Improving the extraction and purification of leaf and phloem sugars for oxygen isotope analyses

Marco M. Lehmann^{1,†,*}, Melanie Egli^{2,†}, Nadine Brinkmann², Roland A. Werner³, Matthias Saurer¹, Ansgar Kahmen²

¹Forest Dynamics, Swiss Federal Institute for Forest, Snow and Landscape Research (WSL), Zuercherstrasse 111, 8903 Birmensdorf, Switzerland

²Department of Environmental Sciences - Botany, University of Basel, Schoenbeinstrasse 6, 4056 Basel, Switzerland

³Institute of Agricultural Sciences, ETH Zurich, Universitaetstrasse 2, 8092 Zurich, Switzerland

[†] Shared first authorship due to equal contribution

*Corresponding author: Dr. Marco M. Lehmann
 WSL Birmensdorf
 HL D35
 8903 Birmensdorf
 Switzerland
marco.lehmann@alumni.ethz.ch
 Office: +41 739 21 99

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Abstract

RATIONALE: The oxygen isotopic composition (here shown as $\delta^{18}\text{O}$ value) of soluble sugars in leaves and phloem tissue holds valuable information about plant functions in response to climatic changes. However, $\delta^{18}\text{O}$ analysis of sugars is prone to error and thoroughly tested methods are lacking.

METHODS: We performed three experiments to test if sample preparation modifies the $\delta^{18}\text{O}$ values of sugars. In experiment 1, we tested effects of oven- vs freeze-drying, while in experiment 2 we focused on the extraction and purification of leaf sugars. In experiment 3, we investigated the exudation and purification of twig phloem sugars as a function of exudation time and different ethylenediaminetetraacetic acid (EDTA) exudation media.

RESULTS: Freeze-drying produced more consistent $\delta^{18}\text{O}$ values than oven-drying for sucrose, but not for phloem sugars. Extraction and purification of leaf sugars can be performed without a significant modification of its $\delta^{18}\text{O}$ values. Yet, purified leaf and phloem sugars had higher $\delta^{18}\text{O}$ values than the fraction of water-soluble compounds highlighting the necessity for purification of extracted sugars. Moreover, the exudation time significantly modulated the $\delta^{18}\text{O}$ values of phloem sugars, which is probably related to changes in sugar composition. EDTA addition did not improve the determination of phloem sugars' $\delta^{18}\text{O}$ values.

CONCLUSIONS: We show that sample preparation of plant sugars for reliable determination of $\delta^{18}\text{O}$ values requires a strict protocol that we provide in this manuscript. We recommend for phloem sugar a maximum exudation time of one hour to reduce the degradation of sucrose and minimise oxygen isotope exchange reactions between the resulting hexoses and water.

Keywords: Assimilates, foliar, fructose, glucose, sucrose

Introduction

The measurement of the oxygen isotopic composition (here given as $\delta^{18}\text{O}$ value) of plant-derived compounds is a widely applied tool for the reconstruction of climatic, hydrological, and physiological changes [1-8]. Variations in $\delta^{18}\text{O}$ values of plant compounds are mainly caused by corresponding variations in $\delta^{18}\text{O}$ values of their source and leaf water [5, 9]. The oxygen isotopic composition of leaf water is imprinted on primary assimilates in the leaves via a gem-diol isotope exchange reaction of carbonyl groups of intermediates with surrounding water [10, 11]. These photosynthetically produced mono- and disaccharides, are translocated via phloem sap to the sink tissues (i.e. leaves, stems, roots, fruits) where they are further metabolised into other organic compounds. The mechanisms that modify $\delta^{18}\text{O}$ values of assimilates during transport are, however, rarely investigated and thus not fully understood. Partly this is because well-established methods for the extraction and purification of freshly assimilated sugars (e.g. bulk sugars, sucrose, fructose, glucose) from leaves or phloem for oxygen isotope analysis are scarce [12, 13].

The methods typically used to extract sugars from leaf material for isotope analysis build on methods described by Wanek, et al. [14] and Richter, et al. [15] for carbon isotope analysis of leaf non-structural carbohydrates, but have not yet been thoroughly tested for their reliability in $\delta^{18}\text{O}$ analyses. The methods involve the suspension of dried and ground material from leaves or other plant tissues in deionised water, heating of the suspension for a certain time, and subsequent centrifugation. The supernatant holds the fraction of water-soluble compounds (i.e. WSC) [16, 17], reflecting a mixture of various compounds (soluble sugars, amino acids, organic acids, and other components) with species-dependent variable composition.

For sugar extraction from a plant's phloem a multitude of methods has been employed in the past. These include, for example, stylectomy using phloem-puncturing aphids [18] or phloem-bleeding techniques based on incisions [19, 20]. The advantage of these methods is the collection of pure phloem sap. However, both approaches have their limitations due to low yields, variation in seasonal availability, and their exclusive use on plant species where phloem bleeding is possible. [21, 22]. To bypass these issues, other studies have applied mild vacuum or centrifugation techniques to extract phloem sap [23, 24], or are based on passive exudation of sugars out of the phloem tissue's sieve tubes into an aqueous exudation medium for several hours [25, 26]. Some of these methods recommend the chelating agent ethylenediaminetetraacetic acid (EDTA) to prevent potential occlusion (clogging) of sieve tubes by callose or other compounds to enable the continuous leakage of phloem sap resulting in higher sugar yields [27-30]. The more efficient exudation could in principal provide more accurate $\delta^{18}\text{O}$ values of phloem sugars. However, EDTA carries its own oxygen isotopic signature and thus needs to be removed before the $\delta^{18}\text{O}$ analysis of a sugar sample. Although phloem sap is dominated by sugars (i.e. sucrose), it additionally contains non-sugar compounds similar to leaf WSC, we therefore refer to phloem exudates as phloem WSC. The concentrations and the $\delta^{18}\text{O}$ values can differ among organic compounds in leaf and phloem WSC [12, 31]. Hence, when the isotopic composition of sugars is of interest [9, 32, 33], the neutral sugar fraction (i.e. sugars) is generally separated from the WSC via additional purification steps such as ion-exchange chromatography [12, 34].

