

Response to the reviewer 2

Manuscript: “A comparative study of plant water extraction methods for isotopic analyses: Scholander-type pressure chamber vs. cryogenic vacuum distillation”, by Zuecco et al.

We would like to thank the reviewer for the time (s)he spent on our manuscript and for the valuable suggestions. We report the reviewer’s comments in black and their response below in blue.

General comments:

The study conducted by Zuecco et al. addresses a very important issue in the ecohydrology community. They investigate two plant water extraction methods and attempt to analyse the differences of the isotopic composition of plant water in relation to the respectively used method. Namely, they compare plant water isotopic compositions extracted with either a Scholander pressure chamber (SPC) or cryogenic vacuum distillation (CVD). They aim at assessing (1) differences in isotopic composition related to the technique used (i.e. SPC or CVD) and additionally (2) want to analyse how differences in the isotopic composition are related to plant species or plant tissue type (from hereon, these two aims will be referred to using the terms aim1 and aim2). While this is a much needed study with a promising topic related to ecohydrology, I have, what I believe, are major concerns regarding the implementation of the method comparison.

While the authors used a fully suberized one- year old shoot to extract water from using the SPC method, they extracted water from several tissues making up a twig/shoot using CVD. These are not the same and therefore cannot be compared. Thus, I feel the authors cannot address aim1. Aim2 addresses differences in relation to tissue type and plant species, but only one sample type is used for SPC. Additionally, the extraction with SPC happened with attached leaves and unpeeled bark. The authors mention in the introduction and the discussion the Geißler et al. 2019 study, but do not take the same precautions when handling the samples (I know that this data set was taken prior to the Geißler manuscript, nevertheless the precautions are valid also for sample handling when not analysing water isotopes (e.g. when analysing xylem sap for nutrients the phloem is peeled off before the shoot is extracted with the SPC)).

We agree with the reviewer that our study design has some limitations, but we still think that our study is very important for the ecohydrological community because, besides Geißler et al. (2019), previous research has never investigated the differences in the isotopic composition of plant water extracted by SPC and CVD and discussed the potentials and limitation of both methods. We are aware that in various studies focusing on nutrients, the bark is peeled off, but in our case, we still considered various options for CVD tissues, one of which comprises the presence of bark. Furthermore, we still think that the twig samples used for CVD-T and CVD-TwB and the ones used for SPC were very similar because they had very similar size (range between 3 and 6 mm), were suberized and were collected at the same position in the tree.

Is it not likely that when leaves are still attached and intact, to extract xylem sap from the leaves especially when applying high pressures (eg for the Ressi samples) and long extraction times (i.e. “until all the water was collected” L139/140)? Especially on the notice that the samples obtained with SPC in situ were different in colour, the authors could have taken another sample were they peeled the bark and stripped the leaves, thus assessing potential contamination of organics, and of the still living cells in attached leaves.

We exclude that there was an influence of leaf water on the isotopic composition of SPC samples because CVD-L samples clearly had an evaporative signature (see the dual-isotope plots in Fig. 4) compared to SPC samples. Furthermore, in the Introduction we reported that SPC is used to retrieve water in xylem conduits, and if we assume that there is no isotopic fractionation between xylem water present in the twig conduits and leaves conduits, then there should not be any difference in the isotopic composition of xylem water obtained from twigs or twigs and leaves together.

That said, I want to emphasize, that the topic and the idea for this study is needed, the community is waiting for this assessment and the results that can come from it, so I would encourage the authors to provide either better argumentation of why they can compare samples not from the same tissue, or an additional dataset, in which they compare the same tissues (e.g. complete shoot extracted with CVD or all plant tissues also extracted with SPC) and then address these aims again.

We thank the reviewer for appreciating our work. We hope that the revised manuscript will clarify all the questions of the two reviewers.

Additionally, I think the manuscript would benefit from a thorough review of a native speaker. Therefore, I will not comment on nor suggest improvements regarding language use or sentence structure.

We thank the reviewer for the suggestion. We will ask a revision of the manuscript by a native speaker.

Specific comments:

INTRODUCTION:

L 41: for completeness sake I would add Marshall et al. (2020) to the vapor equilibration method

We thank the reviewer for the suggested reference, that we will consider in the revised manuscript.

L44: I would suggest giving either a comprehensive list of research in which this method is described/applied or one key publication in which the method is extensively described/first published

As requested, in the revised manuscript, we will add some key references about CVD.

L45/46: “stored in dead and living cells for months or years” please add a reference

We will add a reference.

L49-54: Maybe shortening this section would benefit the reader, as they can look it up in the cited literature

We will shorten this part of the text.

