) columns. The δ^2H measurements of the highest concentrated *n*-alkanes (*n*-C_{29} and *n*-C_{31}) were performed on a GC-^{2}H-pyrolysis-IRMS system, equipped with an Agilent 7890A gas chromatograph (GC) and IsoPrime 100 isotope-ratio-mass spectrometer (IRMS) coupled with a GC5 pyrolysis/combustion interface operating in pyrolysis modus with a Cr (ChromeHD) reactor at 1000°C. The compound-specific δ^2H values were calibrated against a standard alkane mix (*n*-C_{27}, *n*-C_{29}, *n*-C_{33}) with known isotope composition (A. Schimmelmann, University of Indiana), measured twice every six sample injections. Standard deviation of the triplicate measurements was typically ≤5‰. The H^{3+} factor stayed constant during the course of the measurements.

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Additionally, the leaf samples were dried and finely ground in preparation for $\delta^{18}O$ analysis of hemicellulose-derived sugars (modified from Zech and Glaser, 2009) at the Institute of Agronomy and Nutritional Sciences, Soil Biogeochemistry, Martin-Luther-University Halle-Wittenberg. The hemicellulose sugars were hydrolytically extracted for 4 h at 105°C using 4M trifluoroacetic acid (Amelung et al., 1996) and purified via XAD-7 and Dowex 50WX8 columns. Prior to the methylboronic-acid (MBA) derivatization (4 mg of MBA in 400 µl dry pyridine for 1 h at 60°C), the cleaned sugars were frozen and freeze-dried overnight (Knapp, 1979). Compound-specific $\delta^{18}O$ measurements were performed on a Trace GC 2000 coupled to a Delta V Advantage IRMS via an ^{18}O-pyrolysis reactor (GC IsoLink) and a ConFlo IV interface (all devices from Thermo Fisher Scientific, Bremen, Germany). The sample batches were measured along with embedded co-derivatized standard batches, which contained arabinose, fucose, xylose, and rhamnose in different concentrations of known $\delta^{18}O$ value. The $\delta^{18}O$ values of the standard sugars were determined via temperature conversion/elemental analysis-IRMS coupling at the Institute of Plant Sciences, ETH Zurich, Switzerland (Zech and Glaser, 2009). This procedure allows corrections for possible amount dependencies (Zech and Glaser, 2009) and ensures the "Principle of Identical Treatment" (Werner and Brand, 2001). Standard deviations for the triplicate measurements were 0.9‰ and 2.2‰ (average over all investigated samples) for arabinose and xylose, respectively. We focus on arabinose and xylose in this study because they were (i) the dominant peaks in all chromatograms, and (ii) previously found to strongly predominate over fucose (and rhamnose) in terrestrial plants, soils (Hepp et al., 2016).

Figure 1B summarizes isotope data obtained for the 24 analysed leaf samples where all δ values are expressed in per mil as isotope ratios ($R = {}^{18}O/{}^{16}O$ or ${}^{2}H/{}^{1}H$) relative to the Vienna Standard Mean Ocean Water (VSMOW) standard in the common delta notation ($\delta = (R_{sample} - R_{standard})/R_{standard}$; e.g. Coplen, 2011).

2.3 Framework for coupling $\delta^2H_{n\text{-alkane}}$ with $\delta^{18}O_{sugar}$ results

2.3.1 Deuterium-excess of leaf water and relative humidity

The coupled approach is based on the observation that isotope composition of global precipitation plots typically close to the GMWL ($\delta^2H_{precpitation} = 8 \cdot \delta^{18}O_{precipitation} + 10$; Craig, 1961; Fig. 2). The soil water and shallow groundwater, which acts as source water for plants, can often directly be related to the local precipitation. However, especially during daytime, leaf water is typically enriched in heavy isotopes compared to the precipitation due to evapotranspiration through the stomata, therefore plotting to the right of the GMWL (Fig. 2; e.g. Allison et al., 1985; Bariac et al., 1994; Walker and Brunel, 1990). During stable climatic conditions, the leaf water reservoir at the evaporative sites is frequently assumed to be in isotopic steady-state (Allison et al., 1985; Bariac et al., 1994; Gat et al., 2007; Walker and Brunel, 1990), meaning that isotopic composition of transpired water vapour is equal to the isotopic composition of the source water utilized by plants during the evapotranspiration process. The Craig-Gordon model (e.g. Flanagan et al., 1991; Roden and Ehleringer, 1999) approximates the isotope processes in leaf water in δ terms (e.g. Barbour et al., 2004):

$$\delta_e \approx \delta_s + \varepsilon^* + \varepsilon_k + (\delta_a - \delta_s - \varepsilon_k)\,\frac{e_a}{e_i}, \qquad \text{(Equation 1)}$$

where δ_e, δ_s and δ_a are the hydrogen and oxygen isotopic compositions of leaf water at the evaporative sites, source water and atmospheric water vapor, respectively. The equilibrium enrichment (ε^*) is expressed as $(1\text{-}1/\alpha_{L/V}) \cdot 10^3$, where $\alpha_{L/V}$ is the equilibrium fractionation between liquid and vapor in per mil. The kinetic fractionation parameter (ε_k) describes the water vapor diffusion from intracellular

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air space through the stomata and the boundary layer into to the atmosphere, and e_a/e_i is the ratio of the atmospheric to intracellular vapor pressure.

In a δ^2H-$\delta^{18}O$ diagram, the isotope composition of the leaf water as well as the source water can be described as deuterium-excess (d) values by using the equation of Dansgaard (1964), with $d = \delta^2H - 8 \cdot \delta^{18}O$. This allows rewriting Eq. 1, in which hydrogen and oxygen isotopes have to be handled in separate equations, in one equation:

$$d_e \approx d_s + \left(\varepsilon_2^* - 8 \cdot \varepsilon_{18}^*\right) + \left(C_k^2 - 8 \cdot C_k^{18}\right) + \left[d_a - d_s - \left(C_k^2 - 8 \cdot C_k^{18}\right)\right] \cdot \frac{e_a}{e_i}, \quad \text{(Equation 2)}$$

where d_e, d_s and d_a are the deuterium excess values of leaf water at the evaporative sites, source water and atmospheric water vapor, respectively. The kinetic fractionation parameter (ε_k) is typically related to stomatal and boundary layer resistances to water flux (Farquhar et al., 1989). We used the kinetic enrichment factor (C_k) instead of ε_k to be close to paleo studies were direct measurements of such a plant physiological parameter are not available. The kinetic enrichment factor is derived from a more generalized form of the Craig-Gordon model for describing the kinetic isotope enrichment for ^2H and ^{18}O (C_k^2 and C_k^{18}, respectively) (Craig and Gordon, 1965; Gat and Bowser, 1991). If the plant source water and the local atmospheric water vapor are in isotope equilibrium, the term $\delta_a - \delta_s$ in Eq. 1 can be approximated by $-\varepsilon^*$. Thus, Eq. 2 can be reduced to:

$$d_e \approx d_s + \left(\varepsilon_2^* - 8 \cdot \varepsilon_{18}^* + C_k^2 - 8 \cdot C_k^{18}\right) \cdot \left(1 - \frac{e_a}{e_i}\right). \quad \text{(Equation 3)}$$

The actual atmospheric vapor pressure (e_a) and the leaf vapor pressure (e_i) in kPa can be derived from Eqs. 4 and 5 by using T_{air} (Buck, 1981):

$$e_a = 0.61121 \cdot e^{[17.502 \cdot T_{air} / (T_{air} + 240.97)]} \cdot RH_{air} \quad \text{(Equation 4)}$$

$$e_i = 0.61121 \cdot e^{[17.502 \cdot T_{air} / (T_{air} + 240.97)]}. \quad \text{(Equation 5)}$$

When e_i is calculated as in Eq. 5 than the e_a/e_i represents RH_{air} (ranging between 0 and 1, representing 0 to 100% relative humidity). We are aware, that the Craig-Gordon model would require T_{leaf} values for calculating e_i values. However, the RH reconstruction methodological framework presented is attempted to paleo studies for which the T_{leaf} parameter is probably rather difficult to achieve.