If and to what extent the above-described extraction, exudation and purification steps affect the $\delta^{18}\text{O}$ values of WSC or sugars of leaf and phloem tissue has to date not been comprehensively tested. Uncertainties thus exist about the necessity of various extraction or purification steps and/or possible artefacts introduced by these steps. For this purpose, we conducted a series of experiments using laboratory water of different isotope composition and performed isotope and concentration analysis of commercially available C_3 and C_4 sucrose, WSC, and sugars extracted or exuded from leaf and twig phloem material. The aim of this study was to test and establish robust methods for the extraction (i.e. hot water extraction and exudation) and purification (i.e. ion-exchange and sample drying) of leaf and phloem sugars for oxygen isotope analyses. We therefore determined the effects of individual extraction and purification steps on $\delta^{18}\text{O}$ values of sugars by testing (1) different drying methods (i.e. oven- vs freeze-drying) for sucrose and phloem sugar samples; (2) hot water extraction and purification of leaf sugars; and (3) exudation times for phloem WSC with and without EDTA, as well as the subsequent purification of phloem sugars. Moreover, we applied compound-specific concentration analysis to detect potential changes in the phloem sugar composition during the exudation period.

Material and Methods

Experiment 1: Effects of drying method on sugar $\delta^{18}\text{O}$ values

The aim of experiment 1 was to determine how drying a sample with a freeze dryer or a drying oven affects $\delta^{18}\text{O}$ values of sugars. For the experiment, we used three types of commercially available sucrose (two C_4 sucrose samples from Switzerland and USA, and one C_3 sucrose from USA) and exuded beech phloem sugars. We dissolved 200 mg of each of the three sucrose types in 10 mL of three different ^{18}O -labelled water which had approximate $\delta^{18}\text{O}$ values of -30 mUr, -10 mUr, and +30 mUr. The waters were prepared by mixing ^{18}O -enriched water (97 atom %, Sigma-Aldrich, Steinheim, Germany) or ^{18}O -depleted water (by-product derived from deuterium enrichment columns at the Paul Scherrer Institute, Switzerland) with deionised laboratory water ($\geq 18.2 \text{ M}\Omega$, Merck, Darmstadt, Germany), following calculations by Brand and Coplen [35].

For oven drying, aliquots of 50 μL of each sucrose sample were transferred into silver capsules (4x6 mm, Saentis Analytical AG, Teufen, Switzerland) and dried at 60°C for 2 h in an oven (TSW 60 ED, Salvis, Reussbühl, Switzerland). For freeze-drying, again, aliquots of 50 μL of each sucrose sample were transferred into silver capsules (4x6 mm, Saentis), frozen at -20°C , and dried for 2 h at -80°C and 0.01 mbar in a freeze-drier (Beta 2-8 LD plus, Christ GmbH, Osterode am Harz, Germany). The two procedures were repeated to achieve a target weight of 2 mg dried sugars. The silver capsules were then immediately closed and kept at low humidity in a desiccator containing dry silica gel.

Beech phloem sugars were obtained following the protocol of Gessler, et al. [25]. Phloem pieces of mature *Fagus sylvatica* (5 cm^2) were taken from twigs of 1 m length and of 0.5 to 1 cm diameter in a forest (Laegern, Baden, Switzerland) in spring 2014 at midday, briefly rinsed with deionised

water, and immersed in 2 mL deionised water (Merck) for 5 h at room temperature (RT). Subsequently, the phloem piece was discarded and the exuded phloem WSC kept frozen at -20°C . An aliquot of 1 mL of the phloem WSC was then purified via ion-exchange chromatography by passing it through prepared columns of conical and centric 5 mL syringes (B. Braun, Melsungen, Germany) containing pre-conditioned material of Dowex 50WX8 (H^{+} , cation exchange) and Dowex 1X8 (Cl^{-} , anion exchange, both Sigma-Aldrich, St. Louis, USA), following the protocol of Lehmann, et al. [36]. The neutral fraction containing the soluble sugars was eluted with 35 mL deionised water and collected in 50 mL tubes (BD Biosciences, Heidelberg, Germany). Afterwards, samples were frozen, freeze-dried, and the remainder re-dissolved in 1 mL of deionised water. The latter was separately done with the same three ^{18}O -labelled waters as described above. Then, 75 μL of the aqueous solution was transferred into 4x6 mm silver capsules, freeze- or oven-dried (final weight = ca. 5.5 mg), closed, and kept in a desiccator with dry silica gel as described above for sucrose samples.

Experiment 2: Effects of extraction and purification on sugar $\delta^{18}\text{O}$ values

The aim of experiment 2 was to determine potential changes of leaf sugar $\delta^{18}\text{O}$ values during their extraction and purification via SPE cartridges. All individual steps (i.e. extraction, purification, dissolving) of this experiment were conducted separately with three different ^{18}O -labelled water which had approximate $\delta^{18}\text{O}$ values of -50 mUr, -10 mUr, and $+30$ mUr and which were prepared as described earlier in experiment 1.

In a first part of the experiment, we collected sun-exposed *Rhododendron* sp. leaves at 01:00 p.m. on the 9th of March 2016 in the Botanical Garden of the University of Basel, Switzerland. The leaf enzymatic activity was stopped immediately using a microwave at 900 W for 3 min. The material was then oven-dried at 55°C for three days and ground at 30 Hz for 3 min by MM400 ball-mill (Retsch, Haan, Germany). For the extraction, 60 mg of the powder was weighed in 2 mL reaction vials and 1.5 mL deionised water was added and mixed by vortex until the powder was fully suspended [12, 34]. The reaction vials containing the samples were placed in a water bath at 85°C for 30 min to open cells and to facilitate the sugars' dissolution into the water. The samples were then cooled down to RT for another 30 min, centrifuged (2 min, 12100 g, RT), and 1.2 mL of the supernatant (= WSC) was transferred into new reaction vials. The leaf-extracted WSC was then purified via ion-exchange chromatography, based on three OnGuard II H, A, and P SPE cartridges (Dionex, Sunnyvale, CA, USA) stacked on top of each other. The upper H cartridge contains a resin with high selectivity for polyvalent cations, retains e.g. amino acids, calcium, alkaline earth and transition metals, and neutralises highly alkaline samples. The resin of the middle A cartridge retains anionic contaminants such as organic acids and neutralises highly acidic samples. Finally, the resin in the lower P cartridge binds to (poly)phenols, aromatic carboxylic acids, and aromatic aldehydes. Double-cleansed central and conical 5 mL syringes (BD Plastipak, Franklin Lakes, NJ, USA) were connected with the upper H cartridge, and the whole system was rinsed with 30 mL unlabelled deionised water following the protocol by Rinne, et al. [34]. This step ensured the conditioning, enhancing surface contact within each resin, and subsequent removal of potential water-soluble contaminants. For the purification, 1 mL WSC sample solution was transferred into

the syringe, followed by 2x1 mL, and another 2x2 mL deionised water to quantitatively elute sugars [34]. Whenever possible, additional pressure using the syringes' plunger was avoided to preserve optimal purification. The purified sugar solution (6 mL) was collected in 15 mL reaction tubes (Merck or BD Biosciences), frozen, and freeze-dried. The samples were then dissolved again in 1 mL deionised water. Aliquots of non-purified leaf WSC (140 μ L) and of purified leaf sugars (100 μ L) were each transferred into silver capsules (5x9 mm, Saentis), frozen, and freeze-dried overnight (final weight = 0.11 - 0.15 mg) for isotope analysis.