L55ff: I would encourage you to shorten this section to the most relevant conclusion, i.e. that the results are ambiguous and different methods result in different outcomes

We thank the reviewer for the suggestion. However, in contrast to this comment, the first reviewer asked to expand this section, by adding more details about the findings by Millar et al. (2018) and Fischer et al. (2019).

We will revise the text by keeping only the key results.

L69 adding Scholander (1966) to the references would help a comprehensive list (see reference list below)

We thank the reviewer for the suggested references.

L73: what about Ellsworth and Williams (2007) and Magh et al. (2020)? Even though Ellsworth and Williams did use SPC in a different way I think it would be worth mentioning with a statement like the one you make here.

We thank the reviewer for the suggested references, that we will add in the revised manuscript.

STUDY SITE AND SAMPLING:

L88: Reference “Autonomous province of Bozen-Bolzano” is missing URL or in reference list. Please add

We thank the reviewer for noticing the missing URL; we will add it.

L89: what do you mean by “relatively mature”? please specify either the exact age of the stands (for all three sites) or use appropriate description (i.e. either mature or juvenile)

We thank the reviewer for pointing this out. We will remove “relatively”.

FIG1: there are two red points on each photograph, I assume those are supposed to be the sampling sites? If so, one is really close to the river, the other one not. This probably has an influence on the isotopic composition of the samples and should be accounted for and definitely mentioned in the figure description.

We will revise Fig. 1 to make clear where the samples were exactly collected.

L92+L104 Why after sunset? Especially considering water potentials were expected to be low due to the water deficit? Why not pre-dawn like at the Ressi site?

We agree with the reviewer that the water deficit is even lower pre-dawn, but we were interested in carry out the sampling when the transpiration fluxes were supposed to be close to their minimum (either during the early night or pre-dawn). Furthermore, due to logistic issues, in Laas/Lasa and Ahr/Aurino we were able to access the sampling sites only during the daylight and after the sunset.

L105ff soil data are missing here but were added for the other sites. Please add here too.

We will add the soil texture data.

MATERIALS AND METHODS:

L123 again missing the Scholander reference, please add

We will add the reference.

L137 please indicate if the samples were taken close to the river or not. The pressures used for extraction seem to indicate that there was no water deficit at the Laas site, but high deficit at the Ressi site, this could be related to the sampling position close to the river?

The orchards in Laas/Lasa are generally irrigated during dry periods. This irrigation can explain the lower water deficit in Laas/Lasa compared to Ressi, which is mainly covered by a deciduous forest.

L140 how long did it take to sample all of the water for the samples? Were the times different for the different sites? Generally, are the samples all the same size and length?

The duration of the extraction was different among the samples (probably due to the different water deficit conditions), but we kept it as short as possible (less than 10 minutes) to minimize the evaporation. We tried to use samples of similar size for all the species and the three sites.

L148ff The proclaimed target was to compare SPC to CVD. In order to do that it would have been good to extract the same tissues with both methods. Please elaborate why this has been done the way it was. Separating the twigs/shoots into smaller units to answer aim2 could still be done only for CVD if the small sample volume with SPC was the problem.

We still think that the twig samples used for CVD-T and CVD-TwB and the ones used for SPC were very similar because they had very similar size (range between 3 and 6 mm), were suberized and were collected at the same position in the tree. However, as already mentioned by both reviewers, for SPC we cannot exclude a contamination by other tissues, such as phloem. Therefore, we will add a new section presenting the limitations of our study, and will expand the discussion about the capability of the two extraction methods to access different plant water domains.

L153 what does this mean? The trees were too small? Too large? Please elaborate.

Yes, these trees had small diameters and they are located in private land. Therefore, we chose not to use increment borers to avoid additional damages to the trees which were also used for sapflow measurements and during further sampling campaigns. We will add more details in the revised manuscript.

ISOTOPIIC ANALYSIS:

L173-180 please add information about standardization, normalization, and possible corrections

We will add more details about the isotopic analyses.

DATA ANALYSIS:

L 190 I think this sentence could benefit from clarification. Maybe rephrase to something like this: “We report d-excess values enabling us to compare samples from different study areas.”

We thank the reviewer for the suggestion, that we will consider in the revised text.

L206ff This compares different plant tissues extracted by CVD with the whole shoot extracted using SPC. The results are not wrong, but misleading when considering the aim was to assess differences between the extraction methods. The tissues compared here are not the same and differences found from this analysis could also be related to differences in plant tissue instead of extraction method.

Please see the reply given to the comment for L148ff. In the new limitation section, we will thoroughly discuss the capability of the two extraction methods to access different plant water domains, and how to improve our sampling design in future experiments.

DISCUSSION:

L271 “in situ” sounds a little misleading because it is often used in relation to vapor equilibration methods. I suggest replacing it with “in the field”.

