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LETTER TO THE EDITOR

Further experiments comparing direct vapor equilibration and cryogenic vacuum distillation for plant water stable isotope analysis

Recent work compared six plant water extraction approaches for hydrogen and oxygen stable isotope analysis.[1] Previously it was believed that these extraction approaches would provide analytes whose δ^2H and $\delta^{18}O$ values were similar, but the authors found significant differences in the isotopic composition of the produced analytes.[1] We report a short follow-up experiment to specifically explore systematic differences between one form of cryogenic vacuum distillation (hereafter CVD-2 from Millar et al,[1] based on Koeniger et al[2]) and direct vapor equilibration (DVE).[3,4]

Millar et al[1] found that DVE was the best extraction method for accessing the plant transpiration stream in wheat. This was in part because DVE targeted water from locations in the plant where the water potential was highest: the transpiration stream. Furthermore, the authors posited that because the DVE analysis step occurred at room temperature, negligible volumes of organic compounds would be present in the vapor–analyte headspace,[1] thus limiting interference during spectrometric isotopic analysis via off-axis integrated cavity output spectroscopy (OA-ICOS).

So, why are these differences important? Many ecohydrological studies are interested in where plants are sourcing their water, and therefore the transpiration stream water isotopic signatures.[5-7] Thus, accurate targeting of the transpiration stream is critical. Systems such as cryogenic vacuum distillation (CVD) that extract up to 99% of a plant's internal liquid content are not only accessing the transpiration stream, but also intra- and intercellular water, organelle-constrained water, and organic compounds.[1,7] The isotopic signals of these compartments may be dominated by soluble organic compound content unrelated to the transpiration stream. Millar et al[1] found, unexpectedly, that the H and O stable isotopic compositions (δ^2H: $^2H/^1H$, $\delta^{18}O$: $^{18}O/^{16}O$) of the analyte extracted by the CVD-2 system were not significantly different from those of the DVE system.

The lack of significant difference between the CVD-2 and DVE results did not fit with the authors' working hypothesis explaining the differences in results noted between the other tested approaches.[1] They postulated that variance in the co-extracted organic compound content, and the extraction of uniquely targeted liquid pools by each extraction method, was responsible for the differences in isotopic results.[1] Indeed, the CVD-2 system co-extracted substantial amounts of methanol (MeOH) and ethanol (EtOH),[1] which are known to cause errors in OA-ICOS instruments and may even modify isotope ratio mass spectrometry (IRMS) results if in high enough concentrations.[8-11] However, the DVE approach showed limited evidence of spectral interference, as assessed by the spectra fit residuals plot.[1,12]

Here we ask the follow-up question: Was the similarity between DVE and CVD-2 results in the previous method comparison[1] due to small sample numbers? While a low number of samples for comparison is not the only potential driver for differences between the previously tested methods, the scope of this study will focus on increasing the number of samples produced to address concerns about a lack of statistical robustness in the previous work.

We used the same soil, variety of wheat, extraction methodology (temperatures and extraction durations) and isotopic analysis systems (IRMS and OA-ICOS) as in the previous study[1] and refer the reader there for these details. However, in this study two containers (C1 and C2) of spring wheat were grown and only the root crown and first 5 cm of the leaf growth and differentiation zone (LGDZ) were collected for extraction and analysis. The outer sheathes of older leaves were removed for all samples to mitigate contributions from that evaporatively enriched (in ^2H and ^{18}O) material.[13,14] This plant portion was selected for its lower MeOH and EtOH content.[1] We sought to utilize an analyte source that would inherently have limited spectral contamination issues. While MeOH and EtOH are not the only co-extractable compounds, they are the most commonly noted ones in research investigating organic contamination effects on isotope ratio infrared spectroscopy (IRIS).[8-10] This is in part because the OH bonds of these compounds share spectral features with water, which is noted as the likely cause of interference during IRIS analysis.[8] Wheat samples were collected weekly over an 8-week period. During each sampling period eight individual plants were collected for each extraction–analysis approach per growth container, for a total of 32 samples per week. After extraction by CVD-2, the liquid analyte had its isotopic composition measured using an Isoprime IRMS instrument (Elementar UK Ltd, Stockport, UK). The DVE samples were analyzed via an IWA-45EP OA-ICOS instrument (Los Gatos Research Inc., San Jose, CA, USA). The isotope ratios are expressed in per mil (‰) relative to VSMOW.[15,16]

TABLE 1 Means and standard deviations (sd) of δ^2H and $\delta^{18}O$ values from spring wheat samples, extracted by DVE and CVD-2 for container-1 and container-2 from all 8 weeks of sampling. $N = 8$ for each method of extraction per week of sampling. DVE samples analyzed via OA-ICOS and CVD-2 samples analyzed via IRMS

