Response to the editor's and the reviewers' comments

Dear authors,

Thanks again for submitting your revised manuscript. Two reviewers (one new) have provided detailed comments. They recognise that you made improvements to your previous version but both still have major concerns, in particular regarding the methodology and discussion of your work. I'd like to ask you to consider these reviews. With regards to the methodology, there are a few clarifications needed and please also take care of the comments around the weighted lc-excess and the measurement uncertainty around the lc-excess. Your discussion could be strengthened in particular on the potential reasons for the differences found between methods and species, also considering the work by Barbeta et al 2021, Chen et al 2020 and Allen and Kirchner (2021). I look forward to receiving your revised manuscript.

Best wishes,
Josie Geris

We would like to thank the editor and the reviewers for the time they spent on our manuscript and for the valuable suggestions. We report the reviewer's comments in black and their response below in blue. Lines (L) refer to the revised manuscript with no track changes.

Response to reviewer 1

Review Hess Zuecco et al.
Much improved version of the manuscript, thanks.

Abstract. No comments

Introduction:
I think you greatly improved the introduction and I have only some minor comments:
Line 39-40: water representative of transpiration needed in ecohydrological studies. With your findings the method of choice would be SPC. Pick that thread up in the discussion again.
We thank the reviewer for this suggestion, that we implemented at L404.

Line 80-81: Barbeta et al. observed this depletion, but the problem that it was related to CVD and not the plant fractionation was identified by Chen et al. 2020, and Allen and Kirchner 2021). If you could add these references (see below) and put them in context with Barbeta et al. 2019 that would be great.
We thank the reviewer for this suggestion, that we implemented at L87-96. Note that Allen and Kirchner (2021) HESS now became Allen and Kirchner (2022) HYP.

Site descriptions:
Line 109 and 113 what do you want to tell the reader with the Engel et al. in review reference? To look for a better site description in there? I think it is irrelevant and I would delete it, especially because this has not been published yet.

As suggested by the reviewer, we removed the reference since Engel et al. has not been published yet.

Line 125 again, why is it important to have this reference here? I think your site description is sufficient as is, but if you insist on adding a reference here, please add what it is for.
As suggested by the reviewer, we removed the reference at this line.

Table 1 belongs in the Results section, but while looking at it, how do you deal with the tiny deviations from the LMWL (i.e. the small lc values)? See (Landwehr and Coplen 2006) who indicate that lc's smaller than can be detected by the measurement precision are considered not to differ from LMWL (here , it looks like this could be the case for Alder CVDWC, Chestnut CVD-T, Beech SPC and CVD-TwB and -TcT and maybe even more) could you add the calculations for S in regard to the measurement precision of the used instruments (as you write in the MM 2.5permil for delta2H and 0.1permil for delta18O) and then indicate in parentheses which values are and are not different from the LMWL in your case? It would give the reader the chance to directly assess differences.
We thank the reviewer for the useful suggestion. We computed S and modified Table 1 accordingly, we have now moved Table 1 and updated the Results section (we did not add parentheses in Table 1).

Line 136 and again, emphasize what you want to say with adding these references here, or delete them if they are not giving additional information.
In the revised manuscript, we kept only the key references to the experimental catchment.

Material and Methods:
Line 234 the landwehr and Coplen reference is dated to 2004 in your manuscript, however when I checked it again on google scholar and web of science the date is 2006, please change it to the correct date.
We thank the reviewer for finding this mistake, that we have corrected.

Generally, I am missing the explanation of how and why you weighted the means of the measurements? I know it says it in the header of table 1 but it should be part of the MM for completeness sake. What did you define as the mean extracted volume? Did that then differ for each method or each species or each tissue? And if so, what is the benefit of weighting them, the measurement device takes the same amount of sample for injection, right?
The comment of the reviewer on the first round of reviews was not very clear, and we misunderstood his/her suggestion. In the revised manuscript, we decided to report the median isotopic composition, as it was in the original submission. However, we kept the information about the extracted volume because it gives an idea about the plant water amount that were extracted from the various sample types.

Also, I think I was misunderstood in the last revision round when I asked for a volume weighted isotopic composition, I meant if you could do a mixing ratio calculation where (in theory) you would mix the samples CVD_L and CVD_TWB together (weighted by each their volume) and get a mixed isotope signal that would in theory then represent the same tissue as when you extracted one twig

with bark and leaves using the SPC. But since you do not see any co-extraction of water from the leaves in the SPC samples, I can agree how you would most closely compare them to CVD-TwB. However, that does not mean they are the same tissue type.
We are sorry we misunderstood the reviewer's indications on the previous version of the manuscript, we re-read it now at the light of this further comment, and we understand what the reviewer meant. We agree with this new comment by the reviewer that even if the mixing ratio calculation between CVD-L and CVD-TwB returned a mixed isotopic signature close to that extracted from leaves and twigs using the SPC method, we would not have conclusive indications about the possible same tissue type. Therefore, we prefer avoiding speculative analysis and discussion, and did not perform the mixing ratio calculation.

