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Technical note: On uncertainties in plant water isotopic composition following extraction by cryogenic vacuum distillation

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Abstract. Recent studies have challenged the interpretation of plant water isotopes obtained through cryogenic vacuum distillation (CVD) based on observations of large ^{2}H-fractionations. These studies have hypothesized the existence of a H-atom exchange between water and organic tissue during CVD extraction with the magnitude of H-exchange related to relative water content of the sample; however, clear evidence is lacking. Here, we systematically tested the uncertainties in the isotopic composition of CVD-extracted water by conducting a series of incubation and rehydration experiments using isotopically depleted water, water at natural isotopic abundance, and organic materials with and without exchangeable H. We show that the offsets between hydrogen and oxygen isotope ratios and expected reference values (Δ^{2}H and Δ^{18}O) have inversely proportional relationships with the absolute amount of water being extracted, i.e., the lower the water amount, the higher the Δ^{2}H and Δ^{18}O. However, neither Δ^{2}H, nor Δ^{18}O, values were related to sample relative water content. The Δ^{2}H pattern was more pronounced for materials with exchangeable H-atoms than with non-exchangeable H-atoms. This is caused by the combined effect of H-exchange during the incubation and evaporation and sublimation enrichments that depend on absolute water amount. The H-exchange during CVD extraction itself was negligible. Despite these technical issues, we observed that the water amount-dependent patterns were much less pronounced for samples at natural isotope abundance and particularly low when sufficiently high amounts of water were extracted (> 600 µl). Our study provides new insights into the mechanisms causing isotope fractionation during CVD extraction of water. The methodological uncertainties can be controlled if large samples of natural isotope abundance are used in ecohydrological research studies.

1 Introduction

Hydrogen and oxygen isotope ratios (δ^{2}H and δ^{18}O) in plant water are powerful tools for tracing the water movement of ecosystems and providing information on the source water used by plants (Goldsmith et al., 2017; Flanagan and Ehleringer, 1991; Brinkmann et al., 2019; Nehemy et al., 2019). The most widely used approach for determining the δ^{2}H and δ^{18}O of plant water is by first extracting water from plants using cryogenic vacuum distillation (CVD) method (Ehleringer et al., 2000; West

https://doi.org/10.5194/hess-2022-178
Preprint. Discussion started: 9 May 2022

et al., 2006; Ingraham and Shadel, 1992; Orlowski et al., 2013). Since no isotopic fractionation generally occurs during plant water uptake (White et al., 1985; Zimmermann et al., 1967; Poca et al., 2019), it is generally believed that the isotope ratios in stem water (root crown water for grasses) derived from the CVD extraction (δ_{CVD}) are equal to that in the plant source water (δ_{source}; i.e., soil water). However, an increasing number of studies have found that δ_{CVD}, especially δ^2H_{CVD}, does not faithfully reflect plant source water, as shown by a strong negative δ^2H offset ($\Delta^2H = \delta^2H_{CVD} - \delta^2H_{source}$) between CVD extracted water and source water (Lin and Da S. L. Sternberg, 1993; Zhao et al., 2016; Barbeta et al., 2020; Allen and Kirchner, 2022; Newberry et al., 2017). This issue is important because it may invalidate conclusions on plant water uptake patterns and the partitioning of different pools of water in an ecosystem (Allen et al., 2019; Gessler et al., 2022; Goldsmith et al., 2012; Evaristo and Mcdonnell, 2017).

A critical hypothesis for the observed Δ^2H is the spatial heterogeneity of hydrogen isotope composition of water in stems (Zhao et al., 2016; Barbeta et al., 2020), i.e., isotopic differences between stem conductive and nonconductive tissues and isotopic differences from inside to outside along the stem radial direction. This hypothesis was rejected by Chen et al. (2020). The authors rehydrated dry stem samples of saplings of nine plant species in excess of a reference water with a known isotopic composition for 24 hours and then CVD-extracted the water that had soaked into the material. They concluded that the observed negative Δ^2H was not caused by within-stem isotope heterogeneity, but was more likely related to a hydrogen atom (H)-exchange between stem water and the exchangeable H of stem organic tissue that occurs during the CVD extraction. The large majority of exchangeable H reflects the O-bound H atoms in woody tissue, which can exchange with H atoms with the surrounding liquid water and water vapor. Theoretically, about 30 to 50% of the H-atoms of cellulose and its precursors are exchangeable (Schuler et al., 2022; Filot et al., 2006). If the CVD extraction disturbs the previously established isotopic equilibration between organic tissue and tissue water because of the gradually isotopically enriched tissue water during extraction, the potential H-exchange would affect ecohydrological interpretations of Δ^2H (Barbeta et al., 2020; De La Casa et al., 2021). However, Chen et al. (2020) were unable to isolate the H-exchange that occurred exactly during the extraction. This is because their experimental design included two possible H-exchange steps: one that occurs during rehydration (hereinafter referred to as “H-exchange during rehydration”) and a second one that occurs during the extraction itself (hereinafter referred to as “H-exchange during extraction”). Only the latter is the H-exchange of interest, because it is the H-exchange process that theoretically affects the isotopic composition of CVD extracted water from actual plant samples. However, empirical evidence for the occurrence of H-exchange and its effects on δ^2H_{CVD} during rehydration and/or extraction is still lacking.

