Response to the reviewer 1

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.

This paper provides further examination of a promising plant water extraction method for isotopic analyses, Scholander-type pressure chamber water extraction (SPC), and compares results of that method to the conventional plant water extraction method of cryogenic vacuum distillation (CVD). This comparison is necessary to determine possible advantages, disadvantages, and implicit assumptions related to method choice for many ecohydrologic studies. However, I have what I think are valid concerns about the current version.

General feedback:

As mentioned in the introduction, there is an overarching issue within the ecohydrological community where differences in the isotopic composition of plant water have been related to extraction methods. These differences have been attributed to either one or a combination of the following: 1) Inter-lab differences: lab specific protocols, setups, accuracies, efficiencies 2) Inter-method differences: various alterations and various accessibility 2a) alterations of plant material associated with specific methods: e.g., fractionation effects associated with incomplete extractions or specific extraction methods, co-extraction of organic substances 2b) variability in the proportion of plant water domains accessed via different methods: lower residence time domain from xylem tracheary elements (dead cells) participating in transportation of water from soil to leaves versus higher residence time domain of living cells not participating in transportation of water. (I use domains rather than pools since that is the language recently being used for residence time of soil water in ecohydrology. Pools and domains are likely interchangeable and I am not saying one should be used over another, just that the use should be consistent throughout the paper. I will refer to them as domains for the remainder of this review to be consistent.)

For this paper the authors focus on (2a) with d-excess analyses, but there are issues (highlighted in specific comments) with whether d-excess can exclude considerations of (2b). Additionally, (2b) is poorly discussed for samples and analyses throughout paper. There are also some study design limitations for (2b) which would be hard to address by including more samples since sampling conditions will not be consistent, but the limitations should be mentioned for any future studies to address.

We agree with the reviewer that our study design has some limitations, and the manuscript lacks a specific discussion of the domains accessed by each method and sample type, and the possibility of extracting organic compounds by the two methods. In the revised version of the manuscript, we plan to significantly improve the Discussion and add a short section on the study limitations, presenting the gaps that our research could not fill in, and the way to overcome them in future studies. Despite the limitations highlighted by the reviewer, we still think that our study is important 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.

The authors argue that the SPC method needs more investigation into its merits for ecohydrologic studies and that the SPC results need to be compared to conventional method of CVD. Hence, the authors two objectives (L79). However, there are issues with how they can address the first objective because their study design does not have a direct comparison between methods since the sampling materials are not the same.

Granted a shoot and a twig are similar, if not the same in some cases, but the use of these different terms is confusing from the onset.
We agree with the reviewer that the different terminology is a bit confusing. Therefore, we will use the term 'twig' for the SPC samples as well. The age of the twig can slightly differ between the SPC and CVD samples, but the diameters of CVD-T, CVD-TwB and SPC samples were comparable (3-6 mm), as well as the sampling position along the branch.

Furthermore, SPC samples are from one year old shoots with leaves and bark intact whereas CVD samples are from multiple tissue types that make up elements in a one year old shoot, but these elements are never all together (there are no CVD samples of one year old shoots with leaves and bark included).
We agree with the reviewer that this is, indeed, a limitation that will be discussed in the revised manuscript. However, we want to stress that we used, for SPC, the same sampling procedure widely applied by many ecophysiologists to measure the plant water potential.
For CVD, we are aware that this technique is able to extract the bulk plant water from different tissues and cells. Therefore, we decided not to make the comparison too complex by mixing twigs and leaves (by the way, it would have been difficult to collect the same amount/volume of twigs and leaves – and therefore the same plant water – for both SPC and CVD to have comparable samples). Furthermore, in the revised manuscript, we plan to discuss more in details that SPC samples are not comparable with a mix of water derived by CVD-TwB and CVD-L samples, particularly because SPC samples clearly did not show an evaporative signature (see the dual-isotope plots in Fig. 4).

These differences in tissue type for each method seem to be part of the reason for the second objective, but there is inadequate considerations of the (2b): variable access issues. With leaves still intact for SPC extraction, the possibility of extracting leaf water from predominantly xylem conducting cells in leaves seems hard to discount, especially when authors state that water extraction of shoots via SPC ended when they had collected all the water flowing out of the shoots (L139).
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.