Results:
Figure 4: If possible, it would be great if you could change the pink line and text to e.g. grey but that's just a personal preference, I feel the pink is too much, maybe other readers think so too?
Also, it would be reader friendly if you could plot the leaf data as an inset or a separate column and therewith zoom in to the data plotting closer to the LMWL. Especially looking at the Laas data, I think the figure would benefit greatly from a higher resolution on the potentially non-enriched data.
We thank the reviewer for this suggestion, that we implemented in the revised manuscript.

Line 297: I think once you correct for the measurement precision this will not be distinctly different from 0, so I think it is important that you add this S (as standard deviation) according to Landwehr and Coplen 2006.
We thank the reviewer for the comment, and we think it is a good suggestion. We have revised the text based on the new results (please see L310-317).

Discussion:
Figure 8 should go to the last page of the result section.
Yes, we agree with the reviewer, but this issue depend on the LaTeX compiler and the template used for the journal. We will check the position of all figures again before the publication of the manuscript.

I would switch sections 5.1 and 5.2 as it is logical to first read your assessment about the differences and therewith the answer to your main question: do these methods yield different results, and then move on with the method of choice discussion in relevance to ecohydrological questions.
Thank you for this comment. This suggestion makes sense and we considered switching the two sections. However, we think that section 5.1 includes general information, whereas section 5.2 goes in more details addressing the differences between the two techniques and, importantly, the possible implications for isotope-based ecohydrological studies. In order to better stress this focus, we renamed the title of section 5.2 as follows: "Difference between the two techniques and implications for ecohydrological studies".

Line 333 -336: This could also be related to the contamination by phloem, as you did not peel it from the twig. Please add this information here.
Thank you for the comment. We added the information at L354-355.

Line 347: again, I think if you would “correct” the lc-excess values by the precision of your measurement device, these values will be indistinguishable from 0 and therewith indistinguishable from the LMWL.
We thank the reviewer for the comment. Please see the revised text at L364-367.

Lines 351-352: please discuss this also in relation to (Chen et al. 2020, Allen and Kirchner 2021)
Thank you for suggesting these recent works, we are aware of them. The paper by Chen et al. (2020) was heavily criticized due to some possible lack of statistical robustness. However, their results are important as suggest cautiousness when interpreting CVD-extracted stem values due to possible artifacts implicit in the method. We believe that this reference does not fit this part of the discussion as here we reported more depleted isotopic values for both isotopes compared to SPC, without implying fractionation processes addressed by Chen et al. (2020). Still, we mentioned this study at L90-91 of the revised manuscript as it fits better in that part.
The work by Allen and Kirchner (2021) published on HESSD but not HESS was recently published on Hydrological Processes. We added the reference in the discussion at L399-403.

Concluding remarks: no comments

References:
Allen S and Kirchner J 2021. Potential effects of cryogenic extraction biases on inferences drawn from xylem water deuterium isotope ratios: case studies using stable isotopes to infer plant water sources. Hydrol. Earth Syst. Sci. Discuss.: 1–15.
Chen Y, Helliker BR, Tang X, Li F, Zhou Y, and Song X 2020. Stem water cryogenic extraction biases estimation in deuterium isotope composition of plant source water. Proc. Natl. Acad. Sci. 117: 33345–33350.
Landwehr JM and Coplen TB 2006. Line-conditioned excess: a new method for characterizing stable hydrogen and oxygen isotope ratios in hydrologic systems. Int. Conf. Isot. Environ. Stud.: 132–135.