Moreover, the study of Chen et al. (2020) also found a significant positive correlation between Δ^2H and sample relative water content (RWC). They posited that the H-exchange effect was probably dampened at high RWC. This correlation has also been found for both Δ^2H and $\Delta^{18}O$ (i.e., $\delta^{18}O_{CVD} - \delta^{18}O_{source}$) in soil samples (Wen et al., 2021). However, using cellulose in wood as an example, much of the O-bound H is engaged in H-bonding that link cellulose fibrils together, i.e., the non-freely exchangeable “bridging hydrogen” (Sepall and Mason, 1961; Meier-Augenstein et al., 2014), which requires high temperatures

https://doi.org/10.5194/hess-2022-178
Preprint. Discussion started: 9 May 2022

(> 100 °C) for accessing (Schuler et al., 2022). As such, the freely exchangeable H in cellulose is theoretically only about 5% (Sepall and Mason, 1961; Meier-Augenstein et al., 2014). If the magnitude of H-exchange effect during extraction is therefore moderate, the observed Δ^2H would be more readily attributed to isotopic fractionation and mixing that occur after the water is extracted from the sample. If this were to be true, what really matters would not be the water amount relative to the sample amount (i.e., RWC), but rather the absolute amount of water being extracted (hereinafter referred to "absolute water amount (AWA)"). The effects of AWA on extraction uncertainties could influence both Δ^2H and Δ^{18}O through fractionation during CVD extraction. However, no studies have tested the effect of sample water amount on the CVD extraction biases yet and the CVD extraction uncertainties during liquid-vapor phase changes are not well known.

During the CVD extraction, successive isotope fractionation processes occur at each phase change of the water (liquid-vapour-solid-liquid). When liquid sample water is distilled to water vapour, isotope fractionations are expected if incomplete extraction occurs (Orlowski et al., 2013; West et al., 2006). When the water vapour is trapped by liquid nitrogen and turns into solid ice, a sublimation isotope fractionation is also expected. Studies testing the sublimation of pure water ice at low temperatures into a vacuum have shown that the sublimated percentage of the bulk water aliquot was < 10% within 1 h at -100°C and the sublimation rate decreased drastically with the decrease of temperature in the cold trap (Mortimer et al., 2018; Lécuyer et al., 2017). Given the fact that the extracted water is frozen at such a low temperature (-196°C) during CVD extraction, negligible amounts of water should be released into the vapour phase by sublimation during the extraction. Nevertheless, an observable enrichment of the residual ice is still possible as the water lost to the vapour phase is isotopically depleted (Lécuyer et al., 2017; Mortimer et al., 2018). In addition, isotope fractionations related to evaporation and mixing with water vapour in the laboratory probably occur during the end of the CVD extraction (i.e., mixing of the extracted water with air humidity of the lab air when the water traps are removed from the extraction system). Although this can be partly avoided by flushing the extraction line with N_2 gas, isotope fractionations can occur when the extracted and frozen water thaws in the water collection tube and when it is transferred to storage vials. A better understanding of these individual isotope fractionation processes during CVD extraction would not only aid in identifying the potential sources of methodological biases (Schoppach and Klaus, 2019) and improving the instrumentation, but also contribute to better estimates of plant water sources by mitigating their effects.

To investigate these open questions, we systematically tested for biases introduced by CVD extraction of plant water. A series of experiments that involved extracting water from different kinds of materials (with and without exchangeable H) that were incubated or rehydrated using isotopically depleted water and from plant samples at natural isotope abundance were conducted. Specifically, we hypothesized that the Δ^2H and Δ^{18}O are influenced by (i) an H-exchange effect, (ii) tissue AWA and (iii) evaporation and sublimation enrichments. The lower the AWA, the higher the H-exchange effect and evaporation and sublimation fractionations, and the higher the Δ^2H and Δ^{18}O.

Hydrology and Earth System Sciences
Discussions

2. Materials and Methods

2.1 Experiment 1: Testing the overall H-exchange effect

For evaluating the overall effect of H-exchange during both rehydration and extraction on the Δ^2H and Δ^{18}O values of CVD extracted water, we conducted an incubation experiment by adding different amounts of strongly depleted reference water (δ^2H: ca. -460‰; δ^{18}O: ca. -170‰; hereafter referred to as δ_{ref}) to the same amount of different dried materials with and without exchangeable H (Experiment 1, Fig. S1). The isotopically depleted water was used to make potential isotope effects more evident.

The materials with exchangeable H included stem pieces, stem powder, purified stem cellulose powder and twig pieces. The stem materials were obtained from the xylem of a trunk disc of a mature *Larix sibirica* grown in Siberia (27 cm diameter, 4 cm thickness). Stem pieces were prepared by cutting the xylem (a mixture of sap wood and heart wood) of the disc into 4 mm cubes, while the stem powder was prepared by grinding the same material to a homogeneous powder using a steel-ball mill (MM400, Retsch GmbH, Haan, Germany). The stem cellulose was then extracted from ground stem powder (Schuler et al., 2022). The twig pieces were obtained from five young *Larix decidua* trees growing in a forest in Birmensdorf, Switzerland (47.36°N, 8.45°E). Two twigs per tree were collected in the morning, outer bark and phloem removed, and the twig xylem cut into 2 mm pieces.

The materials without exchangeable H were cellulose triacetate and caffeine. The cellulose triacetate ($C_{40}H_{54}O_{27}$; Sigma-Aldrich, St. Louis, MO, USA, Prod. No. 22199) is a white granule. The caffeine material is ≥99% caffeine anhydrous ($C_8H_{10}N_4O_2$; Fluka Chemie AG, Buchs, Switzerland, Prod. No. 27600) in a white crystalline powder form. All H-atoms in cellulose triacetate and caffeine are bound to carbon in a form of methyl group ($-CH_3$) and thus non-exchangeable with vapour or liquid water.