With rearranging Eq. 3, an equation is given to derive relative humidity values (Eq. 6):

$$RH_{air} \approx 1 - \frac{d_e - d_s}{\left(\varepsilon_2^* - 8 \cdot \varepsilon_{18}^* + C_k^2 - 8 \cdot C_k^{18}\right)}. \quad \text{(Equation 6).}$$

Equilibrium fractionation parameters (ε_2^* and ε_{18}^*) can derived from empirical equations of Horita and Wesolowski (1994) by using the climate chamber T_{air} values. The kinetic fractionation parameters (C_k^2 and C_k^{18}) for ^2H and ^{18}O, respectively, are set to 25.1 and 28.5‰ according to Merlivat (1978), who reported maximum values during the molecular diffusion process of water through a stagnant boundary layer. When using supplementary data of Cernusak et al., (2016), ε_k values of broadleaf trees and shrubs over broad climatic conditions can calculated which are well in the range with the used C_k^2 and C_k^{18} values (23.9 ± 0.9 and 26.7‰ ± 1.0 for ε_k^2 and ε_k^{18}, respectively).

If $\delta^2H_{leaf\text{-}water}$ and $\delta^{18}O_{leaf\text{-}water}$ can be reconstructed from the measured δ values of *n*-alkanes and sugars biomarkers, this framework provides a powerful tool to establish relative humidity records from sedimentary archives (Hepp et al., 2017; Zech et al., 2013a).

To reconstruct the isotope composition of leaf water it is assumed that fractionation factors of −160‰ for ^2H of alkanes *n*-C_{29} and *n*-C_{31} (ε^2_{bio}; Sachse et al., 2012; Sessions et al., 1999), and +27‰ for ^{18}O of

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the hemicellulose-derived sugars arabinose and xylose (ε^{18}_{bio}; Cernusak et al., 2003; Schmidt et al., 2001; Sternberg et al., 1986; Yakir and DeNiro, 1990) can be applied:

alkane-based $\delta^2H_{leaf\text{-}water} = (\delta^2H_{n\text{-}alkane} - \varepsilon^2_{bio})/(1 + \varepsilon^2_{bio}/1000)$ (Equation 7)

sugar-based $\delta^{18}O_{leaf\text{-}water} = (\delta^{18}O_{sugar} - \varepsilon^{18}_{bio})/(1 + \varepsilon^{18}_{bio}/1000)$. (Equation 8)

2.3.2 Isotope composition of plant source water

In a δ^2H-$\delta^{18}O$ diagram, the hydrogen and oxygen isotope composition of the plant source water (δ^2H_s and $\delta^{18}O_s$, respectively) can be reconstructed via the slope of the individual leaf water evapotranspiration lines (LEL´s; Craig and Gordon, 1965; Gat and Bowser, 1991). The LEL slope (S_{LEL}) can be derived from Eq. 9:

$$S_{LEL} \approx \frac{\varepsilon_2^* + C_k^2 \cdot \left(1 - \frac{e_a}{e_i}\right)}{\varepsilon_{18}^* + C_k^{18} \cdot \left(1 - \frac{e_a}{e_i}\right)} \approx \frac{\varepsilon_2^* + C_k^2}{\varepsilon_{18}^* + C_k^{18}}, \quad \text{(Equation 9)}$$

where all parameters are defined as in section 2.3.1. The δ^2H_s and $\delta^{18}O_s$ values can then be calculated for each leaf water data point via the intersect between the individual LEL´s with the GMWL. The δ^2H_s and $\delta^{18}O_s$ model results can then compared to the measured $\delta^2H_{tank\text{-}water}$ and $\delta^{18}O_{tank\text{-}water}$ values.

2.4 Modeling and isotope fractionation calculations

The d_e values are modeled using Eq. 3 and measured RH_{air} as input, which can be compared to the calculated deuterium-excess via the measured $\delta^2H_{leaf\text{-}water}$ and $\delta^{18}O_{leaf\text{-}water}$ values. From latter, also the RH_{air} can derived from Eq. 6 and compared to the measured once. In a next step, reconstructed (biomarker-based) deuterium-excess$_{leaf\text{-}water}$ was used as input for Eq. 6 and compared to the measured RH_{air} values. All models represent a simplified approach because $\delta_a - \delta_s$ are approximated by $-\varepsilon^*$ (see section 2.3). In all equations where δ_s and d_s are needed as input the measured $\delta^2H_{tank\text{-}water}$ and $\delta^{18}O_{tank\text{-}water}$ were used for calculations. All other input parameters were set as described in section 2.3.

Model quality was overall assessed by calculating the coefficient of determination $\left[R^2 = 1 - \sum(\text{modeled} - \text{measured})^2 / \sum(\text{measured} - \text{measured mean})^2\right]$ and the root mean square error $\left[\text{RMSE} = \sqrt{\left(\frac{1}{n} \cdot \sum(\text{modeled} - \text{measured})^2\right)}\right]$. The R^2 is not equal to the r^2, which provides here the fraction of variance explained by a linear regression between a dependent (y) and an explanatory variable $[r^2 = 1 - \sum(y - \text{fitted } y)^2 / \sum(y - \text{mean } y)^2]$ (R Core Team, 2015).

The fractionation between the measured leaf biomarkers and leaf water can be described by the following equations (e.g. Coplen, 2011):

$\varepsilon_{n\text{-}alkane/leaf\text{-}water} = (\delta^2H_{n\text{-}alkane} - \delta^2H_{leaf\text{-}water}) / (1 + \delta^2H_{leaf\text{-}water}/1000)$ (Equation 10)

$\varepsilon_{sugar/leaf\text{-}water} = (\delta^{18}O_{sugar} + \delta^{18}O_{leaf\text{-}water}) / (1 + \delta^{18}O_{leaf\text{-}water}/1000)$. (Equation 11)

In order to provide a 1 σ range bracketing the modeled results and calculations they were additionally run with values generated by subtracting/adding the individual σ to the average.

All calculations and statistical analysis were realized in R (version 3.2.2; R Core Team, 2015).

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3 Results and Discussion

3.1 Compound-specific isotope results of leaf wax-derived *n*-alkanes and hemicellulose-derived sugars

The investigated leaf material shows a dominance of C_{29} *n*-alkanes. The dominance of *n*-C_{29} in *Brassica oleracea* and *Eucalyptus globulus* was also reported by Ali et al. (2005) and Herbin and Robins (1968). *Vicia faba* leaf samples additionally revealed a high abundance of C_{31} *n*-alkanes. This agrees with results from Maffei (1996) and enables a robust determination of compound-specific δ^2H values for C_{29} and C_{31}. The $\delta^2H_{n\text{-alkane}}$ values of *Vicia faba* are therefore calculated as weighted mean. Figure 1B illustrates the $\delta^2H_{n\text{-alkane}}$ results along with isotopic data for leaf, xylem and soil water (the latter were originally published in Mayr 2002). In addition, the climate chamber conditions (RH_{air}, RH_{leaf}, T_{air} and T_{leaf}) are displayed (all from Mayr, 2002; Fig. 1A). For more details about the (plant) water isotope results, climate chamber conditions as well as not shown plant physiological properties the reader is referred to Mayr (2002). The $\delta^2H_{n\text{-alkane}}$ values range from -213 to -144‰ over all plant species. As revealed by overlapping notches in the respective boxplots, no statistically significant differences in the median values between the three species can be described (Fig. A1A; McGill et al., 1978). Figure 1B moreover shows that $\delta^2H_{n\text{-alkane}}$ values range largest for *Eucalyptus globulus* compared to the other two plants. However, the low number of samples per plant species prohibits a robust interpretation.