For the second part of the experiment, 15 mg of commercially available C₃ and C₄ sucrose (both from Switzerland) was dissolved in 1.5 mL deionised water and boiled at 85°C for 30 min as described above for leaf sugars. Aliquots of 1 mL of each sucrose solution were purified via ion-exchange as described above for leaf sugars. Subsequently, 100 μ L of the boiled non-purified and purified sucrose solutions were transferred into silver capsules (5x9 mm, Saentis), frozen, and freeze-dried overnight (final weight = 1 mg) for isotope analysis.

Experiment 3: Effects of extraction time and EDTA during exudation and purification on phloem sugar $\delta^{18}\text{O}$ values and concentrations

The aim of experiment 3 was to determine temporal changes in sugar $\delta^{18}\text{O}$ values and in individual sugar concentrations that occur during the exudation and purification of phloem sap, with and without the addition of EDTA. Sun-exposed branches with leaves and twigs of *Fagus sylvatica* var. *pendula* were collected on the 28th of October 2016 in the Botanical Garden of the University of Basel, Switzerland. The branches were cut at ca. 3.5 m height and twigs of 50 cm length and of about 1 to 3 cm diameter prepared. Approximately 5 cm² twig phloem material including the bark were removed with a carpet knife and immediately immersed in a 15 mL reaction tube containing 6 mL of the respective exudation media. The extraction medium was pure deionized water and contained for the different treatments 0 mM EDTA, 5 mM EDTA, or 20 mM EDTA (Gerbu, Heidelberg, Germany; $\delta^{18}\text{O}$ value of EDTA = -2.7 ± 0.3 mUr, n = 5). Samples were first kept in the exudation media for 0.25 h (15 min) to rinse the sample. This step was conducted before the start of the exudation procedure to remove potential contaminants (e.g. sugars) from non-sieve cells that were injured during the preparation of the phloem material. Following this step, all samples were transferred into fresh exudation solutions for defined exudation periods of 1, 2, 3, and 5 hours. After the respective exudation time, the phloem tissue was removed from the solutions and the exudation medium containing the phloem WSC was frozen at -20°C. Aliquots of 1 mL of phloem WSC were purified by SPE cartridges as described in experiment 2. The solutions containing purified sugars (6 mL) were collected in 15 mL reaction tubes, frozen and freeze-dried, and the pellet dissolved again in 140 μ L deionised water for bulk analyses or in 1 mL deionised water for analysis of individual sugar concentrations (see description below for compound-specific analysis). Aliquots of 75 to 140 μ L of the non-purified phloem WSC and purified phloem sugars were transferred into silver capsules (5x9 mm, Saentis), frozen, and freeze-dried overnight (final weight = 0.1 to 0.6 mg) for isotope analysis.

Oxygen isotope analysis of sugar, WSC, and water samples

The $\delta^{18}\text{O}$ analysis of dried sucrose and leaf- or phloem-extracted assimilates packed in silver capsules were performed via pyrolysis at 1450°C into carbon monoxide (CO) with a HTO (Hekatech, Wegberg, Germany) reaction unit at ETH Zürich (Experiment 1) [37, 38] or with a vario PYRO cube (Elementar, Hanau, Germany) at WSL (Experiment 2 and 3) [39], respectively. In both isotope laboratories, the CO gas was transferred via a Conflo III reference gas interface to an IRMS instrument (Finnigan Delta Plus XP, all supplied by Thermo Fisher Scientific, Bremen, Germany). To avoid absorption of gaseous water or rehydration through air humidity by the sugars, the following steps were found to be critical for $\delta^{18}\text{O}$ analysis of sugars. At ETH Zurich, sugar samples and measurement standards were placed together in a pre-warmed Zerobank autosampler carousel (Costech, Cernusco, Italy), followed by drying for one hour at 60°C in an oven. The hot autosampler carousel was then immediately transferred to the Zero blank autosampler (Costech) and flushed for another hour with dry helium (He 5.0, Pangas Dagmersellen, Switzerland) before the measurement sequence was started. At WSL, sugar samples and measurement standards were placed together directly into the autosampler tray of the PYRO cube that is heated at 50°C and flushed continuously with argon until the end of the measurement sequence. $\delta^{18}\text{O}$ values of water samples were measured in both isotope laboratories using a thermal conversion elemental analyser (Thermo Fisher Scientific) coupled to the same IRMS instrumental setup as described above [40]. The applied referencing for both $\delta^{18}\text{O}$ measurements of water and organic samples followed the principles described in the "MPI-BGC" part of Gehre, et al. [40] and in Brand, et al. [41] in both isotope laboratories. The raw $\delta^{18}\text{O}$ values of the samples were corrected for instrumental memory effect and instrumental drift followed by a scaling to a pair of laboratory water or organic standards (benzoic acid and dimethoxybenzoic acid at ETH, sucrose and lactose at WSL) with a known difference in oxygen isotopic composition ("normalisation" according to Coplen et al. 1988). An additional quality control standard (trimethoxybenzoic acid at ETH or cellulose at WSL) for the analysis of solid samples is used to determine the precision and the accuracy of each measurement sequence. The laboratory standards were repeatedly calibrated versus international standards available from IAEA. All presented $\delta^{18}\text{O}$ values are expressed as $\delta^{18}\text{O} [\text{mUr}] = R_{\text{Sample}}/R_{\text{VSMOW}} - 1$, where R_{Sample} is the $^{18}\text{O}/^{16}\text{O}$ ratio of the sample and R_{VSMOW} that of the international standard Vienna Standard Mean Ocean Water (VSMOW) [35]. The typical measurement precision (SD) was 0.2 mUr for water and 0.3 mUr for organics (for a minimum amount of 0.1 mg).