For each material and each different water amount (50, 100, 200, 400, 600, 800 and 1200 µl), 3 replicates were prepared by transferring 200 mg of each material into 12 ml gas-tight glass vials (Exetainer; Labco, Lampeter, UK). Before injecting the reference water into the vials, the materials were oven dried at 60°C for 24 h to remove any remaining moisture in the material. Depending on the form of the material, the low water amounts (e.g., 50 and 100 µl) could not fully wet the material, whereas the high water amounts (e.g., > 400 µl) oversaturated the material. Then, the vials were sealed and the materials were incubated for 24 h at room temperature. According to Chen et al. (2020), this incubation time is thought to lead to a full exchange of H-atoms between materials and reference water. As a control, the experiment was repeated without any material by adding only the range of reference water into the vial. All samples were frozen at -20°C and then extracted with CVD. Δ was calculated as δ_{CVD} - δ_{ref}.

2.2 Experiment 2: Testing the effects of the absolute water amount and relative water content

For separating the H-exchange effects on Δ^2H and Δ^{18}O values occurring during rehydration from those occurring during

https://doi.org/10.5194/hess-2022-178
Preprint. Discussion started: 9 May 2022

extraction, we extracted water from a range of rehydrated stem segments with different sizes (Experiment 2, Fig. S1).

Large stem segments (10×2.5×0.3 cm) were cut from the *L. sibirica* disc (see 2.1) and oven-dried at 60°C for 24 h before the experiment. We firstly determined the saturated water content of the large stem segments by weighing them before and after they were soaked in excess of deionized water for 24 h. The saturated water content was determined for estimating the AWA of the following smaller stem segment samples. The large stem segments were then oven-dried again at 60°C for 24 h and cut into smaller stem segments with different sizes, aiming to generate a range of samples with a narrow RWC (ca. 40%), but varying in their AWA from 50–1200 µl. The sizes of the small segments were determined based on the proportional relationship between the size and the saturated water content of the large stem segments. Three replicates were produced for each size. Then, the small stem segments were separately soaked in excess of the isotopic depleted reference water (ca. 25 ml) in sealed glass vials at room temperature for 24 h, following Chen et al. (2020). During the 24 h rehydration, the isotope ratios of the original reference water equilibrated with the exchangeable H in the small stem segments. Thus, by the end of the rehydration, the isotope ratios of water in the small stem segments are assumed equal to the isotope ratio of the reference water after rehydration ($\delta_{\text{ref after rehyd}}$) and not to be equal to the original reference water (δ_{ref}). Subsequently, the small stem segments were taken out of the reference water, the surface water shaken off, and then immediately sealed in vials and frozen at -20°C to prevent evaporation. A sample of the reference water after the rehydration was taken from each tube for determining $\delta_{\text{ref after rehyd}}$. The AWA and the RWC of each small stem segment sample were calculated by comparing the sample weights before and after the extraction. Here, the RWC is defined as (wet weight - dry weight) / wet weight, its unit is parts per hundred (%). In experiment 2, Δ was calculated as δ_{CVD} -$\delta_{\text{ref after rehyd}}$. This is because it represents the changes in isotope ratios of the water that occurred during the CVD extraction alone.

2.3 Experiment 3: Testing sublimation and evaporation effects

For evaluating the potential effects of sublimation and evaporation during CVD extraction on the isotope ratios of the extracted water, different amounts of reference water (50–1200 µl range as above) were added directly into the U-shaped water collection tubes (i.e., in the cold trap) of the CVD extraction system without sample material and then extracted following a standard procedure (see 2.5). The water in the collection tubes was first frozen by the liquid nitrogen cold trap before the extraction started. The experimental design allows us to exclude isotope fractionations related to distillation and condensation occurring when water in the sample vial was relocated to the collection tube (i.e., U-tube), but captures isotope fractionations related to sublimation of the frozen water under the vacuum and evaporation during thawing of water after CVD extraction (Experiment 3, Fig S1). Δ was calculated as δ_{CVD} - δ_{ref}.

For isolating the evaporation effect from the sublimation effect, we also added different amounts of reference water (50–1200 µl range as above) into 2 ml glass vials inside a climate chamber (PGR15, Conviron, Manitoba, Canada). The samples were then left to evaporate with lids open at 25°C and 50 % relative humidity (RH) for 2 h. The samples in the 2 ml glass vials did not require the CVD extraction. The exposed surface area of the water in the 2 ml glass vials was about 64 mm^2. Three replicates per water amount were performed for both tests (Experiment 3, Fig S1). Notably, CVD extraction was not used for the evaporation test in the climate chamber, thus the Δ was calculated as the difference between the isotope ratios of the reference water after and before the evaporation (i.e., $\delta_{\text{ref after evap}}$ - δ_{ref}).