In addition, with bark still intact at collection site via SPC it is possible that phloem substrates are co-extracted, which is not mentioned when addressing how SPC samples were colorful in discussion(L285). In fact, I am perplexed when they cite Geißler et al. (2019) thoroughly in the introduction yet do not follow the same precautions, removing leaves and bark near collection site, to eliminate the contribution of water from leaves and phloem from live tissues still intact near collection site(L131).
Indeed, we did not follow the procedure described by Geißler et al. (2019), but we conducted the experiment before Geißler et al. (2019). Again, we want to stress that we carried out the SPC sampling procedure as it is usually done to measure the plant water potential.
We also agree with the reviewer that we cannot exclude the co-extraction of organic compounds by SPC, particularly in Ressi, where the water deficit conditions imposed us to apply higher pressure to extract the water samples compared to Laas/Lasa and Ahr/Aurino. We already mentioned this detail at lines 285-286. We will address this as a limitation of the SPC method and of our application.

In methods section 3.2, authors state that "Samples with and without bark were used to test whether the plant water extracted by the SPC method had an isotopic composition more similar to CVD-extracted bulk plant water (i.e., CVD-TwB) or to plant water deprived of phloem tissues (i.e., CVD-T)". This somewhat states the assumptions by the authors that a twigs with bark have more living cells, which is reasonable.

Furthermore, twigs without bark are predominantly dead cells and isotopic compositions of CVD-T samples should be more similar to isotopic composition of SPC samples (under the logic stated in introduction that SPC is collecting water primarily from dead cells). Although this logic holds up, this instance and other areas that discuss lignified tissues are missing the necessary consideration that total extractions of a twigs without bark via CVD could still be extracting water from living xylem parenchyma cells in the complex woody tissue. The proportion of xylem parenchyma varies by species and a recent global synthesis showed that the combined ray and axial parenchyma content for angiosperm trees and shrubs averaged 26.3 ± 12.4% (Morris et al., 2016). A possible contribution of a quarter of the water extracted seems like an important consideration to me, especially as they argue the possibility of the living cells having very different isotopic compositions than water being conducted in xylem conduits (L332). Granted some details of variation in dead cells are included in description of CVD-TcT (L153), but the relative amount of living cells needs to be highlighted more.

We thank the reviewer for providing the reference to Morris et al. (2016) that we will include in the revised manuscript. We also agree with the reviewer about the possibility of CVD to extract a significant amount of water from living parenchyma cells, which is an issue that we will address in more detail in the revised discussion and the new limitation section.

Overall, the authors need to discuss limitations in their design and provide arguments of why they can directly compare samples for first objective (e.g., SPC vs CVD-T). In addition, they need to have a more thorough discussion about the variability of accessing plant water domains with each method, tissue type, and species. In regard to methods, this includes many instances of when they compare to other studies investigating differences between methods and provide inadequate context to what plant water domains are accessed for the comparison. Finally, there needs to be greater clarity in the paper with the use of the word “effects” on isotopic composition of plant water. The paper lacks necessary discussion distinguishing the possible effects that could alter plant material versus effects of accessing different plant material when collecting a water sample, particularly when assessing results and conveying the future directions.

We agree that the current version of the Discussion should be revised by considering more the variability of accessing plant water domains by each method, tissue type and species, and by clarification of the effects on the isotopic composition of plant water. We plan to greatly revise the Discussion section by accounting for the indications of the reviewers, and by adding a section on limitations that will also address the directions for future experiments using SPC.

Specific comments:

L5: could benefit from more explicit mention of what plant water domain SPC is accessing similar to that done by CVD. In other words, why does SPC need more attention other than it being rarely applied?

We will rephrase the sentence highlighting that SPC could be used as a valid technique to extract xylem water that is likely transpiring during the sampling day.

L9: kind of misleading that SPC was done on multiple separate plant tissues when it was done from a one year old shoot comprised of multiple plant tissues. Certainly does not have to be here, but in the discussion it would be helpful to recognize past work on variability of isotopic composition of multiple plant materials done by Zhao et al. (2016) “Significant Difference in Hydrogen Isotope Composition Between Xylem and Tissue Water in Populus Euphratica”

We thank the reviewer for the suggested reference. We will rephrase the sentence to clarify that only CVD was performed on multiple plant tissues.