We thank the reviewer for the suggestion, that we will consider in the revised text.

L277 which advantages are these?

We will clarify the specific advantages, that we reported few lines above.

L280 extraction at 3Mpa likely damages living cells in the leaves/shoot, so it could be possible that the samples did not only consist of xylem sap anymore?

Yes, it is possible that such samples could not include only xylem water, but also water extracted from living cells. We will add this part as a limitation of our study.

L283 how large are these volumes? Would it be possible to calculate a volume weighted isotopic composition of the samples CVD_L and CVD_TWB to get an approximation for the signature of a complete shoot extracted with CVD? Maybe that way, it would be possible to “compare” SPC and CVD with the same plant tissue used. That would only work if the extraction was complete though. Was that calculated? I mean were the CVD samples weighed before and after extraction and then again after drying?

We thank the reviewer for the suggestion. Yes, the samples were weighed before and after the extraction to determine the extraction efficiency for CVD. We observed an average extraction efficiency for the specific CVD system of 98.7% (n=65), whereas the median was 100%. In the revised manuscript, we will provide the computation of this volume-weighted isotopic composition.

L248ff I understand the volume from the SPC samples was too small for spectral contamination analysis, but an assessment for the CVD samples would have been possible? These data would be interesting especially when comparing the CVD_T and CVD_TWB samples.

We agree with the reviewer that it would have been interesting to have spectral contamination data. However, it was not measured, and it will not be possible to perform the analysis now.

L296-300 I feel this is redundant here because it was just discussed in the previous section. I suggest deleting
We agree with the reviewer and we will remove these lines.

L310 I don't understand why it was expected for a CVD_WC sample obtained at breast height (?), this is an assumption, please specify the sampling heights and direction for all samples in the material and methods section) to have the same or a similar isotopic composition as a twig sampled from the crown (which likely has a different height). There has been evidence that this cannot be automatically assumed (de Deurwaerder et al. 2020). Please elaborate.

We are aware and we have not automatically assumed that the various samples had the same isotopic composition. Indeed, we decided to test whether they had the same or similar isotopic signature.

L313ff I don't think this argumentation is valid here. The samples are not comparable because they are not the same.

Please see the reply given to the comment for L148ff and other previous comments.

L319ff Again, this argument bases on the comparison between tissues which are not the same. I agree however, that SPC and CVD are able to extract different water pools/domains and it would be rational to quantify these differences especially when talking about water storage.

We agree with the reviewer and we will expand the discussion about the capability of the two extraction methods to access different plant water domains.

L326-329 redundant as the previous sentence says the same, rephrase or delete

We thank the reviewer for the suggestion. We will rephrase the sentence.

L331 missing reference for water storage times. Please add

We will add a reference about water storage times.

L339/340 how much older? Maybe a few examples would help here. Also, here you use plant water fractions before you used pools, please be consistent with which term you use throughout the manuscript.

As suggested by the first reviewer, we will be more consistent in the terminology throughout the manuscript, by referring to 'plant water domains'.

General recommendations to improve the discussion:

I would suggest discussing the possibility of water pool mixture during transportation, because it is highly likely that water pools exchange and cannot be considered completely separate.

We agree with the reviewer, and we will consider the possibility of water pool mixture transportation in the revised Discussion.

CONCLUDING REMARKS:

This reads like a second abstract/a summary and not like a conclusion. I suggest deleting the summarizing parts and keeping the main message (i.e. lines 363 -371)

We thank the reviewer for the suggestion. We will revise the conclusions as requested.

References used in this review:

Deurwaerder HPT de, Visser MD, Detto M, Boeckx P, Meunier F, Kuehnhammer K, Magh RK, Marshall JD, Wang L, Zhao L, and Verbeeck H 2020. Causes and consequences of pronounced variation in the isotope composition of plant xylem water. *Biogeosciences* 17: 4853–4870.

Ellsworth PZ and Williams DG 2007. Hydrogen isotope fractionation during water uptake by woody xerophytes. *Plant Soil* 291: 93–107.

Magh R-K, Eiferle C, Burzlaff T, Dannenmann M, Rennenberg H, and Dubbert M 2020. Competition for water rather than facilitation in mixed beech-fir forests after dryingwetting cycle. *J. Hydrol.* 587: 124944.

Marshall JD, Cuntz M, Beyer M, Dubbert M, and Kuehnhammer K 2020. Borehole Equilibration: Testing a New Method to Monitor the Isotopic Composition of Tree Xylem Water in situ. *Front. Plant Sci.* 11: 1–14.

Scholander PF 1966. The role of solvent pressure in osmotic systems. *P. Natl. Acad. Sci.* 55: 1407–1414.