2.4 Experiment 4: Extraction tests using samples at isotope natural abundance

In addition, we performed tests with tap water and fresh *L. decidua* twigs with natural isotope abundance to compare with results derived from tests with isotopically depleted reference water. For the tap water (δ_{tap}; δ^2H: -73.91‰, δ^{18}O: -14.43‰), the different amounts of water (i.e., 50, 100, 200, 400, 600, 800 and 1200 µl) were added into the vials and kept frozen at -20°C until CVD extraction (Experiment 4, Fig. S1). The twigs were collected from five young *L. decidua* trees growing in a forest in Birmensdorf, Switzerland (see 2.1). The twig xylem was cut to different lengths (1, 2, 3, 4, 5, 6, 7 and 8 cm) and sealed in vials. The AWA and RWC of each twig sample were determined by weighing before and after CVD extraction. Three replicates were performed per water amount and twig length (Experiment 4, Fig. S1). For the extraction with tap water, the Δ was calculated as δ_{CVD} - δ_{tap}. The isotope ratios of the source water of the *L. decidua* twigs before extraction were unknown, thus the Δ in this case was calculated as the difference between the δ_{CVD} and the average value of δ_{CVD} (i.e., δ_{CVD} - $\delta_{\text{CVD\_ave}}$).

2.5 Water extraction and stable isotope analysis

We extracted water using a CVD setup similar to the one described by Orlowski et al. (2013). A schematic overview of this setup is shown in Fig. S2. During extraction, the pressure inside the system was maintained below 0.05 mbar using a vacuum pump. The samples in the sample tubes were heated in 80°C water and the extracted water was condensed and trapped in the collection tubes by liquid nitrogen. The extraction was maintained for 2 h to achieve a complete extraction (West et al., 2006). After the extraction, the vacuum inside the system was released by adding dry nitrogen gas until atmospheric pressure conditions were re-established. Then the collection tubes with frozen water samples were detached from the system and sealed with rubber plugs. The water in the collection tubes were thawed at room temperature. During this process, the evaporated water vapour usually condensed to form very small water droplets on the inside walls of the collection tube. We consolidated and collected as many of these small water droplets as possible and transferred them into glass vials (350 µl or 2 ml, depending on the extracted water amount; Infochroma AG, Goldau, Switzerland) using a pipette. Syringes and 0.45 µm Nylon filters were used if the extracted water appeared to be turbid. The samples were stored at -20°C before and after the extraction.

The δ^2H and δ^{18}O of water samples were measured with a high temperature conversion elemental analyser coupled to a Delta$^{\text{Plus}}$ XP isotope ratio mass spectrometer (TC/EA-IRMS; Finnigan MAT, Bermen, Germany). The δ^2H and δ^{18}O in the materials without exchangeable H (i.e., cellulose triacetate and caffeine) and δ^{18}O in the bulk organic matter of the materials with exchangeable H were measured with a vario PYRO cube (Elementar Analysensysteme GmbH, Langenselbold, Germany)

https://doi.org/10.5194/hess-2022-178
Preprint. Discussion started: 9 May 2022

Hydrology and Earth System Sciences
Discussions

coupled to a Delta^{Plus} XP IRMS. Isotope ratios are reported in per mille (‰) relative to Vienna Standard Mean Ocean Water (VSMOW). Calibration versus the international standards was achieved by analysis of a range of certified water of different isotope ratios, resulting in a precision of analyses of 2 ‰ for δ^2H and 0.3 ‰ for δ^{18}O. The non-exchangeable δ^2H (δ^2H_{ne}) of the materials with exchangeable H was determined by pre-treating the materials with a high-temperature water vapour equilibration method according to Schuler et al. (2022). The δ^2H and δ^{18}O values of the materials used in the experiments are shown in Table S1.

2.6 Statistical analyses

To determine if the sample water δ^2H and δ^{18}O values were significantly different before and after the CVD extraction, we performed one-sample t-tests for Δ^2H and Δ^{18}O values to test if they were significantly different from 0. We fit relationships between Δ^2H and Δ^{18}O as a function of AWA using inversely proportional models and the relationships between Δ^2H and Δ^{18}O as a function of RWC using linear models for the best description of the relationships. All statistics were performed using R version 4.0.4 (R Core Team, 2021).

3. Results and Discussion

3.1 H-exchange occurs between sample exchangeable H and water during rehydration

Experiment 1 revealed an inversely proportional relationship between the AWA and the Δ^2H and Δ^{18}O values of the water derived from material with or without exchangeable H (Fig. 1). In other words, the lower the AWA, the higher the Δ^2H and Δ^{18}O values. Noticeably, Δ^2H of the water derived from materials with exchangeable H showed a more pronounced pattern, which reached about 150‰ at 50 µl AWA, compared to those without exchangeable H (Fig. 1a). In contrast, the Δ^2H pattern of the water derived from materials without exchangeable H was similar to that of the pure reference water, which reached about 30‰ at 50 µl AWA (Fig. 1b). Also, Δ^{18}O values of materials with and without exchangeable H showed a decrease from 36‰ at 50 µl to 4.7‰ at 1200 µl (Fig. 1c and d).

The results of experiment 1 suggest that the H-exchange during rehydration is mainly responsible for the large Δ^2H differences between materials with and without exchangeable H at the low water amounts. This can likely be explained by an isotope mass balance between the exchangeable H in the samples (natural isotope abundance) and H atoms in the reference water (isotopically depleted), with the impact of the exchangeable H of a constant sample size (200 mg) becoming less noticeable as the amount of water increases (from 50 to 1200 µl). Our results thus provide empirical evidence that H-exchange occurs during rehydration of material and that this artefact must be considered in rehydration experiments (Chen et al., 2020; Zhao et al., 2022).