Response to reviewer 2

This paper addresses very important issue in ecohydrology and particularly for water isotope-based plant water source studies. The authors compare two methods to extract plant water: Scholander-type pressure chamber water extraction (SPC) and the most commonly used method, cryogenic vacuum distillation (CVD). I believe this study is much needed, as the community is looking for a standardized protocol in plant water source studies methods to potentially solve the generalized mismatch between soil and plant water found in many studies. However, I have major concerns about the methodology used and the current version of the paper that should be addressed before publication.
Firstly, from my point of view the reasons explaining why SPC and CVD show different isotopic composition are not profoundly discussed. Particularly, I miss two very important and current references addressing this issue that should be taken into consideration and discussed doubtlessly: (1) Barbeta et al. (2021) New Phyt, uses a novel method (special centrifuge) to separate vessel water from other water in the stem and compare these two differentiate water pools with ‘bulk’ water extracted by CVD; (2) Chen et al. (2020) PNAS compared CVD extractions to water vapour

transpiration measurements (with several comments associated in PNAS and Hess). Both studies show a more enriched value of the xylem and transpired water compared to CVD (like Zuecco et al.) but associate this offset to different reasons (1) heterogeneity of water pools/domains inside the stem (2) the H exchange between organic compounds (mostly cellulose) and water during CVD water extraction. I encourage the authors to take into consideration the discussion of these papers and I believe this can be of much help to solve some of the concerns that the previous reviewers and myself have.

We thank the reviewer for the comment and suggestion. In the revised manuscript, we discussed the findings by Barbeta et al. (2021) and Chen et al. (2020) at L87-94, L373-375 and L399-403.

Additionally, I have some concerns about the methodology used:

• I don't understand the reasons behind sampling at sunset. If the authors wanted to have the maximum hydration of the tissues (in order to get enough water for analysis) they should have conducted the sampling campaign at predawn. If they wanted to sample sap water that was transpired they should have conducted the sampling campaign at the moment of maximum transpiration (mid-morning if the conditions where very dry). I cannot understand of what conditions would be representative the sunset sampling. Maybe it was just because logistic reasons but this should be either well discussed or considered a limitation of the study. Particularly, I think about the many studies, p. ex. Pfaust et al. (2015) Tree Phys, showing daily dehydration-rehydration cycles inside the stems (with a key function of parenchyma rays in this paper). In this regard, Barbeta et al. 2021 showed also an exchange between xylem and other 'tissue' water in the stems. With this comment I mean that xylem water collected by SPC could be influenced by other water domains in the stem depending on the time of the day.

We thank the reviewer for his/her comment. Sampling at the sunset was carried out for logistic reasons, but we agree that it could represent a limitation of the study. So, we added new sentences at L439-446.

• In the same line as the other referees, I wonder about the contamination from phloem or other living cells during SPC sap extraction, particularly when the authors say that the extracted water was coloured (to me an indicator that there is mixing of other water in the stem apart from sap). I think no more analysis can be conducted in the samples from this study but if the authors want to promote this method this is an important issue to solve. Maybe it would be necessary not to reach too high pressures (so, the method would not be valid in very dry conditions) and analyse for organic compounds as a proxy of other tissues contamination. In this regard, in both Geißler et al. (2019) and Barbeta et al. (2021) xylem water was always less contaminated by organics than bulk (CVD). I would also suggest to the authors to make a test conducting SPC with and without leaves, bark and phloem and check differences in water isotopes and the presence of organic compounds.

We thank the reviewer for his/her comment. We already mentioned in section 5.3 that the contamination from phloem or other living cells represents a potential limitation of our experimental setup, and furthermore, the quantification of organic compounds should be performed in future studies.

• The sampling of CVD-WC or CVD-TwB, as now addressed, does not give any relevant information to the study. I would suggest to remove these results or justify better their significance.

We thank the reviewer for this comment. Our main goal was to compare the two techniques for water extractions from different plant tissues and, as such, we think it is useful to present a wide range of plant tissues not to leave any curiosity in the reader. Therefore, we kept the wood cores and twigs with bark samples in the paper.

• Do you have any soil data from these or previous campaigns? It would be good to see if soil water fitted better to SPC or CVD samples. Anyway, the overlap of SPC values to the LMWL (lower lc-excess) would indicate to me that the isotopic values for SPC are closer to the water that plants uptake and transpire than the CVD ones.
In principle, we agree with the reviewer that it would be interesting to see how the different water samples extracted through the two techniques compare to soil water samples. However, the degree of similarity and overlapping between xylem and soil water isotopic composition does not always give clear indications about the sources of plant transpiration for a variety of reasons, including possible fractionation of xylem samples and/or shallow soil water samples; presence of other sources for tree transpiration in addition to soil water; uncertainty in collecting representative soil water samples (see Beyer and Penna, 2021). We do have soil water samples for some of the study sites but, for these reasons, we believe that including them into the analysis would increase the complexity of the interpretation of the comparison experiment between two techniques and, ultimately, make the main message of the paper more cloudy.

• Extraction times: Did you take note of the extraction times for each sample? It would be good to check if there is a relation between extraction time and evaporation. However, this relationship, could be also associated to a more enriched sap water inside the vessels (Martín-Gómez et al. 2016. Tree Phys)
Unfortunately, we do not have data about all extraction times, and therefore, we cannot add such analysis in our manuscript.