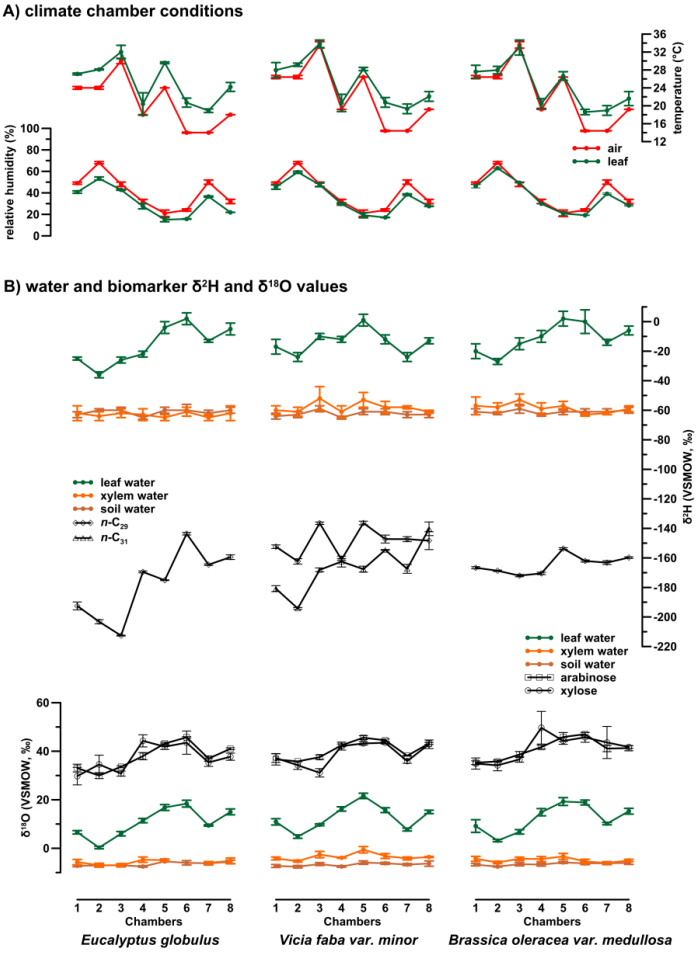

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Fig. 1: A: Climate chamber conditions (leaf temperature and relative humidity in green and air temperature and relative humidity in red). Error bars represent analytical standard deviation of the respective measurements (see section 2.2 and Mayr, 2002). B: Plant water (leaf water, xylem water and soil water) isotope compositions (in green, orange and brown, respectively) and the isotope composition of the investigated leaf biomarkers (leaf wax *n*-alkanes *n*-C_{29} and *n*-C_{31} as open diamonds and triangles, respectively; hemicellulose-derived sugars: arabinose and xylose as open squares and circles, respectively) for the three plants *Eucalyptus globulus, Vicia faba* and *Brassica oleracea* grown in the climate chambers.

The investigated leaf samples yielded substantially higher amounts of arabinose and xylose compared to fucose and rhamnose. This is in agreement with sugar patterns reported for higher plants (D'Souza et al., 2005; Hepp et al., 2016; Jia et al., 2008; Prietzel et al., 2013; Zech et al., 2012, 2014a) and hampers a robust data evaluation of fucose and rhamnose. The $\delta^{18}O$ values of the investigated pentoses arabinose and xylose range from 30 to 47‰ and 30 to 50‰, respectively, and are shown along with isotopic data for leaf, xylem and soil water (Mayr 2002) in the bottom of Fig. 1B. No considerable difference in the $\delta^{18}O$ values of arabinose and xylose can be seen in the $\delta^{18}O$ pentose data. This is in line with findings from Zech and Glaser (2009), Zech et al. (2012), Zech et al. (2013b) and Zech et al. (2014b) but contradicting with slightly more positive $\delta^{18}O_{arabinose}$ values compared to $\delta^{18}O_{xylose}$ values reported by Zech et al. (2013a) and Tuthorn et al. (2014). Overall, the two sugars display very similar results (Fig. 1B; $r^2 = 0.7$, $p < 0.001$, $n = 24$). The $\delta^{18}O$ values of arabinose and xylose can therefore be combined as a weighted mean (as $\delta^{18}O_{sugar}$ values) for further data interpretation. The $\delta^{18}O_{sugar}$ values are not significantly different between the three investigated plant species (Fig. A1B).

A comparison of compound-specific isotope results of leaf hemicellulose-derived sugars and leaf wax-derived *n*-alkanes with leaf, xylem, soil and tank water (compare Fig. 1B and Fig. 2) reveals that soil and xylem water plot close to the tank water, whereas leaf water shows a clear heavy-isotope enrichment due to evapotranspiration. This enrichment strongly differs between the climate chambers, depending mainly on T and RH conditions. The biomarker results furthermore follow the leaf water with a certain offset (ε_{bio}). The offset between soil and xylem water compared to the tank water in Fig. 2 is most likely caused by partial evaporation of tank water from the soil during the experiment.

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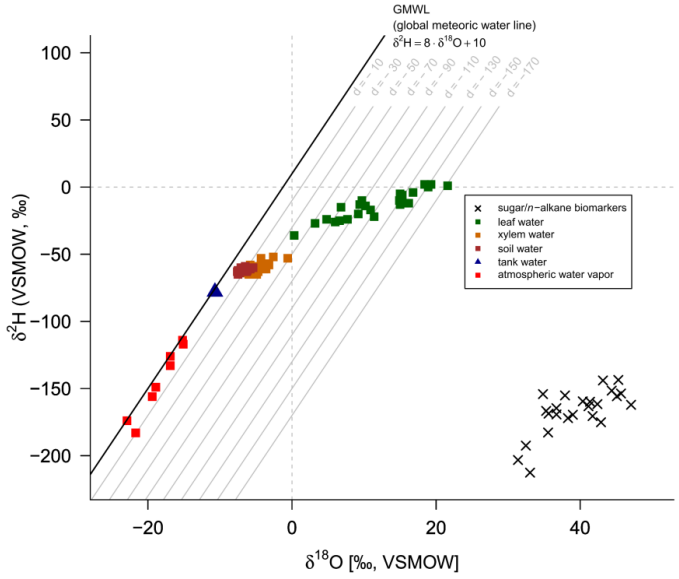

Fig. 2: δ^2H-$\delta^{18}O$ diagram illustrating the isotope composition of the biomarkers, comprising δ^2H values of the leaf wax *n*-alkanes (C_{29} for *Eucalyptus globulus* and *Brassica oleracea*; weighted mean of C_{29} and C_{31} for *Vicia faba*) and $\delta^{18}O$ values of the hemicellulose-derived sugars arabinose and xylose (black crosses) and the measured isotope compositions of leaf water (green squares), xylem water (orange squares), soil water (brown squares), atmospheric water vapor (red squares) and the tank water used for irrigation (blue triangle), which plot very close to the global meteoric water line.

3.2 Do *n*-alkane and sugar biomarkers reflect the isotope composition of leaf water?

The $\delta^2H_{n\text{-alkane}}$ dataset reveals a significant correlation with $\delta^2H_{leaf\text{-}water}$ ($r^2 = 0.45$) using all plant species with $p < 0.001$ (Fig. 3A). A slope of 1.1 and an intercept of -152‰ furthermore characterize the relationship. It seems that each plant type shows a different $\delta^2H_{n\text{-alkane}}$ to $\delta^2H_{leaf\text{-}water}$ relation, with the highest slope for *Vicia faba* and the lowest for *Brassica oleracea*. However, we argue that the number of replicates for each plant species is simply too low to interpret this finding robustly. A highly significant correlation is also observed for the correlation between $\delta^{18}O_{sugar}$ and $\delta^{18}O_{leaf\text{-}water}$ ($r^2 = 0.84$, $p < 0.001$; Fig. 3B). The regression reveals a slope of 0.74 and an intercept of 30.7‰.