Moreover, the Δ^2H of the stem and twig pieces were higher than that of the stem and stem cellulose powder when AWA > 600 µl (Fig. 1a), whereas no differences were observed for Δ^{18}O (Fig. 1c). At a first glance, this is unexpected because woody pieces should be less prone to H-exchange due to lower surface area and because the OH-groups are locked in the matrix of the woody structure (Sepall and Mason, 1961). A possible explanation for the observed Δ^2H discrepancies might be an isotopic difference in the exchangeable H of pieces and powdered samples before the start of the experiment. However, if this would be true, the effect should be more visible at lower water amounts rather than higher water amounts, as observed in this study. We therefore can conclude that the sample matrix may also influence H-exchange in rehydration experiments, but that the underlying mechanisms remain speculative.

Unexpectedly, Δ^2H values of the pure reference water and of material without exchangeable H, as well as Δ^{18}O values, all show a dependency on AWA (Fig. 1b–d). This observation indicates that Δ values can be biased even when no exchangeable H in plant material is available and suggests that other factors than H-exchange play an important role in biasing isotopic results of CVD-extracted water.

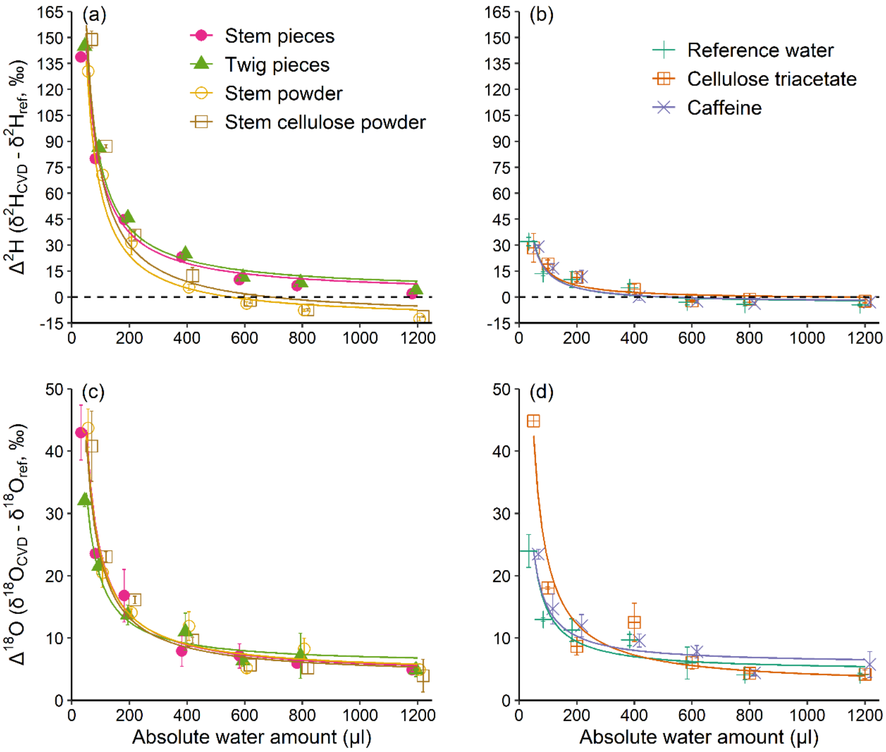


Figure 1: Absolute water amount effects on Δ^2H and Δ^{18}O values (top and bottom panels, respectively) of water derived from materials with and without exchangeable H (left and right panels, respectively) of a constant weight (200 mg). Material with exchangeable H differ in their structural composition (pieces vs. powder). "Reference water" reflects a control performed with water but without any material. $\Delta = \delta_{CVD} - \delta_{ref}$ (see 2.1). The dashed lines represent $\Delta = 0$. The curves are inversely proportional fits. Mean values ± 1 SD are shown (n = 3).

3.2 Absolute water amount not relative water content cause isotope fractionation during CVD extraction

To better understand the unknown isotopic effects during extraction, we performed experiment 2, where we separated the

https://doi.org/10.5194/hess-2022-178
Preprint. Discussion started: 9 May 2022

effects between rehydration and extraction on CVD extracted water along a similar AWA gradient (50–1600 µl), but in a narrow RWC range (36–45%). The experiment confirms results of experiment 1 (Fig. 1b and d), showing that Δ values of water extracted from stem material that have been previously rehydrated change along a similar inversely proportional pattern along an AWA gradient (Fig. 2a and c). Given that we excluded rehydration as a potential effect in experiment 2, but that we still observed isotopic effects on CVD extracted water H (similar to experiment 1 for material without exchangeable H), we conclude that the remaining Δ^2H and Δ^{18}O pattern is caused by additional isotope fractionations rather than an H-exchange effect with material during extraction.

This finding is in contrast to the conclusions made by Chen et al. (2020), stating that stem water CVD extraction error could originate from a dynamic H exchange between sample and water during extraction. The reason for the lack of H-exchange effect during the 2 h CVD extraction itself is probably because the duration for the H-exchange was too short to fully exchange. Previous research shows that a large portion of the water in the sample is expected to be extracted within the first 30 min of extraction (Orlowski et al., 2013; West et al., 2006). Thus, there may not be a strong H-exchange occurring between the water and sample tissue, especially when a large portion of exchangeable H atoms are not freely accessible at the extraction temperature of 80°C (Sepall and Mason, 1961).