• I agree that the fact that the isotope composition of SPC samples is not enriched like the CVD leaves, suggest that during the SPC water extraction no strong contamination from leaves water was happening. However, I would suggest the authors an additional use for the CVD-leaves isotopic composition. By building a regression line of the CVD-leaves it's possible to retrieve the origin of the leaf evaporative water line; and check the possible water sources of the leaves (Barbeta et al. 2021).
We think the reviewer refers to the recent work by Benettin et al. (2021), and not by Barbeta et al. (2021). Benettin et al. (2021) used a backward evaporation model to map leaf water back to its individual precipitation event water sources. This is an extremely interesting approach that can open new research ways in isotope ecohydrology. However, Benettin et al. (2021) provided only a preliminary proof of concept and this method deserves to be carefully tested with specifically planned experimental designs that, we believe, are not offered by our current dataset. Some authors included in this research are currently working with Benettin to test this approach under specific conditions. Furthermore, we have too few leaf water samples, particularly for Ahr/Aurino and Ressi, to apply a meaningful linear regression analysis.

• There is a lack of analysis and discussion about species-specific differences between the methods. For example, for beech the difference between SPC and CVD is smaller than in the other species.

Could these differences be associated to wood anatomy (vessel area, wood density, parenchyma volume fraction…) or wood properties (quantity of lignin…)? Please check numerically and discuss it.
We agree with the reviewer that it would be interesting and of practical importance to analyze whether the differences in the isotope values returned by the two extraction techniques might be related to species characteristics, such as wood anatomy or wood property. However, this would require an array of wood data that we do not have. Relying on information and data reported in the literature would not be appropriate to address these aspects in a robust way and speculative discussion would follow. We prefer avoiding this.

Apart from these general comments, that will necessarily change the content and structure of the paper in several parts (please include them along all the document), I have some minor comments outlined in the text:
L18. CVD-leaves is not comparable to SPC in twigs.
Thank you for the comment. We removed the parentheses.

L21: soil water sources
We added “soil”.

L21: rephrase xylem water transpiring during the sampling day. Maybe just ‘sap water’ or ‘xylem water’
Done.

L36-37. For soils I would not say ‘a little’. Update the references for plant water extraction techniques
Thank you for the comment. We rephrased the sentence.

L45. Update the techniques (Barbeta et al. 2021, Zhao et al. 2016)
Done.

L48. Also, water within cell walls.
Done.

L48 and L52. Consider that water inside living cells might not be just different because of the age of the water but also because of fractionation inside the plant (aquaporins)
Thank you for the comment. We integrated the sentence at L51.

L86. Which other ecophysiological method could be used for this purpose?
Thank you for the comment. However, the aim of our study was to compare SPC and the widely adopted CVD and, therefore, we focus on these two methods in the introduction.

L92. Any of these studies checked if the isotopic composition of SPC extracted water fitted with soil water?

Yes, the mentioned studies considered the isotopic composition of both soil water and plant water extracted by SPC. However, we think this detail is not of great important for our introduction.

L99. Urgently needed to find the best method to sample transpiration water.
Thank you for the suggestion, that we have implemented in the text.

L101. Consider again differences not only associated to different ages but also to internal cell fractionation. In Barbeta et al. 2021, they prove that the stem water (both xylem and other tissue water) is replaced within 3 days of well water conditions. Besides, they observe a more or less constant offset between sap and bulk water.
Thank you for the suggestion. Please see the revised text at L113.

Table 1 and analysis. Are there species-specific differences in the quantity of water collected in the SPC and CVD twig samples?
Thank you for the comment. We have added the results of the test at L278-281.

Table 1. Did you take note of the pressure and time of the extraction? If you did, I would include the values in the table.
Unfortunately, we do not have notes for pressure and extraction times of each sample, and therefore, we cannot report these details in the table.

L150. Rephrase 'bad injections', change for "problems with the analyser"?
Thank you for the comment. We rephrased with "injection issue".

L162. Please explain "SPC contains a cutting board…" consider removing it or putting the sentence in context
We removed this part of the sentence.

L162. Consider rephrasing "The set up consisted…" one or more leaves alone? I guess you meant "one branch/twig with one or more leaves…"
Thank you for the comment. We rephrased the sentence.

L172. It would be good to have the water deficit conditions of every site (i.e. water potential of every species). Table 1?
We agree with the reviewer that this an interesting detail. Unfortunately, we did not take notes of water deficit for all samples.