Compound-specific concentration analysis of individual phloem sugars

Concentrations of individual phloem sugars in pure water without added EDTA (0 mM EDTA) were measured with high-performance liquid chromatography (HPLC) coupled via LC Isolink interface to a Delta V Advantage IRMS instrument (all supplied by Thermo Fisher Scientific), following the protocol of Rinne, et al. [34]. While the setup is used for carbon isotope analysis, the conversion of the compound into CO_2 is quantitative and can, in consequence, be well used for concentration analysis. In brief, an aliquot of 1 mL phloem WSC was purified via SPE cartridges (see experiment 3). The individual eluted phloem sugars were separated on a Dionex CarboPac PA20 analytical column (Thermo Fisher Scientific) at 20°C using 2 mM NaOH as mobile phase with a flow rate of 250 $\mu\text{L min}^{-1}$ and injection volume of 20 μL . Subsequently, the individual sugars

(i.e. myo-inositol, sucrose, glucose, and fructose) are quantitatively oxidised in a reactor using sodium persulfate ($\text{Na}_2\text{S}_2\text{O}_8$) under acidic conditions (H_3PO_4) at 99°C to CO_2 , which is removed from the water phase with helium gas, dried, and administered to the IRMS instrument. The relationship between the CO_2 concentration of standard solutions and the corresponding mass-spectrometer peak areas is highly linear ($r^2 > 0.95$), resulting in concentration determination with a relative precision better than 5%. For fructose, peak overlaps with an unidentified minor compound in the HPLC chromatograms were observed, hence limiting an accurate concentration determination of fructose. We suspect the unknown compound to be raffinose because of similar retention times as fructose, but raffinose typically occurs only in low abundances^[34]. Myo-inositol co-elutes with pinitol and the two cannot be distinguished using HPLC^[34], but GC-MS analysis of phloem sap suggests myo-inositol as the main compound in beech phloem sap^[26].

Statistics

One or two-way ANOVAs, T-tests, and Tukey-HSD post-hoc analyses were used to test for isotopic ($\delta^{18}\text{O}$ value) and concentration differences across treatments with various ^{18}O -labelled water, different samples (sucrose, leaf WSC/sugars, and phloem WSC/sugars), EDTA solutions, and exudation times. All statistical analysis and figures were made in R version 3.5.1^[42].

Results and Discussion

Freeze-drying is more efficient than oven-drying for $\delta^{18}\text{O}$ analysis of sugars

Experiment 1 revealed that the effect of laboratory water on the $\delta^{18}\text{O}$ values of sucrose samples depends on the drying method (Fig. 1). $\delta^{18}\text{O}$ values of the different oven-dried sucrose samples varied by 6 mUr among the different ^{18}O -labelled water treatments ($P < 0.001$). In contrast, $\delta^{18}\text{O}$ values of freeze-dried sucrose samples varied only by 1 mUr, although differences were still significant among the treatments ($P < 0.005$). The observed $\delta^{18}\text{O}$ variations in the sugar samples across the three ^{18}O -labelled water treatments can generally be a result of two effects: (1) remaining water in the sample due to an incomplete drying process^[43] or (2) exchange of anomeric oxygen atoms between carbonyl groups of the reducing monosaccharides and surrounding water molecules during the oxy-cyclo-tautomerisation of mutarotation^[44]. For sucrose samples, the second effect can be excluded due to the lack of free carbonyl groups and given that no additional other compounds have been detected in the samples by HPLC-IRMS that would allow for isotope exchange with water. Acetal bridges connect both monosaccharide units in the sucrose molecule (i.e. fructose and glucose) between their anomeric carbon atoms, which results in mutual protection of the cyclic hemiacetal groups and therefore prevents mutarotation^[45]. Low oxygen exchange of sucrose with medium water is supported by previous observations^[12, 46, 47]. $\delta^{18}\text{O}$ variations of sucrose samples are thus mainly caused by remaining water in the sample after drying. The higher $\delta^{18}\text{O}$ variations of oven-dried sucrose samples can likely be explained by a higher formation of amorphous sugars than crystalline sugar. Amorphous sugars reflect a liquid that can hold more water than the amount of absorbed water remaining bound to the hygroscopic surface of crystalline sugars^[43]. One should also consider that the effect of oxygen derived from water on sugars depends on the isotopic difference between the two compounds. The effect should theoretically be highest

between ^{18}O -depleted water (-30 mUr) and sugars (ca. 30 mUr). Indeed, the strongest effect of 0.5 mUr was observed between the laboratory water and the ^{18}O -depleted water (i.e. difference of 20 mUr) for freeze-dried C_4 sucrose (Switzerland, Fig. 1). This results in a maximum error of 2.5% in the sugar sample that is derived from oxygen of water due to incomplete drying or exchange into sugar molecules. The effect of the water on $\delta^{18}\text{O}$ values of sugars is thus negligible if always the same laboratory water is applied during sample processing. In summary, freeze-drying of dissolved sugar samples seems to most effectively remove the aqueous solvent and thus improves the precision of the oxygen isotope analysis. Given the observed results, samples of all the following experiments were freeze-dried.

Effects on $\delta^{18}\text{O}$ values caused by the extraction and purification of leaf sugars

Experiment 2 revealed an ^{18}O -enrichment of 5.1 to 5.5 mUr of purified sugars compared to non-purified WSC extracted from leaves of *Rhododendron* sp. across all ^{18}O -labelled water treatments ($P < 0.001$; T-test, Fig. 2A). Most likely, these differences occur because the purified leaf samples contain only sugars and sugar alcohols^[48], which are known to be one of the most ^{18}O -enriched compounds in plants^[12]. Purification removed other leaf components present in the WSC such as amino and organic acid molecules that are subject to different biosynthesis reactions implying different oxygen isotope fractionations^[31]. Importantly, the extraction and purification method itself did not affect the $\delta^{18}\text{O}$ values of sugars. This became evident as generally no $\delta^{18}\text{O}$ difference was observed between non-purified and purified sucrose samples ($P > 0.05$, T-test; Fig. 2B, C). Similar to experiment 1, the ^{18}O -labelled water treatment with a range of 80 mUr caused significant but only small effects of about 1 mUr for both sucrose samples ($P < 0.001$), independent of purification. This demonstrates again the minor importance of laboratory water on sugar samples and that the chosen purification method by commercially available SPE cartridges is a reliable method for oxygen isotope analysis of leaf sugars.

No effects of EDTA but strong effects of exudation time on phloem sugar $\delta^{18}\text{O}$ values

Experiment 3 was conducted to test how EDTA addition affects $\delta^{18}\text{O}$ values of exuded phloem WSC and sugars of *Fagus sylvatica* var. *pendula*. We found that non-purified phloem WSC with 0, 5, or 20 mM EDTA exudation media clearly differed in their $\delta^{18}\text{O}$ values ($P < 0.001$; Fig. 3A). The decreasing pattern of $\delta^{18}\text{O}$ values with increasing EDTA concentration is caused by the presence of EDTA in the analysed samples, with pure EDTA having a low $\delta^{18}\text{O}$ value of -2.7 mUr. The effect of EDTA on phloem sample $\delta^{18}\text{O}$ values disappeared after purification of the samples (Fig. 3B), showing that the negatively charged EDTA molecules can well be removed by the anion-exchange SPE cartridge (i.e. OnGuard II A, Dionex). In consequence, $\delta^{18}\text{O}$ values of exuded phloem sap using EDTA media are only meaningful if samples are purified. However, the EDTA addition did neither reduce the $\delta^{18}\text{O}$ variation in exuded and purified phloem sugars ($P < 0.05$, Fig. 3B) nor prevent sucrose degradation regardless of EDTA concentrations in the exudation media (data not shown). Thus, we conclude that the addition of EDTA is not advisable for oxygen isotope analysis of purified phloem sugars.