Moreover, we found an increasing linear trend in Δ^2H with RWC ($r^2 = 0.28$, $p = 0.02$, Fig. 2b), but not for Δ^{18}O ($P = 0.08$, Fig. 2d). The trend in Δ^2H can be explained by the fact that samples with a smaller AWA (< 400 µl) had a ca. 2 % higher RWC compared to those with a higher AWA (> 400 µl). This indicates that the Δ^2H and Δ^{18}O values are dependent on AWA rather than on RWC of the sample. It should be noted that the data in Fig. 2 were visually separated with 400 µl AWA, the resulting data groups have no statistical meaning, a linear mixed regression was therefore not performed. Our result is not consistent with Chen et al. (2020), who found a significant positive correlation of stem RWC with the Δ^2H in a rehydration experiment and further recommended determining sample RWC for correcting CVD artefacts. However, our results are well supported by the recent study of Zhao et al. (2022), who CVD-extracted water from rehydrated samples of 12 woody plant species and analysed Δ values. The RWC of their samples ranged from 30–60 % and no significant relationships were found between both Δ^2H and Δ^{18}O and RWC. Therefore, the relatively smaller sample RWC range in our study (ca. 10%) compared to that in Chen et al. (2020), i.e., ca. 20% for most samples, could not be the reason for the inconsistency of the two studies. Further, given that AWA is typically not reported, we could not test whether the AWA or RWC caused the effect in previous studies. We highlight that the AWA rather than the RWC of a sample should be considered to potentially correct for Δ offsets and that future studies should report AWA in order to quantify the CVD-introduced isotopic biases across laboratories.

https://doi.org/10.5194/hess-2022-178
Preprint. Discussion started: 9 May 2022

Hydrology and Earth System Sciences
Discussions

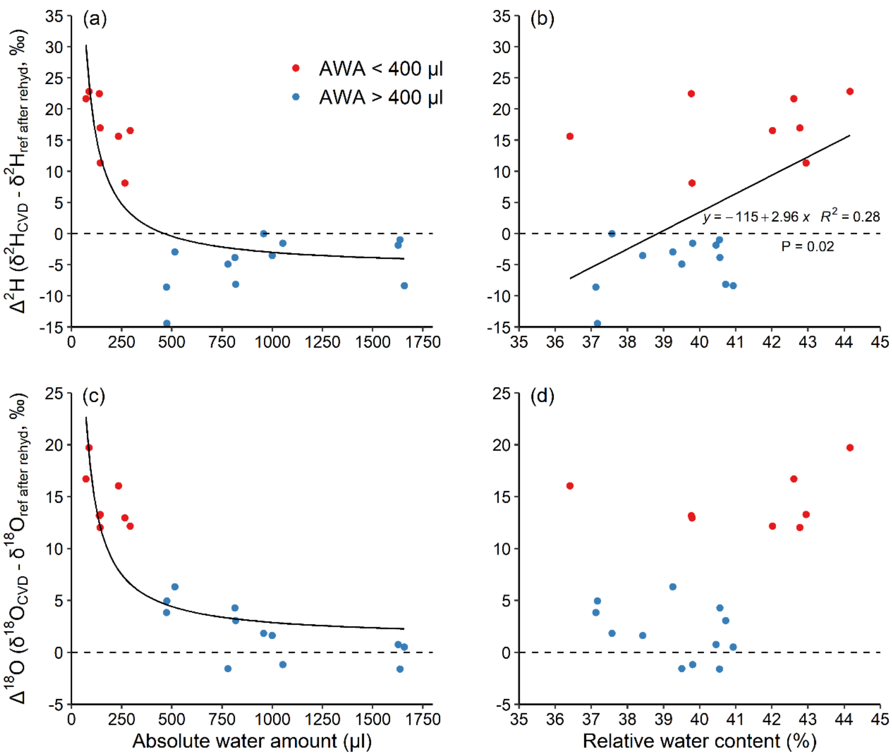


Figure 2: Effects of absolute water amount and relative water content (left and right panels, respectively) on Δ^2H and $\Delta^{18}O$ of water derived from the stem segments (top and bottom panels, respectively). The stem segments were rehydrated in excess of the reference water followed by a CVD extraction. $\Delta = \delta_{CVD} - \delta_{ref\ after\ rehyd}$ (see 2.2). The relationship between AWA and Δ was fit with an inversely proportional function; the relationship between RWC and Δ was fit with a linear regression. The dashed lines represent Δ = 0. Different colours were used to visually separate data with water amount < 400 µl (red) and > 400 µl (blue).

3.3 On the processes causing isotope fractionations during CVD extraction

In order to better understand the processes leading to isotope fractionation during CVD, we performed additional experiments. Interestingly, experiment 3 shows that both Δ^2H and $\Delta^{18}O$ values of reference water, that was directly transferred to the cold trap (i.e., U-tubes), also followed the inversely proportional pattern as a function of AWA (Fig. 3a). At the lowest AWA, Δ^2H reached about 19‰, while $\Delta^{18}O$ reached about 9‰. For AWA > 600 µl, the average Δ^2H and $\Delta^{18}O$ were -1.5‰ and -2.3‰, respectively and both were significantly different from 0 (t = -3.13 and -3.34, df = 8, $p < 0.01$). We think the negative Δ^2H and $\Delta^{18}O$ values for the AWA > 600 µl were purely caused by the analytical uncertainty, because no incomplete extraction could occur given that the reference water was added directly into the collection tube. The same inversely proportional pattern was observed when water was evaporated under controlled conditions in a climate chamber, whereby Δ^2H reached about 46‰ and $\Delta^{18}O$ reached about 23‰ at the lowest AWA. For AWA > 600 µl, average Δ^2H was about 3‰ and significantly different from 0 (t = 12.15, df = 8, $p < 0.01$). No significant difference from 0 was found for $\Delta^{18}O$ (t = 0.23, df = 8, $p = 0.8$). By