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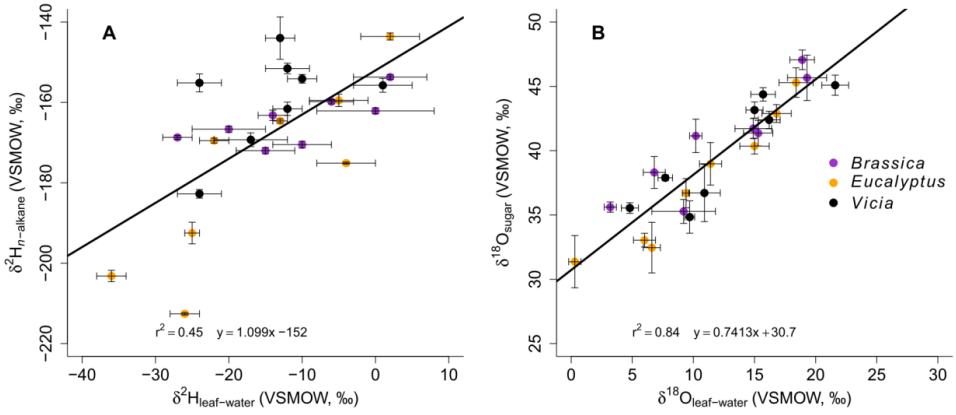

Fig. 3: Scatterplots depicting the relationships between the compound-specific biomarker isotope composition and the respective leaf water values (A: $\delta^2H_{n\text{-alkane}}$ vs. $\delta^2H_{leaf\text{-}water}$; B: $\delta^{18}O_{sugar}$ vs. $\delta^{18}O_{leaf\text{-}water}$). *Brassica oleracea, Eucalyptus globulus* and *Vicia faba* samples are shown in purple, orange and black, respectively. Error bars of the δ values represent standard deviation of repeated measurements (see section 2.2 and Mayr, 2002).

Since it is well known that measured leaf water is not always equal to the specific water pool in which the *n*-alkanes are biosynthesized (e.g. Tipple et al., 2015), the correlation reveals a rather low r^2 (Fig. 3A). Furthermore, NADPH is acting also as hydrogen source during *n*-alkane biosynthesis, which is clearly more negative than the biosynthetic water pool (Schmidt et al., 2003), further contributing to a weakening of the $\delta^2H_{n\text{-alkane}}$ to $\delta^2H_{leaf\text{-}water}$ correlation. The correlation between the deuterium contents of leaf wax *n*-alkanes and leaf water presented here is still well in range with the literature. Feakins and Sessions (2010) presented *n*-alkane (C_{29} and C_{31}) and leaf water δ^2H data from typical plant species (excluding grasses) along a southern California aridity gradient, revealing that only δ^2H of *n*-C_{29} is significantly correlated with leaf water (r^2 = 0.24, p < 0.1, n = 16; based on the associated supplementary data). Another field dataset from the temperate forest at Brown's Lake Bog, Ohio, USA revealed significant correlations between δ^2H of *n*-C_{29} and *n*-C_{31} with leaf water of the species *Prunus serotina*, *Acer saccharinum*, *Quercus rubra*, *Quercus alba,* and *Ulmus americana* (r^2 = 0.49, p < 0.001, n = 38; r^2 = 0.59, p < 0.001, n = 29; as derived form the supplement material of Freimuth et al., 2017). Data from a controlled climate chamber experiment using two tree species show a highly significant relationship between leaf wax *n*-alkanes δ^2H and leaf water (with C_{31} of *Betula occidentalis* and C_{29} of *Populus fremontii*; r^2 = 0.96, p < 0.001, n = 24; derived from supplementary data of Tipple et al., 2015). It is conformed that leaf wax *n*-alkanes of dicotyledonous plants largely incorporate the leaf water isotope signal, while in monocotyledonous plants (e.g. grasses) the *n*-alkanes are more strongly affected by the source water due to the leaf growth at the intercalary meristem (Kahmen et al., 2013). The observed slope of the $\delta^{18}O_{sugar}$ to $\delta^{18}O_{leaf\text{-}water}$ relationship (Fig. 3B) could serve as indicator for a leaf water (heavy-isotope enrichment) signal transfer damping of approximately 26%.

The theory behind the signal damping is adopted from the cellulose research (e.g. Barbour and Farquhar, 2000). Barbour and Farquhar (2000) related the extent of the signal damping to the proportion of unenriched source water, which contribute to the local synthesis water pool and to the proportion of exchangeable oxygen during cellulose synthesis. The here observed damping of 26% is well in the range with values reported for cellulose synthesis in *Gossypium hirsutum* leaves (between

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365 35 and 38%; Barbour and Farquhar, 2000), for *Eucalyptus globulus* leaf samples (38%; Cernusak et al., 2005) and for five C_3 and C_4 grasses (25%; Helliker and Ehleringer, 2002). Recently, Cheesman and Cernusak (2017) provided damping factors for leaf cellulose synthesis based on plant data grown under same conditions at Jerusalem Botanical Gardens published by Wang et al. (1998), ranging between 4 and 100% with a mean of 49%, revealing large variations among and between ecological groups
370 (namely conifers, deciduous, evergreen and shrubs). A large range of damping factors associated with leaf cellulose was also reported by Song et al. (2014) for *Ricinus communis* grown under controlled conditions. A common disadvantage of the above-mentioned studies is the absence of direct measurements of the proportion of depleted source water contribution to the local synthesis water (as noticed by Liu et al., 2017), which largely contribute to the extent of the damping factor (Barbour
375 and Farquhar, 2000). However, when transferring cellulose results to pentoses, such as hemicellulose-derived arabinose and xylose, it should be noted that they are biosynthesized via decarboxylation of the carbon at position six (C6) from glucose (Altermatt and Neish, 1956; Burget et al., 2003; Harper and Bar-Peled, 2002). Waterhouse et al. (2013) showed that the oxygen atoms at C6 position in glucose moieties, used for heterotrophic cellulose synthesis, are strongly affected by the exchange with local
380 water (up to 80%). Based on these findings, it can be suggested that the influence of the non-enriched source water during the synthesis of leaf hemicelluloses is rather small.

3.3 Fractionation factors between biomarkers and leaf water

In order to explore possible species-specific effects on the fractionation between the biomarkers and
385 the leaf water, boxplots of the individual plant species of $\varepsilon_{n\text{-alkane/leaf-water}}$ and $\varepsilon_{\text{sugar/leaf-water}}$ values are shown in Fig. 4. Median $\varepsilon_{n\text{-alkane/leaf-water}}$ values are -155‰ for *Brassica oleracea*, -164‰ for *Eucalyptus globulus* and -149‰ for *Vicia faba* (Fig. 4A), with an overall mean value of -156‰ (ranging from -133 to -192‰). Median $\varepsilon_{\text{sugar/leaf-water}}$ values of +27.0‰ for *Brassica oleracea*, +26.6‰ for *Eucalyptus globulus*, +26.8‰ for *Vicia faba* are shown in Fig. 4B. The overall $\varepsilon_{\text{sugar/leaf-water}}$ average value of the
390 three investigated species is +27.3‰ (ranging from +23.0 to +32.3‰). In both plots, no systematic difference between the individual species seems to be observable.