We subsequently focus our discussion on the samples that experienced no EDTA addition (i.e. 0 mM EDTA). Similar to leaf sugars, purification of sugars in phloem WSC in 0 mM EDTA caused an ^{18}O -enrichment of 2.6 to 6.6 mUr across all exudation times (Fig. 3A, B). Thus, other compounds in exuded phloem sap bear a strong influence on $\delta^{18}\text{O}$ values and should therefore be removed before isotope analysis of phloem sugars. We also found that $\delta^{18}\text{O}$ values of purified phloem sugars in 0 mM EDTA showed a strong dependence on exudation time (Fig. 3B, $P < 0.001$), with a step-wise decrease of 3.7 mUr over 5 h. For a better understanding of this effect we analysed the concentration of the individual phloem sugars in 0 mM EDTA. During the rinsing period with deionised water for 0.25 h, the concentrations for individual sugars were highest (Fig. 4), with sucrose concentrations twice as high as those of fructose and glucose. Possibly, the rinsing step was already causing an intense exudation of phloem sugars into the sample that mixed with the unwanted sugars derived from non-sieve cells, with potentially different $\delta^{18}\text{O}$ values compared to those in phloem sap due to the metabolic conversion of sugars^[26]. While we cannot fully resolve this issue here, we recommend testing the timing of the rinsing step more thoroughly in future applications. Potentially, a more conservative method such as a short dipping of the sample into deionised water or rinsing of the surface with deionised water using a wash bottle might be already sufficient to remove contaminants.

For longer exudation times (> 0.25 h), total concentrations of all individual compounds sharply decreased ($P < 0.001$), with the lowest yield (i.e. total sum of sugars and sugar alcohol) after 2 h of exudation. Also the sugar chemical composition changed with progressing exudation time, with decreasing relative amounts of sucrose and increasing relative amounts of glucose, fructose and myo-inositol. The changes in the sugar composition during the exudation period most likely explain the $\delta^{18}\text{O}$ changes in the bulk phloem sugar fraction (Fig. 3B). For instance, sucrose is known to be ^{18}O -enriched compared to hexoses and sugar alcohols^[12]. The high sucrose content compared to other sugars in the bulk phloem sugar fraction at 0.25 h thus explains the higher ^{18}O -enrichment of the sample compared to other time points, where sucrose was less abundant. The increasing amount of hexoses may have led to an additional ^{18}O -depletion via oxygen isotope exchange of the reducing sugars with water or due to an addition of water to hexoses during hydrolysis of sucrose^[12]. This is supported by the substantial $\delta^{18}\text{O}$ variations of 11.9 mUr across all ^{18}O -labelled water treatments for both drying techniques in twig phloem sugars of beech in experiment 1 ($P < 0.001$; Fig. 1). Similar to experiment 3, the samples have been exuded for 5 h and likely contained also a relatively high amount of hexoses that have incorporated the ^{18}O -label, independent of the drying procedure. In addition, myo-inositol, which has also been found in phloem samples, shows only minor isotopic variations during a growing season^[48, 49]. Such compounds do not exchange their oxygen atoms as they lack the respectively needed carbonyl group for it^[50, 51], but can be ^{18}O -depleted compared to hexoses and sucrose^[12]. An increase in their concentration may thus additionally cause an ^{18}O -depletion in the bulk phloem sugar fraction.

The reasons for the breakdown of sucrose remains speculative but could be related to pH, microbial activity, or enzymatic activity of free invertase^[25, 52]. Hence, future studies trying to improve oxygen isotope analysis of phloem sugars should therefore focus on stopping the degradation of

sucrose during exudation. A potential starting point could be the reduction of exudation time for phloem sugars to about one hour, given that sucrose was much less abundant in longer exudation times and the relative concentration of other sugars and sugar alcohol in the phloem sample increased (Fig. 4). However, already during rinsing (at 0.25 h), we found high amounts of fructose and glucose, which may indicate that the breakdown of sucrose already started. High amounts of fructose and glucose could, however, also be derived from non-sieve cells due to incision of the twig phloem tissue. Other possibilities to improve the oxygen isotope analysis of phloem sugars for future investigations might involve the addition of ethanol or methanol into the exudation media to stop enzymatic and microbial activity, changes in temperature and pH during exudation, changes in methodology (e.g. centrifugation or mild vacuum), or in phloem tissue collection (e.g. direct extraction and purification of sugars from dried and ground material).

Summary and conclusions

We found that individual extraction (i.e. hot water extraction and exudation) and purification (i.e. ion-exchange and sample drying) steps can affect $\delta^{18}\text{O}$ values of plant sugars. Reliable $\delta^{18}\text{O}$ measurements of plant sugars are, however, possible if the necessary precautions are taken. We summarised our improved method in an annotated scheme (Fig. S1), demonstrating how leaf and phloem sugars can be isolated and purified for $\delta^{18}\text{O}$ analysis. Extraction and purification of leaf sugars can be performed without a significant modification of its $\delta^{18}\text{O}$ values. Yet, purified leaf and phloem sugars had higher $\delta^{18}\text{O}$ values than the fraction of water-soluble compounds, which is explained by the differential composition and isolation of sugars is advisable. Moreover, phloem exudation with EDTA addition strongly biases the $\delta^{18}\text{O}$ values of phloem sap and the chelating agent needs to be removed before any isotope analysis, which can be easily achieved by ion-exchange chromatography as shown in this study. However, we found no beneficial effect of EDTA on phloem sugars, neither on their $\delta^{18}\text{O}$ values nor on their chemical composition, and its application is therefore not needed. While we cannot present an optimal method for phloem sugars, our results provide a better understanding of the underlying problems during exudation and purification. We found that sucrose concentrations strongly decreased with increasing exudation time and that mainly hexoses and sugar alcohol are exuded if the exudation time exceeded one hour; longer exudation times are therefore not advisable. Finally, our results show that freeze-drying should be favoured, and oven-drying avoided, if sugars are prepared for $\delta^{18}\text{O}$ analysis.