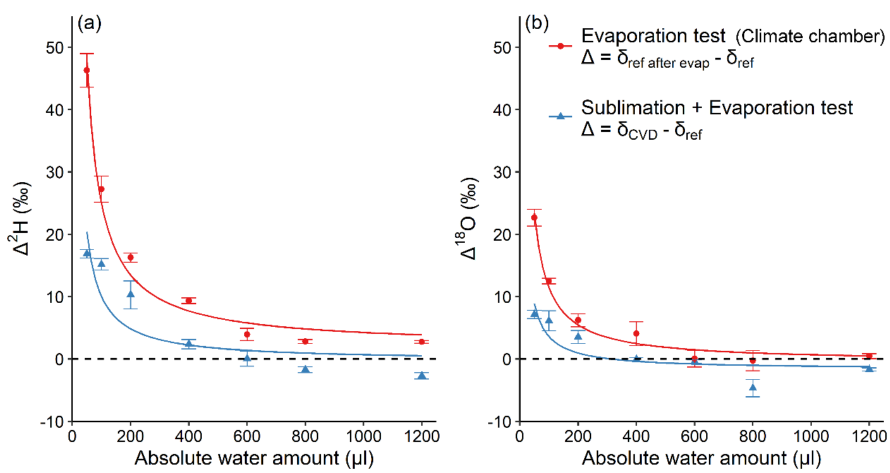
https://doi.org/10.5194/hess-2022-178
Preprint. Discussion started: 9 May 2022

Hydrology and Earth System Sciences
Open Access
Discussions

comparing these results with the one of 50 μl of reference water that was extracted from the sample tube (Fig. 1b), the sublimation and evaporation isotope fractionations account for 59 % and 38 % of the observed Δ^2H and $\Delta^{18}O$, respectively. The unaccounted isotope fractionation may originate from distillation and condensation, when water in the sample tube was relocated to the water collection tube.

Two potential reasons can explain this water amount-dependent pattern of Δ. On the one hand, a bi-directional exchange between water droplets and water vapour may lead to an isotopic equilibration between the two sources, similar to observations at the leaf level (Lehmann et al., 2020; Goldsmith et al., 2017). This process is expected to occur when the extracted water drop was thawing inside the water collection tube after extraction. The equilibration effect was presumably large on our extracted water, because of a high isotopic discrepancy between the water vapour of the laboratory (at natural isotope abundance) and the isotopically depleted reference water. The effect is expected to increase as the water pool size decreases, because the smaller the water droplets, the larger the ratio of water vapour volume to water droplets volume in the water collection tube. Therefore, isotope ratios of smaller water amounts were subject to greater influence of the isotopic composition of the laboratory water vapour. On the other hand, the sublimation, evaporation and/or exchange with surrounding water vapour occurs at the surface of water drops (Stewart, 1975). The smaller the water drop, the larger the ratio of the enriched surface water to the total water drop volume. Thus, the mixing effect of enriched surface water and the rest of the water body would be greater for smaller water drops.

Taken together, our results clearly show that the inversely proportional pattern of Δ along AWA is partly influenced by the isotope fractionations during water sublimation and evaporation during CVD extraction. We therefore conclude that isotope fractionations rather than H-exchange effects with plant material during extraction itself should be considered as factors during CVD extraction of plant water. We also want to highlight that the observed isotope fractionations likely depend on the setup of the CVD extraction system and further laboratory comparisons for plant water extraction are urgently needed for constraining this extraction bias.



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Preprint. Discussion started: 9 May 2022

Figure 3: Absolute water amount effects on Δ^2H (a) and $\Delta^{18}O$ (b) of the reference water that was directly added into the water collection tube before CVD extraction (blue), or evaporated in a climate chamber (red). For the former, $\Delta = \delta_{CVD} - \delta_{ref}$; for the latter, $\Delta = \delta_{ref\ after\ evap} - \delta_{ref}$ (see 2.3). The dashed lines represent $\Delta = 0$. The curves are inversely proportional fits. Mean values ± 1 SD are shown (n = 3).

3.4 Relevance for isotope fractionations during CVD extraction for samples at natural isotope abundance

The large isotopic discrepancy between the strongly depleted reference water and the plant tissue organic matter allowed us to investigate the processes and mechanisms driving isotope fractionation during CVD extraction. However, plant water at natural isotope abundance is typically more enriched compared to our reference water (δ^2H: ca. -460‰; $\delta^{18}O$: ca. -170‰) and therefore the observed isotopic effects of this study are likely less pronounced at natural isotope abundance. This was true, as shown by the results of experiment 4; the inversely proportional pattern was less pronounced or absent with plant samples (average values in δ^2H and $\delta^{18}O$ of extracted water: -58.16‰ and -7.84‰, respectively), or when tap water (δ^2H: -73.91‰; $\delta^{18}O$: -14.43‰) was used (Fig. 4). The Δ^2H showed an overall variation between approximately -7‰ and 10‰, while $\Delta^{18}O$ showed an overall variation between approximately -1‰ and 2‰. These values are much smaller compared to those obtained from water of stem segments rehydrated by the strongly depleted reference water (Fig. 2) and the pure reference water extraction (Fig. 1), which showed an increase to 30‰ at the lowest AWA. We also found that for both fresh *L. decidua* twigs and pure tap water extractions, the water amount dependent pattern was evident for $\Delta^{18}O$, but not for Δ^2H (Fig. 4a and c).