L18: granted you preface with likely, but how do you know that pressure applied via SPC is not affecting any living cells and only dead cells? Pressure is applied through leaves, so aren’t some living cells impacted, albeit

minorly (especially considering your differences in CVD-L and SPC)? in your results you suggest cell walls were broken leading to co-extraction of organic substances by SPC.
We cannot exclude that we co-extracted organic compounds by SPC, particularly in Ressi where the water deficit conditions imposed us to apply higher pressure to extract the water samples compared to the Laas/Lasa and Ahr/Aurino sites. We already mentioned this detail at lines 285-286. We will address this as a limitation of the SPC method and of our application.

L35: mentioning these ecohydrologic studies is necessary, but your introduction currently lacks the broader context of issues within the community. CVD has been the conventional method for so long with assumptions that root water uptake is nonfractionating process for the most part and that total plant water via CVD has been considered representative of transpiration due to water in plant tissue assumed to be in equilibrium or well-mixed. The well-mixed assumption has been recently questioned by ecohydrologic separation studies (many of which you cite here) and discussion around plant water domains similar to soil water domains. For instance, you mention Barbeta et al. (2019) in the end of the discussion, but I feel like it would also help to include some of the ideas/issues from that paper here to help preface why SPC should be investigated more.
We thank the reviewer for this suggestion, and we will revise the Introduction by considering the findings by Barbeta et al. (2019).

L38: in addition to not altering plant material, these studies also want isotopic composition of plant water that is representative of transpiration which requires techniques that don't alter plant material and involves criticizing what plant water is accessed with each method (water from live versus dead cells).
We will reword this part.

L58: Millar et al. (2018) do categorize methods by what plant water domains(or pools in their case) are accessed and being more explicit about that here would help provide context to readers of why SPC is important to further investigate.
We will expand the sentences reporting the findings by Millar at al. (2018).

L61: what plant water domains were accessed with Fischer et al. (2019) various methods?
In the revised manuscript, we will expand the sentences reporting the findings by Fischer at al. (2019).

L71: what about the work by Ellsworth and Williams, 2007–"Hydrogen isotope fractionation during water uptake by woody xerophytes" which used modified SPC chamber to understand possible fractionation during root water uptake
We thank the reviewer for pointing this out; we will include the reference to Ellsworth and Williams (2007).

L75: This mentioned comparison by Geißler et al. (2019) does have important nuances of how they performed SPC method and the sample material preparation.
We agree with the reviewer, and we will expand the sentences reporting the findings by Geißler et al. (2019).

L93: what is the rationale in after sunset? Why not when water deficit is lower, i.e., pre-dawn to mid-morning, especially if there are issues in acquiring enough volume via SPC? "at the downstream in the Ahr/Aurino study area" is unclear – photo of site has two sites with one more downstream than the other which is also much closer to the stream.
We agree with the reviewer that the water deficit is even lower pre-dawn, but we were interested in carrying 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.

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

Table 1: unclear what plant tissue/material was used for SPC, adding this detail to the table description would be helpful
We thank the reviewer for the indication, that we will integrate in the revised table.

L129: what position in canopy were one year old shoots taken from? Range in size of diameter extracted like that mentioned in 3.2 for CVD-T and CVD-TwB (L146)? Was canopy position and aspect similar for all sample types being more closely compared (SPC, CVD-T, CVD-TwB)?
Yes, the position of SPC, CVD-T and CVD-TwB samples was the same along the branch. The diameters of the twigs used for the samplings varied between 3 and 6 mm.

L131: what is the rationale in leaving the leaves on and bark intact near collection site? This does not follow rationale and guidance of Geißler et al. (2019) use of the SPC method to collect xylem water. Also, figure 2 has several leaves on the shoot in the chamber making the statement of "one or more leaves sealed inside chamber" somewhat unclear and added clarity would be beneficial.
As suggested by the reviewer, we will revise the sentence to improve the clarity. We are aware that we did not adopt same set up as Geißler et al. (2019), but we conducted the experiment before Geißler et al. (2019). Please, also refer to the replies that we have provided to the general comments.

L136: directly is a little vague, how does that differ from pipettes? Was the pressure chamber put on its side so that water could fall into vials directly via the help of gravity?
Yes, the SPC was put on its side to help the collection by gravity.