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References

- [1] Farquhar GD, Barbour MM, Henry BK, in *Stable isotopes: the integration of biological, ecological and geochemical processes* (Ed.: Griffiths H), BIOS Scientific, Oxford, 1998, pp. 27-62.
- [2] Barbour MM, Stable oxygen isotope composition of plant tissue: a review, *Functional Plant Biology* 2007;34:83-94.
- [3] Helliker BR, Ehleringer JR, Grass blades as tree rings: environmentally induced changes in the oxygen isotope ratio of cellulose along the length of grass blades, *New Phytologist* 2002;155:417-424.
- [4] Moreno-Gutierrez C, Dawson TE, Nicolas E, Querejeta JJ, Isotopes reveal contrasting water use strategies among coexisting plant species in a Mediterranean ecosystem, *New Phytologist* 2012;196:489-496.
- [5] Kahmen A, Sachse D, Arndt SK, Tu KP, Farrington H, Vitousek PM, Dawson TE, Cellulose $\delta^{18}\text{O}$ is an index of leaf-to-air vapor pressure difference (VPD) in tropical plants, *Proceedings of the National Academy of Sciences of the United States of America* 2011;108:1981-1986.
- [6] Keitel C, Adams MA, Holst T, Matzarakis A, Mayer H, Rennenberg H, Gessler A, Carbon and oxygen isotope composition of organic compounds in the phloem sap provides a short-term measure for stomatal conductance of European beech (*Fagus sylvatica* L.), *Plant Cell and Environment* 2003;26:1157-1168.
- [7] Hepp J, Tuthorn M, Zech R, Mugler I, Schlutz F, Zech W, Zech M, Reconstructing lake evaporation history and the isotopic composition of precipitation by a coupled $\delta^{18}\text{O}$ - $\delta^2\text{H}$ biomarker approach, *Journal of Hydrology* 2015;529:622-631.
- [8] Blees J, Saurer M, Siegwolf RTW, Ulevicius V, Prevôt ASH, Dommen J, Lehmann MM, Oxygen isotope analysis of levoglucosan, a tracer of wood burning, in experimental and ambient aerosol samples, *Rapid Communications in Mass Spectrometry* 2017;31:2101-2108.
- [9] Treydte K, Boda S, Pannatier EG, Fonti P, Frank D, Ullrich B, Saurer M, Siegwolf R, Battipaglia G, Werner W, Gessler A, Seasonal transfer of oxygen isotopes from precipitation and soil to the tree ring: source water versus needle water enrichment, *New Phytologist* 2014;202:772-783.
- [10] Sternberg LDSL, DeNiro MJD, Biogeochemical implications of the isotopic equilibrium fractionation factor between the oxygen-atoms of acetone and water, *Geochimica Et Cosmochimica Acta* 1983;47:2271-2274.
- [11] Yakir D, DeNiro MJ, Oxygen and hydrogen isotope fractionation during cellulose metabolism in *Lemna gibba* L., *Plant Physiology* 1990;93:325-332.
- [12] Lehmann MM, Gamarra B, Kahmen A, Siegwolf RTW, Saurer M, Oxygen isotope fractionations across individual leaf carbohydrates in grass and tree species, *Plant, Cell & Environment* 2017;40:1658-1670.

- [13] Offermann C, Ferrio JP, Holst J, Grote R, Siegwolf R, Kayler Z, Gessler A, The long way down-are carbon and oxygen isotope signals in the tree ring uncoupled from canopy physiological processes?, *Tree Physiology* 2011;31:1088-1102.
- [14] Wanek W, Heintel S, Richter A, Preparation of starch and other carbon fractions from higher plant leaves for stable carbon isotope analysis, *Rapid Communications in Mass Spectrometry* 2001;15:1136-1140.
- [15] Richter A, Wanek W, Werner RA, Ghashghaie J, Jaeggi M, Gessler A, Brugnoli E, Hettmann E, Gottlicher SG, Salmon Y, Bathellier C, Kodama N, Nogues S, Soe A, Volders F, Soergel K, Bloechl A, Siegwolf RTW, Buchmann N, Gleixner G, Preparation of starch and soluble sugars of plant material for the analysis of carbon isotope composition: a comparison of methods, *Rapid Communications in Mass Spectrometry* 2009;23:2476-2488.
- [16] Bögelein R, Lehmann MM, Thomas FM, Differences in carbon isotope leaf-to-phloem fractionation and mixing patterns along a vertical gradient in mature European beech and Douglas fir, *New Phytologist* 2019;222:1803-1815.
- [17] Streit K, Rinne KT, Hagedorn F, Dawes MA, Saurer M, Hoch G, Werner RA, Buchmann N, Siegwolf RTW, Tracing fresh assimilates through *Larix decidua* exposed to elevated CO₂ and soil warming at the alpine treeline using compound-specific stable isotope analysis, *New Phytologist* 2013;197:838-849.
- [18] Fisher DB, Frame JM, A guide to the use of the xxuding-stylet technique in phloem physiology, *Planta* 1984;161:385-393.
- [19] Keitel C, Matzarakis A, Rennenberg H, Gessler A, Carbon isotopic composition and oxygen isotopic enrichment in phloem and total leaf organic matter of European beech (*Fagus sylvatica* L.) along a climate gradient, *Plant, Cell & Environment* 2006;29:1492-1507.
- [20] Pate J, Shedley E, Arthur D, Adams M, Spatial and temporal variations in phloem sap composition of plantation-grown *Eucalyptus globulus*, *Oecologia* 1998;117:312-322.
- [21] Gessler A, Schrempp S, Matzarakis A, Mayer H, Rennenberg H, Adams MA, Radiation modifies the effect of water availability on the carbon isotope composition of beech (*Fagus sylvatica*), *New Phytologist* 2001;150:653-664.
- [22] Cernusak LA, Arthur DJ, Pate JS, Farquhar GD, Water relations link carbon and oxygen isotope discrimination to phloem sap sugar concentration in *Eucalyptus globulus*, *Plant Physiology* 2003;131:1544-1554.
- [23] Devaux M, Ghashghaie J, Bert D, Lambrot C, Gessler A, Bathellier C, Ogee J, Loustau D, Carbon stable isotope ratio of phloem sugars in mature pine trees throughout the growing season: comparison of two extraction methods, *Rapid Communications in Mass Spectrometry* 2009;23:2511-2518.
- [24] Cernusak LA, Pate JS, Farquhar GD, Diurnal variation in the stable isotope composition of water and dry matter in fruiting *Lupinus angustifolius* under field conditions, *Plant Cell and Environment* 2002;25:893-907.
- [25] Gessler A, Rennenberg H, Keitel C, Stable isotope composition of organic compounds transported in the phloem of European beech - Evaluation of different methods of phloem