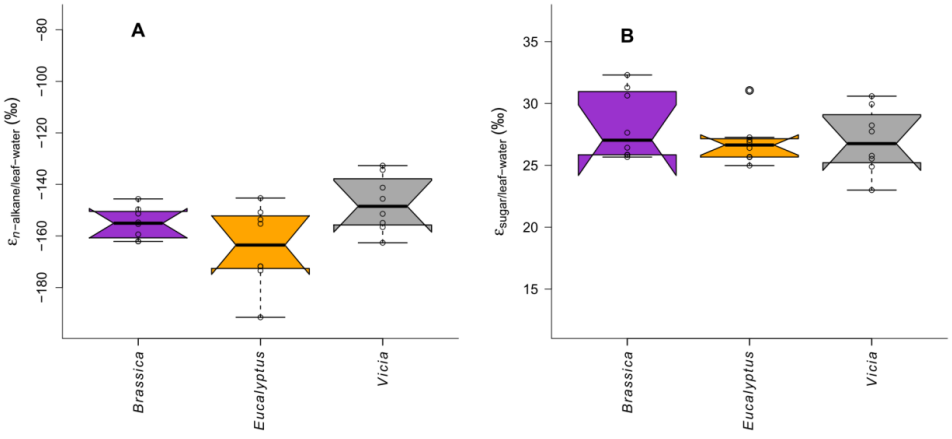

Fig. 4: Boxplots comprising the plant-specific fractionation between the biomarkers and the leaf water (A: $\varepsilon_{n\text{-alkane/leaf-water}}$ according Eq. 10; B: $\varepsilon_{\text{sugar/leaf-water}}$ according to Eq. 11). *Brassica oleracera, Eucalyptus*
395 *globulus* and *Vicia faba* samples are shown in purple, orange and black, respectively. Boxplots show median (thick black line), interquartile range (IQR) with upper (75%) and lower (25%) quartiles, lower and upper whiskers, which are restricted to 1.5 · IQR. Outside the 1.5 · IQR space, the data points are

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marked with a dot. The notches are extend to $\pm 1.58 \cdot IQR/\sqrt{n}$, by convention and give a 95% confidence interval for the difference of two medians (McGill et al., 1978).

The boxplots of $\varepsilon_{n\text{-alkane/leaf-water}}$ reveal that the median of the three investigated plant species can be statistically not distinguished, due to overlapping notches (Fig. 4A). It should be noted that due to the low sample number from each species, the 95% confidence interval is larger than the interquartile range in some cases. However, it seems that at least small species-specific differences cannot be ruled out. Our $\varepsilon_{n\text{-alkane/leaf-water}}$ values resemble well the data from a laboratory study (Kahmen et al., 2011), reporting a median value of -162‰ for *n*-C_{25}, *n*-C_{27} and *n*-C_{29} of *Populus trichocarpa*. Furthermore, they are well comparable to climate chamber data of *Betula occidentalis* (*n*-C_{31}) and *Populus fremontii* (*n*-C_{29}) from Tipple et al. (2015), reporting a median $\varepsilon_{n\text{-alkane/leaf-water}}$ value of -155‰. In addition, field experiments reveal similar median values of -151‰ (for *n*-C_{29}) and -142‰ (for *n*-C_{31}) from typical plant species (excluding grasses) from southern California (Feakins and Sessions, 2010) and -144‰ (for *n*-C_{29}, of the species *Prunus serotina*, *Acer saccharinum*, *Quercus rubra*, *Quercus alba* and *Ulmus americana*) from the temperate forest at Brown's Lake Bog, Ohio, USA. The large range in $\varepsilon_{xylem\text{-water/leaf-water}}$ values from our study (-192 to -133‰) is also obvious in the respective laboratory and field studies (-198 to -115‰, derived from *n*-C_{29} and *n*-C_{31} data from Feakins and Sessions, 2010; Kahmen et al., 2011a; Tipple et al., 2015; Freimuth et al., 2017). This could point to a specific water pool being used rather than bulk leaf water during biosynthesis (Sachse et al., 2012; Schmidt et al., 2003). In more detail, alkane synthesis takes place by modifying/expanding fatty acids in the cytosol, while fatty acids are synthesized in the chloroplasts (Schmidt et al., 2003). Thus, the cytosol as well as chloroplast water is one hydrogen source. However hydrogen can additionally be added to the alkanes and fatty acids by NADPH which originates from different sources (photosynthesis and pentose phosphate cycle, Schmidt et al., 2003). It is therefore challenging to measure directly the water pool in which the alkanes are biosynthesized (Tipple et al., 2015). Moreover, biosynthetic and metabolic pathways in general (Kahmen et al., 2013; Sessions et al., 1999; Zhang et al., 2009), the carbon and energy metabolism of plants more specifically (Cormier et al., 2018) and the number of carbon atoms of the *n*-alkane chains (Zhou et al., 2010) may have an influence on the fractionation. Our $\varepsilon_{n\text{-alkane/leaf-water}}$ values correlate with T_{air} (Fig. A2A), whereas the correlation with RH_{air} (Fig. A2B) is not significant. This could point to a relationship between $\varepsilon_{xylem\text{-water/leaf-water}}$ and plant physiological processes (affecting various plants differently).

The $\varepsilon_{sugar/leaf\text{-water}}$ values (Fig. 4B) do not correlate significantly with T_{air}, but significantly with RH_{air} (Fig. A2C and D). A temperature dependence of the $\varepsilon_{sugar/leaf\text{-water}}$ is not supported by this experiment, in contrast to results from Sternberg and Ellsworth (2011), where a temperature effect on oxygen fractionation during heterotrophic cellulose biosynthesis is observed. The here observed fractionation between hemicellulose-derived sugars and leaf water, with regard to $\varepsilon_{sugar/leaf\text{-water}}$ values, is well in range with values reported for sucrose (exported from photosynthesizing leaves) and leaf water, which was shown to be +27‰ (Cernusak et al., 2003). Also the cellulose biosynthesis is associated with an heavy-isotope enrichment of around +27‰ compared to the synthesis water as shown in growth experiments (Sternberg et al., 1986; Yakir and DeNiro, 1990). The relatively uniform fractionation is explained via the isotope exchange between the carbonyl oxygens of the organic molecules and the surrounding water (cf. Schmidt et al., 2001). This equilibrium fractionation effect was indeed described earlier by the reversible hydration reaction of acetone in water by Sternberg and DeNiro (1983) to be +28, +28 and +26‰ at 15, 25 and 35°C, respectively. However, the observed range of approximately 9‰ (Fig. 4B) could indicate that partially more than the oxygen equilibrium fractionation between organic molecules and medium water have to be considered. Presumably, isotopic as well as sucrose

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synthesis gradients within the leaf have to be taken into account when interpreting leaf sugar oxygen isotopic compositions and their correlation to leaf water (Lehmann et al., 2017). Lehmann et al. (2017) reported on a fractionation between sucrose and leaf water of +33.1‰. Based on this they proposed a conceptual scheme how such gradients can lead to discrepancies between the isotopic composition of the bulk leaf water and the synthesis water, while the latter is incorporated into the carbohydrates, and thus fractionation determination based on bulk leaf water can exceed the common average of +27‰. Also Mayr et al. (2015) found a fractionation between aquatic cellulose $\delta^{18}O$ and lake water larger than this value of around +29‰.

3.4 Strong control of relative humidity over deuterium-excess of leaf water

The correlations between leaf water-based and measured RH_{air} and modeled d_e and measured deuterium-excess$_{leaf\text{-}water}$ are illustrated in Fig. 5A and B, respectively.