L139: rough timeframe? Longer or varied exposure to air/evaporation could impact comparison of results. All water implies that some water from leaves is incorporated in sample alongside shoot water which impacts how direct the comparisons can be between SPC and CVD-L/CVD-T/CVD-TwB
The sample collection by SPC was carried in less than 10 minutes for all the samples since the start of the pressure application. Furthermore, the plant water flowing out of the twigs was immediately trapped in the vial. Since the samplings were carried out during nighttime, we exclude a significant effect of evaporation on the isotopic composition of the samples. Indeed, average lc-excess were -1.0, -3.1 and 1.0 for SPC samples collected in Ressi, Ahr/Aurino and Laas/Lasa, respectively).

L148: what about living ray and axial parenchyma cells in xylem? similar or related reasoning for leaves is lacking in L145.
We will revise the sentence to improve the clarity about why we collected leaves samples as well for CVD extraction.

L150: was phloem removed for wood core samples? How much of active xylem/sapwood was used in wood core and did wood core include heartwood?
Yes, the phloem tissue was removed, and heartwood was not included. We will integrate this detail in the text.

L186: there are two additional LMWL sources missing here that are mentioned in figure 4 caption
We decided to depict the three LMWLs with the same color (pink) to improve the clarity of the figure and report the different equation for the three study sites in the caption.

L192: if you are trying to address whether method is fractionating water (or if you are accessing fractionated water) why not also do site specific lc-excess to more directly account for the local inputs? Especially with Ressi having very different LMWL this seems hard to gain much from the comparison with d-excess.
We decided to use d-excess for a direct comparison between the samples collected at the three study sites. As suggested by the reviewer, we will include a comparison based on lc-excess values in the revised manuscript.

L195: what do you mean by "effects"? Are the effects of the method altering isotopic composition? Or are they accessing different plant water domains? Or possibly both?
We thank the reviewer for pointing this out. In the revised manuscript, we will improve the clarity of the sentence.

L231: Not true for CVD-L samples. In general, CVD-L samples seem to be poorly integrated in discussion and results. I believe that their differences could help highlight that SPC with leaves attached are accessing different plant water domain in leaf than domains accessed by CVD for CVD-L.
We agree with the reviewer that the data of CVD-L samples were not well integrated in the results and the discussion. In the revised manuscript, we are providing a better inclusion of CVD-L samples in the text.

Figure 4: I think it would be helpful to include the GMWL on these plots to aid in visualizing how d-excess values are generated for each site.
We thank the reviewer for the suggestion, but we think that including the GMWL would affect the clarity of the plots. Furthermore, displaying the GMWL would not add much information to the findings that can be derived from the dual-isotope plots.

L277: does this include comparable in plant water domains extracted?
In this case, we were comparing the methods only in terms of simplicity and costs for plant water extraction. About the comparability in terms of plant water domains, we will expand and revise other parts of the Discussion.

L278: no specific mention of sampling times for each site/species sampled before this statement. Recommend putting SPC extraction times in methods
It will be done as suggested by the reviewer.

L286: what about phloem contributions via SPC extraction in addition to destruction of plant cell walls? According to methods the bark near the cut surface of one-year old shoot was not removed to limit contribution of phloem to extracted "water". What about contributions from water transporting and non-water transporting leaf cells? So, does your use of SPC method contradict opening statement of the discussion by breaking cell walls? Or does the contribution of phloem need greater consideration with your use of SPC method?
We will consider more the possible contribution of phloem as a limitation of the method and our application. We will discuss this limitation more in detail in the revised manuscript.

L298: based off introduction of CVD and SPC accessing different amount of plant water, is it really a "drawback" that they aren't comparable? I guess it is unclear if you are trying to state the SPC is an alternative for simplicity or if you are also considering SPC as an alternative for accessing water representative of transpiration vs total water via CVD?
We agree with the reviewer that this sentence is not very clear. We will revise the text reporting that SPC could be considered as an alternative for accessing water representative of transpiration.

L301: consider revising this sentence to be more clear, particularly the section starting in "indicating" is confusing as is. At a broader scope, being that leaves were still attached, then does this support that SPC is not accessing the evaporatively enriched pool of water in leaves that is found in the living leaf cells?
Yes, the very different signature of SPC samples compared to CVD-L supports the statement that SPC does not access the evaporated water domains that can be found in the living leaf cells.