- sap collection and assessment of gradients in carbon isotope composition during leaf-to-stem transport, *Plant Biology* 2004;6:721-729.
- [26] Gessler A, Brandes E, Keitel C, Boda S, Kayler ZE, Granier A, Barbour M, Farquhar GD, Treydte K, The oxygen isotope enrichment of leaf-exported assimilates - does it always reflect lamina leaf water enrichment?, *New Phytologist* 2013;200:144-157.
- [27] Rennenberg H, Schneider S, Weber P, Analysis of uptake and allocation of nitrogen and sulphur compounds by trees in the field, *Journal of Experimental Botany* 1996;47:1491-1498.
- [28] Letham DS, The separation of plant cells with ethylenediaminetetraacetic acid, *Experimental Cell Research* 1960;21:353-360.
- [29] King RW, Zeevaart JA, Enhancement of phloem exudation from cut petioles by chelating-agents, *Plant Physiology* 1974;53:96-103.
- [30] Groussol J, Delrot S, Caruhel P, Bonnemain JL, Design of an improved exudation method for phloem sap collection and its use for the study of phloem mobility of pesticides, *Physiologie Vegetale* 1986;24:123-133.
- [31] Schmidt HL, Werner RA, Rossmann A, ^{18}O pattern and biosynthesis of natural plant products, *Phytochemistry* 2001;58:9-32.
- [32] Cernusak LA, Wong SC, Farquhar GD, Oxygen isotope composition of phloem sap in relation to leaf water in *Ricinus communis*, *Functional Plant Biology* 2003;30:1059-1070.
- [33] Gessler A, Brandes E, Buchmann N, Helle G, Rennenberg H, Barnard RL, Tracing carbon and oxygen isotope signals from newly assimilated sugars in the leaves to the tree-ring archive, *Plant, Cell & Environment* 2009;32:780-795.
- [34] Rinne KT, Saurer M, Streit K, Siegwolf RTW, Evaluation of a liquid chromatography method for compound-specific d^{13}C analysis of plant carbohydrates in alkaline media, *Rapid Communications in Mass Spectrometry* 2012;26:2173-2185.
- [35] Brand WA, Coplen TB, Stable isotope deltas: tiny, yet robust signatures in nature, *Isotopes in Environmental and Health Studies* 2012;48:393-409.
- [36] Lehmann MM, Rinne KT, Blessing C, Siegwolf RTW, Buchmann N, Werner RA, Malate as a key carbon source of leaf dark-respired CO_2 across different environmental conditions in potato plants, *Journal of Experimental Botany* 2015;66:5769-5781.
- [37] Kornel BE, Gehre M, Hofling R, Werner RA, On-line $\delta^{18}\text{O}$ measurement of organic and inorganic substances, *Rapid Communications in Mass Spectrometry* 1999;13:1685-1693.
- [38] Kornel BE, Werner RA, Gehre M, Standardization for oxygen isotope ratio measurement - Still an unsolved problem, *Rapid Communications in Mass Spectrometry* 1999;13:1248-1251.
- [39] Weigt RB, Braunlich S, Zimmermann L, Saurer M, Grams TE, Dietrich HP, Siegwolf RT, Nikolova PS, Comparison of $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values between tree-ring whole wood and cellulose in five species growing under two different site conditions, *Rapid Communications in Mass Spectrometry* 2015;29:2233-2244.
- [40] Gehre M, Geilmann H, Richter J, Werner RA, Brand WA, Continuous flow $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$ analysis of water samples with dual inlet precision, *Rapid Communications in Mass Spectrometry* 2004;18:2650-2660.

- [41] Brand WA, Coplen TB, Aerts-Bijma AT, Bohlke JK, Gehre M, Geilmann H, Groning M, Jansen HG, Meijer HAJ, Mroczkowski SJ, Qi HP, Soergel K, Stuart-Williams H, Weise SM, Werner RA, Comprehensive inter-laboratory calibration of reference materials for $\delta^{18}\text{O}$ versus VSMOW using various on-line high-temperature conversion techniques, *Rapid Communications in Mass Spectrometry* 2009;23:999-1019.
- [42] R Core Team, R: A language and environment for statistical computing. Version 3.5.1., *R foundation for statistical computing, Vienna, Austria*, <https://www.r-project.org/> 2019
- [43] Stahl PH, in *Feuchtigkeit und Trocknen in der pharmazeutischen Technologie*, Steinkopff, Heidelberg, 1980, pp. 20-59.
- [44] Rittenberg D, Graff C, A comparison of the rate of mutarotation and ^{18}O exchange of glucose, *Journal of the American Chemical Society* 1958;80:3370-3372.
- [45] Vollhardt KPC, Schore NE, *Organic chemistry Structure and function (6th ed.)*, W.H. Freeman and Co., New York: W.H. Freeman and Co., 2011.
- [46] Zech M, Saurer M, Tuthorn M, Rinne K, Werner RA, Siegwolf R, Glaser B, Juchelka D, A novel methodological approach for $\delta^{18}\text{O}$ analysis of sugars using gas chromatography-pyrolysis-isotope ratio mass spectrometry, *Isotopes in Environmental and Health Studies* 2013;49:492-502.
- [47] Sternberg LDSL, DeNiro MJ, Savidge RA, Oxygen isotope exchange between metabolites and water during biochemical reactions leading to cellulose synthesis, *Plant Physiology* 1986;82:423-427.
- [48] Rinne KT, Saurer M, Kirdyanov AV, Bryukhanova MV, Prokushkin AS, Churakova OV, Siegwolf RTW, Examining the response of needle carbohydrates from Siberian larch trees to climate using compound-specific $\delta^{13}\text{C}$ and concentration analyses, *Plant, Cell & Environment* 2015;38:2340-2352.
- [49] Churakova OV, Lehmann MM, Siegwolf RTW, Saurer M, Fonti MV, Schmid L, Timofeeva G, Rinne-Garmston KT, Bigler C, Compound-specific carbon isotope patterns in needles of conifer tree species from the Swiss National Park under recent climate change, *Plant Physiology and Biochemistry* 2019;139:264-272.
- [50] Lehmann MM, Goldsmith GR, Schmid L, Gessler A, Saurer M, Siegwolf RTW, The effect of ^{18}O -labelled water vapour on the oxygen isotope ratio of water and assimilates in plants at high humidity, *New Phytologist* 2018;217:105-116.
- [51] Lehmann MM, Goldsmith GR, Mirande-Ney C, Weigt RB, Schonbeck L, Kahmen A, Gessler A, Siegwolf RTW, Saurer M, The ^{18}O -signal transfer from water vapour to leaf water and assimilates varies among plant species and growth forms, *Plant Cell and Environment* 2020;43:510-523.
- [52] Schneider S, Gessler A, Weber P, vonSengbusch D, Hanemann U, Rennenberg H, Soluble N compounds in trees exposed to high loads of N: A comparison of spruce (*Picea abies*) and beech (*Fagus sylvatica*) grown under field conditions, *New Phytologist* 1996;134:103-114.