These results indicate that the magnitude of the Δ pattern derived from the CVD extraction is dependent on isotope ratios of plant water being extracted. However, water evaporation tests using water with different isotope ratios (δ^2H range: -14.7‰ to -57.5‰; $\delta^{18}O$ range: 3.4‰ to -7.8‰) conducted by Hu et al. (2009) showed that the δ^2H and $\delta^{18}O$ values of the initial water have little influence on water evaporation isotope fractionation. In other words, for water with different initial isotope ratios evaporating in the same conditions, the changes in δ^2H or $\delta^{18}O$ would be almost the same. Therefore, the initial water isotope ratios would not be the reason for the significant Δ difference between the reference water and the tap water extraction.

As discussed earlier, the sample water can exchange with the water vapour in the laboratory. It is therefore possible that the isotope ratios of the laboratory water vapour are closer to that of the tap water and twig water, but differ greatly compared to the depleted reference water. Therefore, the influence of laboratory water vapour on the sample water with natural isotopic abundance did not appear significantly through the Δ values, although may be hidden in the relatively large variability. Moreover, our results also suggest that the isotope effects related to AWA observed in laboratory experiments using strongly depleted water may be minor under natural conditions. In addition, we did not find a significant correlation between RWC and Δ^2H or $\Delta^{18}O$ for the *L. decidua* twigs at a RWC range between 45% and 57% (Fig. 4b and d). These results suggest again that the Δ^2H and $\Delta^{18}O$ of the CVD extracted water is dependent on AWA rather than on RWC.

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Preprint. Discussion started: 9 May 2022

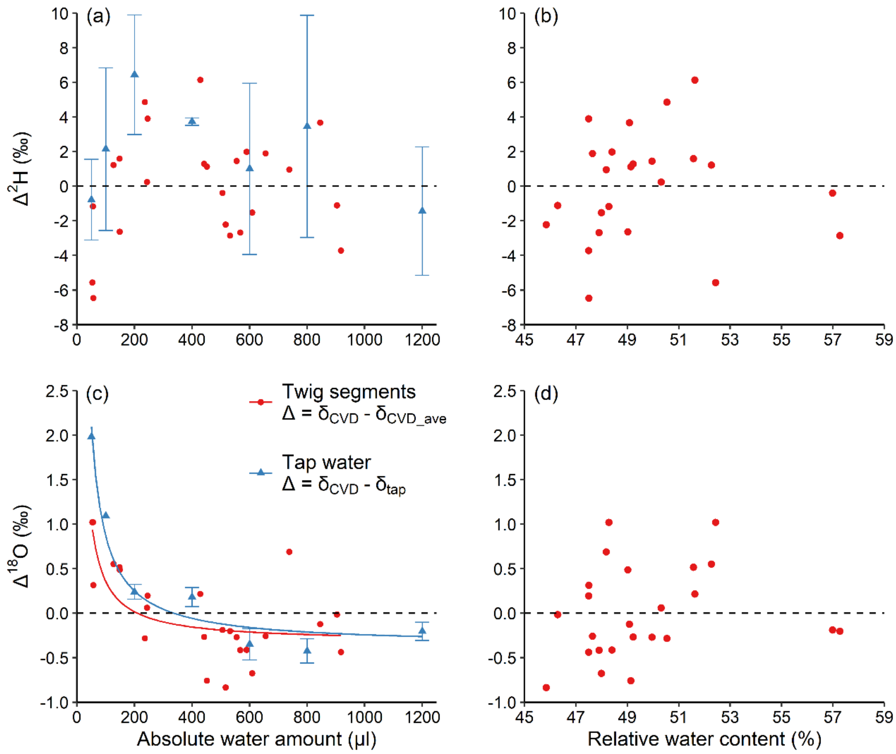

Figure 4: Absolute water amount and relative water content effects (left and right panels, respectively) on Δ^2H and Δ^{18}O (top and bottom panels, respectively) of water at natural isotope abundance. For water of Larix decidua twig xylem, $\Delta = \delta_{CVD} - \delta_{CVD\_ave}$; while for the tap water, $\Delta = \delta_{CVD} - \delta_{tap}$ (see 2.4). The relationship between water amount and Δ was fit with an inversely proportional function. The dashed lines represent Δ = 0. For (a) & (c), mean values ± SD are shown (n = 3).

4. Conclusions

In conclusion, we provide strong evidence that H-exchange with organic H in the sample, despite significantly influencing the δ^2H of water during the sample rehydration process, is not the main driver of the broadly observed negative Δ^2H value during CVD extraction. Instead, we identified a significant CVD artefact when water is present in small amounts, particularly when δ^2H and δ^{18}O of the water were below natural isotope abundance. This is linked to the increase of sublimation and evaporation enrichments with the decrease of water amount, as well as the mixing between the extracted water and laboratory water vapour, rather than the effect of sample RWC on H-exchange. We therefore recommend extracting more than 600 µl of water, especially for studies using labelled water, to avoid large enrichment biases. Our results have implications for studies using stable isotopes of water in plant tissue to determine plant water sources (e.g., Evaristo and Mcdonnell (2017)), trace water through soils with high organic material content (e.g., Koeniger et al. (2016); Sprenger et al. (2016)), and reconstruct climate patterns using tree ring tissue (e.g., Loader et al. (2007); Lehmann et al. (2021)).

Author contribution

HD, PS, GRG, and MML designed the experiments and HD carried them out. HD prepared the manuscript with contributions from all co-authors.

Competing interests

The authors declare that they have no conflict of interest.

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