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Interactive comment on "A comparative study of plant water extraction methods for isotopic analyses: Scholander-type pressure chamber vs. cryogenic vacuum distillation" *by* Giulia Zuecco et al.

Anonymous Referee #1

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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:

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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.

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

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of these different terms is confusing from the onset. 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).

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 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). 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.

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.

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?

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)

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“Significant Difference in Hydrogen Isotope Composition Between Xylem and Tissue Water in Populus Euphratica”

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.

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 non-fractionating 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.

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).

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.

L61: what plant water domains were accessed with Fischer et al. (2019) various methods?

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

L75: This mentioned comparison by Geißler et al. (2019) does have important nuances of how they performed SPC method and the sample material preparation.

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.

Table 1: unclear what plant tissue/material was used for SPC, adding this detail to the table description would be helpful

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)?

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.

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?

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

L148: what about living ray and axial parenchyma cells in xylem? similar or related reasoning for leaves is lacking in L145.

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?

L186: there are two additional LMWL sources missing here that are mentioned in figure 4 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.

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?

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.

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.

L277: does this include comparable in plant water domains extracted?

L278: no specific mention of sampling times for each site/species sampled before this statement. Recommend putting SPC extraction times in methods

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?

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?

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?

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.

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.

L313: what plant water is sampled (predominantly transporting cells or non-transporting 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.

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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

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).

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?

L345: why the focus on these methods? Is it because they also access different plant water domains than CVD?

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?

L357: first, “high transpiration moments”âĂŤWas 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.

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.

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?

L113: a prolongated or prolonged

L276: maybe “without extensive laboratory work” would be better here and similar places since each method inherently has different specific laboratory work

L329: “reach the leaves very rapidly” reads a little better.

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., Ziemińska, 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.

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