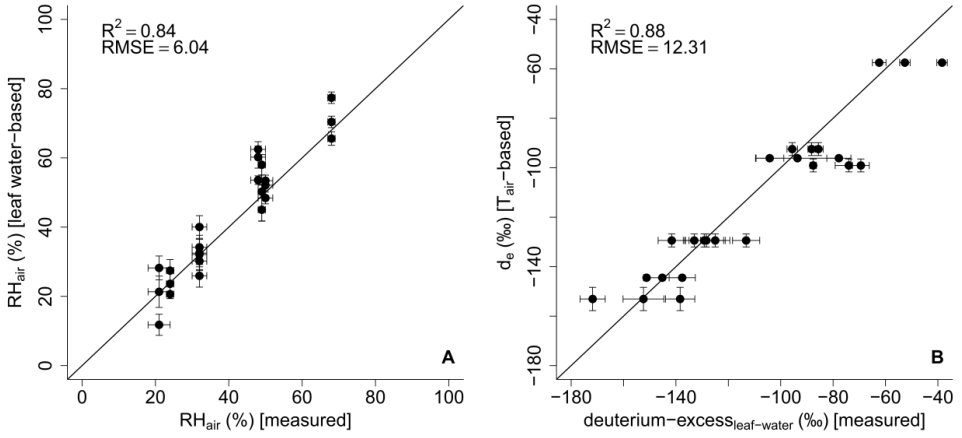

Fig. 5: Scatterplots illustrating the correlation between leaf water-based and measured air relative humidity (RH_{air}), modeled vs. measured leaf water deuterium-excess (T_{air}–based). Black lines indicate the 1:1 relationship. R^2 and RMSE are calculated as described in section 2.4, while the RMSE values have the dimensions of the respective variables. Error bars for the measured RH values represent analytical standard deviations (see Mayr, 2002). See section 2.4 for the uncertainties of the calculated and modeled results.

Evidence for the strong control of relative humidity on deuterium-excess of leaf water comes from multivariate regression analysis between the measured deuterium-excess$_{leaf\text{-}water}$ values versus RH_{air}, and T_{air}. The results reveal that the deuterium-excess$_{leaf\text{-}water}$ significantly correlates with RH_{air} of the climate chambers ($p < 0.001$), with an r^2 of 0.92. The strong control of RH on the deuterium-excess of leaf water is furthermore supported by the significant correlations between calculated versus measured RH_{air} values (Fig. 5A). This is in line with the strong correlation between modeled d_e based on T_{air} and measured deuterium-excess$_{leaf\text{-}water}$ values (Fig. 5B).

Overall, the modeled d_e values show a high agreement with measured deuterium-excess of leaf water despite without being too positive, which can be expected from the literature. This is because bulk leaf is less enriched than the leaf water at the evaporative sites, which is however, the output of the Craig-Gordon-based leaf water enrichment model (e.g. Allison et al., 1985; Barbour et al., 2004; Cernusak et al., 2016; section 2.3). Especially under low relative humidity conditions, the discrepancy between Craig-Gordon model results and the measured values is shown to be more pronounced, associated

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with higher transpiration fluxes and higher isotope heterogeneity within the leaf water due to a non-uniform closure of the stomata (Flanagan et al., 1991; Santrucek et al., 2007). An overestimation of the Craig-Gordon models can hardly be observed here (Fig. 5B). However, based on the accepted leaf water enrichment theory (e.g. Cernusak et al., 2016), higher transpiration rates (e.g. under low humidity conditions) should still lead to a larger discrepancy between Craig-Gordon modelled and measured leaf water, because the back diffusion of enriched leaf water from the evaporative sites should get lower the higher the transpiration flux is.

It should be noted, that there is the possibility to build up a more detailed model with Eq. 2. With this, a model is given to derive d_e values with the usage of d_a and d_s, which can compared to the measured deuterium-excess$_{leaf\text{-}water}$ values. However, when modeling d_e without the simplification $\delta_a - \delta_s \approx -\varepsilon^*$ the R^2 results to 0.86 and RMSE equals 13.07‰ compared to the presented 0.88 and 12.31‰. Furthermore, in Eq. 5 T_{air} can be replaced by T_{leaf}. With this, Eq. 2 results to de values based on leaf temperature. This would take into account that the Craig-Gordon model requires for the e_i parameter the temperature of the evaporating surface rather than the air temperature. However, with this model extension the R^2 and the RMSE results to 0.55 and 23.54‰, respectively. By rearranging Eq. 2, RH values can be modeled which can compared to RH_{air} as well as RH_{leaf} values (e_a/e_i multiplied by 100 with T_{leaf}). The respective model characteristics are again lower for the RH_{leaf} case (R^2 = 0.27 and RMSE = 11.84%) than for the RH_{air} comparison (R^2 = 0.81 and RMSE = 6.56%). Still Eq. 6 provides better results, as presented in this paragraph (R^2 = 0.84 and RMSE = 6.04%). This discussion is in line the differences between T_{leaf} vs. T_{air} and RH_{leaf} vs. RH_{air} conditions in the climate chambers. They reveal the same trends and magnitude, but T_{leaf} is consequently higher than T_{air} along with higher RH_{leaf} values compared to RH_{air} (Fig. 1A; Mayr, 2002). Summarized we therefore argue, that the model presented in Eq. 6 (including the simplifications of $\delta_a - \delta_s \approx -\varepsilon^*$ and using T_{air} in Eq. 5) is able to reconstruct RH_{air} values based on $\delta^2H_{leaf\text{-}water}$ and $\delta^{18}O_{leaf\text{-}water}$ values.

3.5 Coupling $\delta^2H_{n\text{-}alkane}$ and $\delta^{18}O_{sugar}$ – potential and limitations

One of the advantages of the proposed coupled $\delta^2H_{n\text{-}alkane}$-$\delta^{18}O_{sugar}$ approach is a more robust reconstruction of the isotope composition of the source water, which can often be directly linked to the local precipitation signal (Hepp et al., 2015, 2017; Tuthorn et al., 2015; Zech et al., 2013a). Therefore, Fig. 6 shows boxplots for measured leaf water, biomarker-based (reconstructed) leaf water, measured source water (tank water; see section 2.1), biomarker-based source water (using reconstructed leaf water as origin for the LEL´s) and leaf-water-based source water values (using measured leaf water as origin for the LEL´s). Source water isotope compositions were calculated via the slopes of the LEL´s and the GMWL. The figure shows that the *n*-alkane and sugar biomarkers reflect leaf water rather than tank water used for irrigation. For δ^2H, neither the range nor the median of the $\delta^2H_{leaf\text{-}water}$ are well captured by the alkane-based leaf water values. However, the overlapping notches do not support a statistical difference in the median values (Fig. 6A). The medians are still on average 13‰ more positive than the measured $\delta^2H_{tank\text{-}water}$. A higher agreement between measured and modeled values is observed from leaf water-based δ^2H_s compared to $\delta^2H_{tank\text{-}water}$. The average offset is reduced to 2‰ and the range is reduced by approximately 70‰, compared to the biomarker-based reconstruction.

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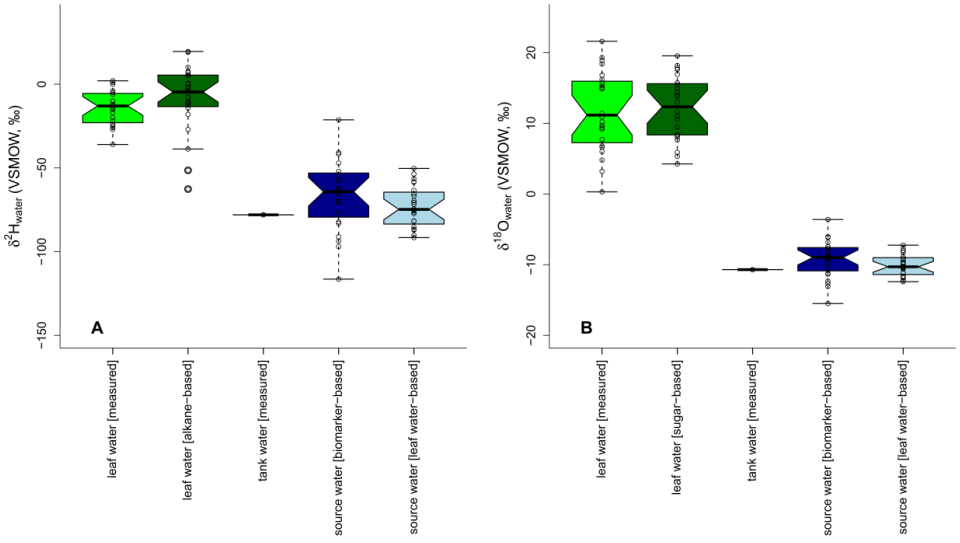