L305: are you stating that the methods didn't fractionate plant water or that you didn't access fractionated water via both methods? Or both? I think this needs to be more clear for readers.
We meant both. Of course, this is a delicate point as we do not have unconfutable experimental evidence of the superimposition of the two issues, but we will revise this sentence to improve its clarity.

L306: I think it would be beneficial to point out that these values for Ressi are high with respect to other sites because the LMWL of Ressi plots above the GMWL in dual isotope space.
We will include this suggestion in the revised text.

L313: what plant water is sampled (predominantly transporting cells or nontransporting cells?) via direct vapor equilibration and microwave extraction and how does that help contextualize the comparisons of SPC results to CVD results? Although Zhao et al. (2018) attributed results to only fractionation effects rather than additionally considering access of different plant water domains, they also reported similar more negative d2H values for CVD-stem, CVD-core, CVD-root compared to d2H values of xylem sap sampled via needles.
In the revised manuscript, we will revise this part, while focusing more on the plant water domains accessed by the different methods.

L321: "sampling material" alone lacks distinction of different plant water domains and how the proportion of those domains possibly vary for each sampling material used
We will revise the sentence as suggested by the reviewer.

L332: Mask? Isn't the possibility of accessing different plant water domains a reason for comparison between methods and your objectives? It seems to me that the study design is what precludes you from a more direct comparison (sampling material not the exact same for both methods).
We will revise the sentence to include the possibility that SPC and CVD access different plant water domains. We do not think that our study design precludes the comparison between the two methods, as already stated in our first reply to the reviewer.

L342: any future directions on why there may be differences between species? Do all species have similar proportions of living and dead cells in various plant tissue samples?
In the new limitation section, we will provide more details about the future directions and other comparisons that could be made by using SPC.

L345: why the focus on these methods? Is it because they also access different plant water domains than CVD?
Since we and Geißler et al. (2019) have not compared SPC to other methods, besides CVD, we think that a wider comparison test is highly needed and useful to the ecohydrological community. This is the main novelty of our work that we will stress better in the revised version.

L346: similar to other comments, is altering the right word choice to solely be used here? Are you not arguing that you are accessing different plant water pools too?

We will revise the sentence to improve its clarity.

L357: first, “high transpiration moments” was the plant transpiring a lot after sunset? Second, why is isotopic difference between methods moreover a limitation? It seems like more of a justification to examine the possible information SPC extracted water would provide in many ecohydrologic studies.
We will remove “high transpiration moments” since our samplings were carried out during nighttime. Furthermore, we will rephrase the sentence to explain the access to different plant water domains.

L364: reads like this was a goal to have them comparable. similar to L298, seems at odds with introduction statement and possible advantage of SPC being more representative of transpired plant water than conventional CVD. Might be better to lead with “SPC accesses only part of the plant water fraction that CVD does and is therefore not an alternative to CVD in terms of plant water accessed.” At the root of the issue is the word choice of alternative is unclear as mentioned in L298 comment.
We agree with the reviewer, and we will revise the sentence as suggested.

Technical corrections:
L22: would it be better to state “longer” time rather than “long” time? Could be semantics, but seems like there is some ambiguity with what is meant by long time. One month? One year? Multiple years?
We will use “long” rather than “longer”.

L113: a prolongated or prolonged
We will replace “prolongated” with “prolonged”.

L276: maybe “without extensive laboratory work” would be better here and similar places since each method inherently has different specific laboratory work
We agree with the revision.

L329: “reach the leaves very rapidly” reads a little better.
We agree with the revision.

References (not included in pre-print and referred to in this review):
Ellsworth, P.Z., Williams, D.G., 2007. Hydrogen isotope fractionation during water uptake by woody xerophytes. Plant Soil 291, 93–107.
Morris, H., Plavcová, L., Cvecko, P., Fichtler, E., Gillingham, M.A.F., Martínez-Cabrera, H.I., Mcglinn, D.J., Wheeler, E., Zheng, J., Ziemi´nska, K., Jansen, S., 2016. A global analysis of parenchyma tissue fractions in secondary xylem of seed plants. New Phytol. 209, 1553–1565.
Zhao, L., Wang, L., Cernusak, L.A., Liu, X., Xiao, H., Zhou, M., Zhang, S., 2016. Significant Difference in Hydrogen Isotope Composition Between Xylem and Tissue Water in Populus Euphratica. Plant Cell Environ. 39, 1848–1857.