L174. Did you consider not to flow all the water out of the twig samples with SPC? Did you do this for any physiological reason or because you were not collecting enough water?
We collected all the water flowing out from the twigs because the volumes were not enough for the isotopic analyses.

L175. Wood anatomy, particularly xylem anatomy could explain also the different extraction times? For example, in Ressi, did you find differences between the two species?

We agree with the reviewer that it would be interesting to analyze whether the differences in the isotope values returned by the two extraction techniques might be related to species characteristics, such as wood anatomy. However, this would require an array of wood data that we do not have. Relying on information and data reported in the literature would not be appropriate to address these aspects in a robust way and speculative discussion would follow. Therefore, we prefer avoiding this.

L182. What does it mean fractionated in this sentence? Please rephrase or explain
We removed "fractionated".

L185-186. Rephrase this part also. I don't understand the 'use' of CVD total plant water
We removed "total".

L192-195. I would put this paragraph in the introduction or discussion, not in methods
We moved the sentence to L389-393.

L238. Evaporation + other fractionation processes
We added "other fractionation processes".

Figure 4. Leaf evaporative water line. The legend for the colours is lacking
Unfortunately, we have too few samples to apply the simple linear regression, or the method proposed by Benettin et al. (2021). Furthermore, please see our previous reply to the specific comment on the leaf water evaporation line.
Thank you for noticing that the legend for the colours was missing; we fixed this adding a sentence in the caption of the figure.

Figure 5 and 6 are redundant to lc-excess in Figure 7. Consider to remove or move them to Supplementary Material
We disagree with the reviewer because Figures 5 and 6 are important to determine which CVD samples are more similar to the SPC samples. So, we decided to keep them in the revised manuscript.

L293. Again, I don't think you can compare CVD-leaves with SPC.
Here we do not compare CVD-L with SPC samples. Furthermore, one of the previous reviewers asked to add more comments on the CVD-L data presented in the manuscript.

Discussion: you write the study limitations in two different sections. I would suggest to reorganise all the discussion and refocus your main message with the suggestions I wrote in the first part of the review.
We think that the three sections of the manuscript present different arguments. Furthermore, we heavily revised the discussion following the suggestions of the two previous reviewers, and based on their comments we added a specific section on the limitations of our study. As suggested by the reviewer, we discussed the findings of Barbeta et al. (2021) and Chen et al. (2020) in the newly revised manuscript.

L342-343. Explain better that statement or remove it. I think, even though CVD from twigs, trunk and branches are closer to LMWL, this is not unequivocal prove of no fractionation. If you look into the results in detail you can see that the offset for d2H is generally larger than for d18O, and not drawing an evaporative line, which could indicate a fractionation caused for a different process than evaporation (mixing, biochemical processes, etc.). Obviously, the leaves are the most fractionated/evaporated samples. Please clarify the term fractionation along the document.
We thank you the reviewer for this comment, we agree with her/him that samples plotting on or close to the LMWL do not necessary imply that no fractionation occurred. Throughout the manuscript we carefully checked if the term "fractionation" was used appropriately. Here, we rephrased as follows: "Our results showed that twig samples (with leaves) obtained by SPC, differently from the CVD-L samples, did not show any offset compared to the LMWL of each site (Fig. 4)".

L348. Please rephrase. With this sentence I could understand that all the methods could be valid. Again, clarify fractionation.
Please see the rephrased sentence at L367-370.

L369. Remove the sentence into () or the word "particularly"
Done.

References:

Barbeta, A., Burlett, R., Martín-Gómez, P., Fréjaville, B., Devert, N., Wingate, L., Domec, J.-C. and Ogée, J. (2021), Evidence for distinct isotopic compositions of sap and tissue water in tree stems: consequences for plant water source identification. New Phytol. https://doi.org/10.1111/nph.17857

Chen Y, Helliker BR, Tang X, Li F, Zhou Y, Song X. 2020. Stem water cryogenic extraction biases estimation in deuterium isotope composition of plant source water. Proceedings of the National Academy of Sciences, USA 117: 33345– 33350.

Martín-Gómez P, Serrano L, Ferrio JP (2017) Short-term dynamics of evaporative enrichment of xylem water in woody stems: implications for ecohydrology. Tree Physiology, 37, 511-522, http://doi.org/10.1093/treephys/tpw115

Pfautsch S, Renard J, Tjoelker MG, Salih A. 2015. Phloem as capacitor: radial transfer of water into xylem of tree stems occurs via symplastic transport in ray parenchyma. Plant Physiology 167: 963–971.

Zhao L, Wang L, Cernusak LA, 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 & Environment 39: 1848– 1857