Fig. 6: Boxplots showing the measured leaf water in comparison to the biomarker-based leaf water, tank water, source water calculated with biomarker-based leaf water values and source water based on measured leaf water (A: $\delta^2H_{leaf\text{-}water}$; B: $\delta^{18}O_{leaf\text{-}water}$). Source water isotope compositions were calculated via the slopes of the LEL´s (either with biomarker-based or measured leaf water values) and the GMWL. Boxplots show median (thick black line), interquartile range (IQR) with upper (75%) and lower (25%) quartiles, lower and upper whiskers, which are restricted to 1.5 · IQR. Outside the 1.5 · IQR space, the data points are marked with a dot. The notches are extend to $\pm 1.58 \cdot IQR/\sqrt{n}$, by convention and give a 95% confidence interval for the difference of two medians (McGill et al., 1978).

For $\delta^{18}O$, the sugar-based leaf water values are in agreement with the measured ones with regard to the median values, as supported by the largely overlapping notches (Fig. 6B). The range of the reconstructed leaf water is in the order of 6‰ smaller than for the measured $\delta^{18}O_{leaf\text{-}water}$ dataset. All reconstructed $\delta^{18}O_s$ values, regardless whether they are biomarker- or leaf water-based, are comparable to the measured $\delta^{18}O_{tank\text{-}water}$. While the biomarker-based datasets depict an average offset of 2‰, the leaf water-based values only differ by 0.3‰ from the tank water $\delta^{18}O$ values, referring to the median.

The overall larger range of modeled δ^2H_s and $\delta^{18}O_s$ compared to measured $\delta^2H_{tank\text{-}water}$ and $\delta^{18}O_{tank\text{-}water}$ can related to uncertainties in S_{LEL} modeling (see equations in section 2.3.2). Bariac et al. (1994) mentioned that they found no agreement between the intersect of modeled LEL´s with the GMWL and the plant source water. Allison et al. (1985) explained such results with changing environmental conditions, leading to various LEL´s with a locus line not necessarily passing the δ^2H_s and $\delta^{18}O_s$ data point, in a system that approaches rapidly new steady-state conditions.

Finally, the alkane and sugar-based leaf water values were used to reconstruct RH_{air}. The measured RH_{air} is well captured by the biomarker-based air relative humidity values ($R^2 = 0.54$; Fig. 7). Overall, a lower coefficient of determination of the biomarker-based model results compared to the leaf water-based reconstructions (compare black with grey data points in Fig. 7) is observed. This can be attributed to the uncertainties in leaf water reconstructed using $\delta^2H_{n\text{-}alkane}$ and $\delta^{18}O_{sugar}$ datasets as

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discussed in section 3.2. The limitations regarding deuterium arose from the rather weak relationship between the δ^2H of the *n*-alkanes and the leaf water, probably linked with the large range in the fractionation between *n*-alkanes and leaf water ($\varepsilon^2_{n\text{-alkane/leaf-water}}$). The applied equation to reconstructed $\delta^2H_{leaf\text{-}water}$ by using $\delta^2H_{n\text{-}alkane}$ and a constant biosynthetic fractionation of -160‰ (Eq. 10) was considered to be suitable (Sachse et al., 2012; Sessions et al., 1999), but introduce also some uncertainty for the final relative humidity reconstruction. With regard to oxygen, the relatively large variations in $\varepsilon_{sugar/leaf\text{-}water}$ of 9‰ have to be considered (Fig. 4B), because in the $\delta^{18}O_{leaf\text{-}water}$ reconstructions a fixed value of +27‰ is used (Eq. 11). Such a uniform biosynthetic fractionation is an approximation which may not always be fulfilled, as shown in the literature (e.g. Sternberg and Ellsworth, 2011; Lehmann et al., 2017). Especially the underestimation of the biomarker-based RH_{air} values under the 68% relative humidity conditions, as well as the large range in reconstructed RH_{air} values for the 48, 49, 50% RH_{air} chambers can be attributed to the leaf water reconstruction uncertainties. It should be mentioned that using Eqs. 7 and 8 to calculate leaf water isotope composition based on the biomarkers via a biosynthetic fractionation values implies that the fractionation process in principle can be treated as single process with a unique source. While this approximation can be questioned (see discussion in section 3.2), the overall correlation between biomarker-based and measured RH_{air} highlights the potential of the approach (Hepp et al., 2017; Tuthorn et al., 2015; Zech et al., 2013a), also for future paleo-applications.

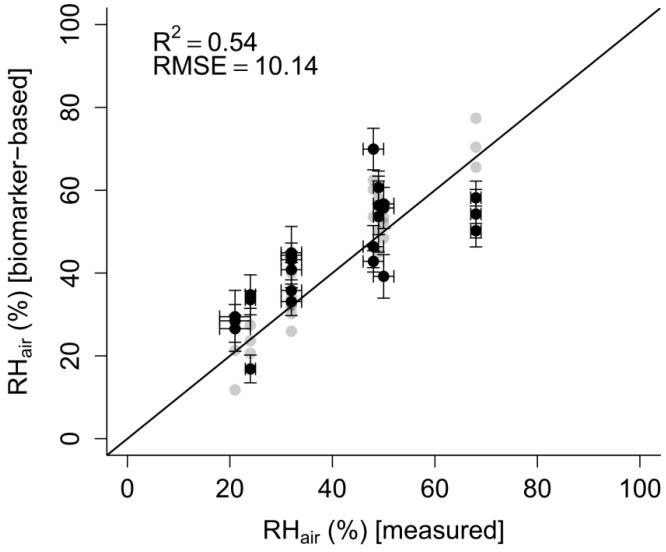

Fig. 7: Scatterplot depicting the relationship between biomarker-based (modeled) and measured air relative humidity (RH). The black line indicates the 1:1 relationship. R^2 and RMSE was calculated as described in section 2.4, while the RMSE values have the dimensions of the respective variables. Error bars for the measured values represent analytical standard deviations (see Mayr, 2002). For uncertainty calculation of the modeled properties, see section 2.4. In addition, the leaf water-based air relative humidity results are shown in light grey for comparison.

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4 Conclusions

The climate chamber results and discussion suggest that leaf wax-derived *n*-alkane and hemicellulose-derived sugar biomarkers are valuable $\delta^2H_{leaf\text{-}water}$ and $\delta^{18}O_{leaf\text{-}water}$ recorders, respectively. The coupling of $\delta^2H_{n\text{-}alkane}$ and $\delta^{18}O_{sugar}$ results allows moreover a robust RH_{air} reconstruction of the chambers in which the plants were grown, by using a simplified Craig-Gordon equation. With regard to the research questions, we conclude as follows:

(i) Alkanes with the chain-length *n*-C_{29} predominated and occurred at abundances suitable for compound-specific δ^2H measurements in the leaf samples from all investigated species (*Eucalyptus globulus*, *Vicia faba* var. *minor* and *Brassica oleracea* var. *medullosa*). For *Vicia faba*, additionally *n*-C_{31} could be evaluated robustly. $\delta^{18}O_{sugar}$ values could be obtained for the hemicellulose-derived monosaccharides arabinose and xylose.