Figures

Figure 1: $\delta^{18}\text{O}$ values ($\delta^{18}\text{O}_{\text{VSMOW}}$) of (A) oven-dried and (B) freeze-dried sucrose samples and twig phloem sugars from *Fagus sylvatica*. All samples were dissolved in water with different $\delta^{18}\text{O}$ values (i.e. -30 mUr, -10 mUr, +30 mUr) to test for analytical memory effects. Based on one-way ANOVA and Tukey HSD post-hoc, capitals letters indicate significant differences in $\delta^{18}\text{O}$ values across sugar types, while small letters indicate differences in $\delta^{18}\text{O}$ values of a sugar resulting from water treatments with different $\delta^{18}\text{O}$ values. Bars indicate means $\pm$ 1 SD (n = 4).

Figure 2: $\delta^{18}\text{O}$ values ($\delta^{18}\text{O}_{\text{VSMOW}}$) of purified and non-purified leaf sugars from *Rhododendron* sp. (A) and pure sucrose samples (B, C). All samples were dissolved in water with different $\delta^{18}\text{O}$ values (i.e. -50 mUr, -10 mUr, +30 mUr). Based on one-way ANOVA and Tukey HSD post-hoc, small letters indicate different $\delta^{18}\text{O}$ values (tested by T-test) between non-purified and purified samples and capital letters indicate differences in $\delta^{18}\text{O}$ values of a sugar resulting from water treatments with different $\delta^{18}\text{O}$ values. Bars indicate means $\pm$ 1 SD (n = 4).

Figure 3: $\delta^{18}\text{O}$ values ($\delta^{18}\text{O}_{\text{VSMOW}}$) of non-purified phloem WSC (A) and purified phloem sugars (B). Twig phloem samples of *Fagus sylvatica* var. *pendula* of 5 cm² were immersed in 0 mM (i.e. deionised water), 5 mM, and 20 mM EDTA exudation media for 0.25 h for rinsing. Samples were subsequently transferred to new vials with the same EDTA exudation media for 1 to 5 h. Based on one-way ANOVA and Tukey HSD post-hoc, small letters indicate significant differences in the $\delta^{18}\text{O}$ values of phloem assimilates over time within each EDTA concentration. Bars indicate means $\pm$ 1 SD (n = 4).

Figure 4: Amount of individual phloem sugars per mL of the 0 mM EDTA (i.e. deionised water) exudation solution over 5 h exudation time. Twig phloem samples of *Fagus sylvatica* var. *pendula* of 5 cm² were first rinsed in 0 mM (i.e. deionised water) by immersion for 0.25 h. Samples were subsequently transferred to new vials and immersed in deionised water for 1 to 5 h. Based on one-way ANOVA and Tukey HSD post-hoc, small letters indicate significant differences in the total sum of the individual sugars and sugar alcohol concentrations, which is further indicated by the numbers. Analysis of fructose is biased by peak overlaps with unknown compounds. Mean + 1 SD (n = 4).

Supplementary data

Figure S1: An annotated scheme illustrating extraction and purification of leaf and phloem sugars with minimum effects on sugar $\delta^{18}\text{O}$ values. Used abbreviations: WSC, water soluble compounds; RT, room temperature (approx. 20°C); Py-IRMS, an analytical system that includes a pyrolysis unit coupled to an isotope ratio mass spectrometer.

Figure 1: $\delta^{18}\text{O}$ values ($\delta^{18}\text{OVSMOW}$) of (A) oven-dried and (B) freeze-dried sucrose samples and twig phloem sugars from *Fagus sylvatica*. All samples were dissolved in water with different $\delta^{18}\text{O}$ values (i.e. -30 mUr, -10 mUr, +30 mUr) to test for analytical memory effects. Based on one-way ANOVA and Tukey HSD post-hoc, capitals letters indicate significant differences in $\delta^{18}\text{O}$ values across sugar types, while small letters indicate differences in $\delta^{18}\text{O}$ values of a sugar resulting from water treatments with different $\delta^{18}\text{O}$ values. Bars indicate means $\pm$ 1 SD ($n = 4$).

200x248mm (300 x 300 DPI)

Figure 2: $\delta^{18}\text{O}$ values ($\delta^{18}\text{OVSMOW}$) of purified and non-purified leaf sugars from *Rhododendron* sp. (A) and pure sucrose samples (B, C). All samples were dissolved in water with different $\delta^{18}\text{O}$ values (i.e. -50 mUr, -10 mUr, +30 mUr). Based on one-way ANOVA and Tukey HSD post-hoc, small letters indicate different $\delta^{18}\text{O}$ values (tested by Tt-test) between non-purified and purified samples and capital letters indicate differences in $\delta^{18}\text{O}$ values of a sugar resulting from water treatments with different $\delta^{18}\text{O}$ values. Bars indicate means ± 1 SD (n = 4).

106x248mm (300 x 300 DPI)

Figure 3: δ¹⁸O values (δ¹⁸O_{VSMOW}) of non-purified phloem WSC (A) and purified phloem sugars (B). Twig phloem samples of *Fagus sylvatica* var. *pendula* of 5 cm² were immersed in 0 mM (i.e. deionised water), 5 mM, and 20 mM EDTA exudation media for 0.25 h for rinsing. Samples were subsequently transferred to new vials with the same EDTA exudation media for 1 to 5 h. Based on one-way ANOVA and Tukey HSD post-hoc, small letters indicate significant differences in the δ¹⁸O values of phloem assimilates over time within each EDTA concentration. Bars indicate means ± 1 SD (n = 4).

241x241mm (300 x 300 DPI)

Figure 4: Amount of individual phloem sugars per ml of the 0 mM EDTA (i.e. deionised water) exudation solution over 5 h exudation time. Twig phloem samples of *Fagus sylvatica* var. *pendula* of 5 cm² were first rinsed in 0 mM (i.e. deionised water) by immersion for 0.25 h. Samples were subsequently transferred to new vials and immersed in deionised water for 1 to 5 h. Based on one-way ANOVA and Tukey HSD post-hoc, small letters indicate significant differences in the total sum of the individual sugars and sugar alcohol concentrations, which is further indicated by the numbers. Analysis of fructose is biased by peak overlaps with unknown compounds. Mean + 1 SD (n = 4).

129x114mm (300 x 300 DPI)