(ii) Both the $\delta^2H_{n\text{-}alkane}$ and $\delta^{18}O_{sugar}$ values yielded highly significant correlations with $\delta^2H_{leaf\text{-}water}$ and $\delta^{18}O_{leaf\text{-}water}$ ($r^2 = 0.45$ and 0.85, respectively; $p < 0.001$, $n = 24$). Mean fractionation factors between biomarkers and leaf water were found to be -156‰ (ranging from -133 to - 192‰) for $\varepsilon_{n\text{-}alkane/leaf\text{-}water}$ and +27.3‰ (ranging from +23.0 to +32.3‰) for $\varepsilon_{sugar/leaf\text{-}water}$.

(iii) RH_{air} can be derived robustly by using the measured leaf water isotope composition ($\delta^2H_{leaf\text{-}water}$ and $\delta^{18}O_{leaf\text{-}water}$) and a rearranged Craig-Gordon model, ($R^2 = 0.84$; $p < 0.001$; RMSE = 6%).

(iv) Reconstructed source water isotope composition (δ^2H_s, $\delta^{18}O_s$) is in range with the measured tank water ($\delta^2H_{tank\text{-}water}$, $\delta^{18}O_{tank\text{-}water}$). However, modeled δ^2H_s and $\delta^{18}O_s$ show a clearly larger range compared to $\delta^2H_{tank\text{-}water}$ and $\delta^{18}O_{tank\text{-}water}$. The uncertainties for source water determination are thus considerably higher compared to the relative humidity reconstructions. Still, the coupled δ^2H-$\delta^{18}O$ approach enables a back calculation of the plant source water. Uncertainties with regard to relative humidity reconstructions via biomarker-based leaf water isotope composition arose from leaf water reconstructions and model uncertainties, as shown in the conclusions ii) and iii). Overall, the biomarker-based and measured RH_{air} correlation with R^2 of 0.54 ($p < 0.001$) and RMSE of 10% highlights the great potential of the coupled $\delta^2H_{n\text{-}alkane}$-$\delta^{18}O_{sugar}$ paleohygrometer approach for reliable relative humidity reconstructions.

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Author contributions

J. Hepp and M. Zech wrote the paper; C. Mayr was responsible for the climate chamber experiment together with W. Stichler and provided the leaf samples and the data; M. Zech and R. Zech were responsible for compound-specific isotope analysis on the biomarkers; J. Hepp, M. Tuthorn and I. K. Schäfer did laboratory work and data evaluation of the biomarker compound-specific isotope analysis; B. Glaser, D. Juchelka, K. Rozanski and all co-authors contributed to the discussion and commented on the manuscript.

Appendix

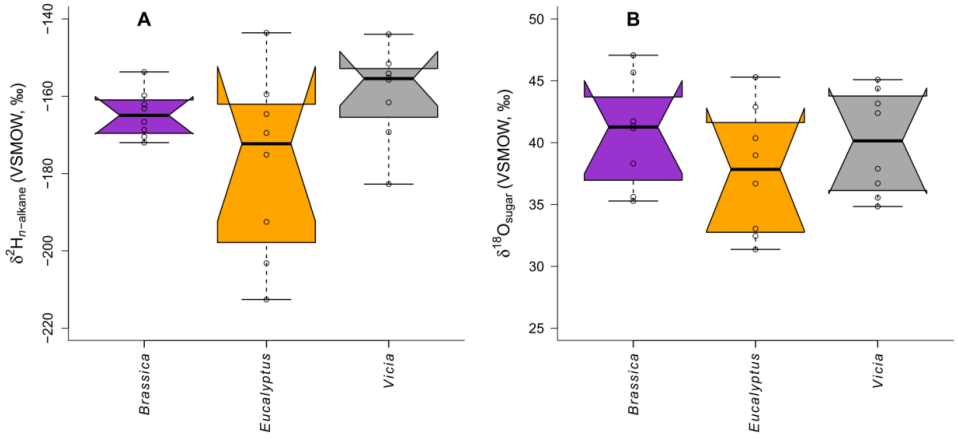

Fig. A1: Boxplots comprising the plant-specific $\delta^2H_{n\text{-alkane}}$ (A) and $\delta^{18}O_{sugar}$ values (B). *Brassica oleracera, Eucalyptus globulus* and *Vicia faba* samples are shown in purple, orange and black, respectively. Boxplots show median (thick black line), interquartile range (IQR) with upper (75%) and lower (25%) quartiles, lower and upper whiskers, which are restricted to 1.5 · IQR. Outside the 1.5 · IQR space, the data points are marked with a dot. The notches are extend to ± 1.58 ·IQR/$\sqrt{n}$, by convention and give a 95% confidence interval for the difference of two medians (McGill et al., 1978).

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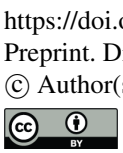

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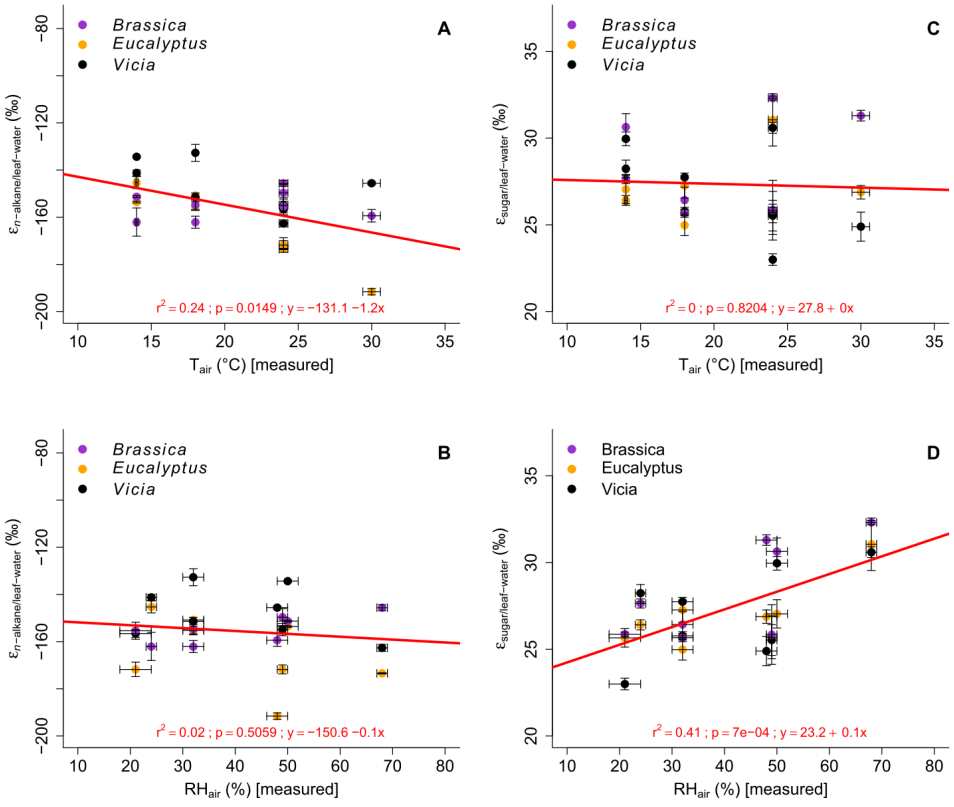

Fig. A2: Scatterplots of the fractionation between the biomarkers and leaf water vs. air temperature, air relative humidity (A and B: $\varepsilon_{n\text{-alkane/leaf-water}}$ according Eq. 10; C and D $\varepsilon_{sugar/leaf-water}$ according Eq. 11). *Brassica oleracera, Eucalyptus globulus* and *Vicia faba* samples are shown in purple, orange and black, respectively. Error bars for the measured values represent analytical standard deviations of repeated measurements (see section 2.2 and Mayr, 2002). For uncertainty calculation of the ε values, see section 2.4.

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