Container-1: δ^2H values																
Extraction/ analysis method	δ^2H value															
	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6		Week 7		Week 8	
	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)
DVE	−75.3	3.7	−89.5	6.4	−95.7	4.3	−96.9	7.6	−101.9	10.2	−102.5	8.6	−95.5	10.3	−110.1	7.0
CVD-2	−109.8	2.8	−104.6	1.9	−115.9	4.3	−116.5	3.2	−112.5	4.1	−118.3	2.2	−122.1	4.1	−121.0	3.5
Container-1: $\delta^{18}O$ values																
Extraction/ analysis method	$\delta^{18}O$ value															
	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6		Week 7		Week 8	
	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)
DVE	−4.4	1.3	−7.7	1.3	−8.9	0.5	−9.3	0.6	−9.8	1.1	−9.7	1.2	−8.6	1.7	−10.4	1.5
CVD-2	−11.6	0.6	−10.2	0.5	−12.9	0.7	−13.6	0.6	−12.5	1.2	−13.4	0.4	−13.3	1.0	−12.7	1.0
Container 2: δ^2H values																
Extraction/ analysis method	δ^2H value															
	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6		Week 7		Week 8	
	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)
DVE	−77.1	7.5	−102.5	7.9	−96.4	5.2	−108.0	5.5	−90.6	5.8	−95.1	5.8	−84.9	7.8	−96.3	4.0
CVD-2	−108.0	7.7	−104.9	4.4	−125.0	2.5	−120.4	3.0	−117.3	4.3	−115.5	2.8	−117.9	5.9	−110.9	3.0
Container 2: $\delta^{18}O$ values																
Extraction/ analysis method	$\delta^{18}O$ value															
	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6		Week 7		Week 8	
	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)	Mean (‰)	Sd (‰)
DVE	−5.1	1.2	−11.6	4.0	−9.4	0.9	−11.2	0.4	−9.0	0.9	−8.1	0.4	−4.9	1.0	−7.9	0.8
CVD-2	−11.4	1.2	−11.2	0.7	−15.5	0.6	−15.0	0.4	−13.7	1.0	−12.4	0.5	−12.1	1.7	−9.5	0.7

Statistical analysis of the isotopic data was carried out with R 3.3.2. software.[17] To check for isotopic composition differences, we compared the DVE and CVD-2 isotopic results on a weekly and container-sampled-from basis. For example, the DVE results from week-1 C1 samples were compared with the CVD-2 results from the week-1 C1 samples; similarly for the C2 samples, and for all 8 weeks. For variance analysis between the DVE and CVD-2 data, where both sets of data were from normal distributions, Student's T-test was applied[18] at a 95% confidence interval ($p \leq 0.05$). Where one or both sets of data were from non-normal distributions, the non-parametric Mann–Whitney–Wilcox test[19,20] was used to determine significant differences ($p \leq 0.05$). Previous work has noted that there is currently no method for obtaining a 'reference' signal for plant-extracted water in studies examining method trueness.[21,22] As such we can make no claims in regard to result trueness, but rather discuss which pools of water within the plant are probably being targeted.

Table 1 presents the means and standard deviations (sd) of the δ^2H and $\delta^{18}O$ values for each method of extraction–analysis. For the plant analyte δ^2H and $\delta^{18}O$ values from both containers, the CVD-2 extraction method consistently produced more negative values than those produced by DVE (Figure 1). For example, the maximum difference between methods for the C1 δ^2H values occurred during week-1 sampling: CVD-2 produced an analyte that was 34‰ more negative than DVE. The average difference between the CVD-2 and DVE δ^2H values from C1 across all weeks of sampling was −19‰. For the C1 $\delta^{18}O$ values, the maximum difference between the methods again occurred during week-1 sampling: CVD-2 produced results that were 7.3‰ more negative than DVE. On only one occasion were the DVE results more negative than the CVD-2 results: week-2 C2 $\delta^{18}O$ values (DVE: −11.6‰, sd = 4.0‰, $n = 8$; CVD-2: −11.2‰, sd = 0.7, $n = 8$). Significant differences ($p \leq 0.05$) were found between the stable isotope results produced by the DVE and CVD-2 methods for all weeks of sampling, except for the C2 week-2 δ^2H and $\delta^{18}O$ results.

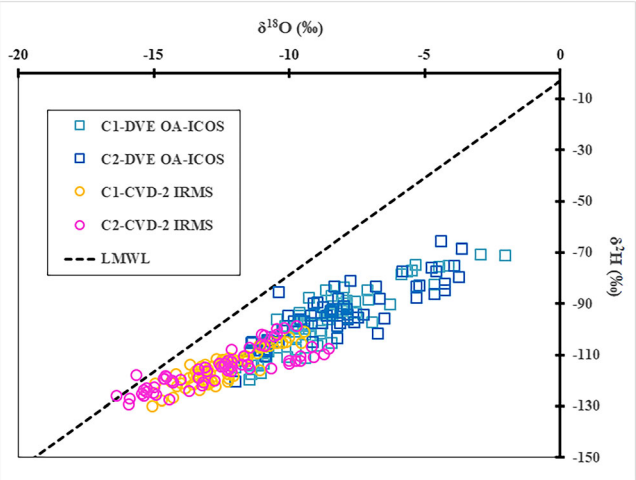

FIGURE 1 Dual isotope plot of extracted plant analyte δ^2H and $\delta^{18}O$ values from DVE and CVD-2 methods, for all weeks of sampling and for both growth containers. Local meteoric water line (LMWL) equation: $\delta^2H = 7.7 \times \delta^{18}O - 1.2‰$[23] [Color figure can be viewed at wileyonlinelibrary.com]

We believe that the explanation for these differences is that the DVE and CVD-2 approaches target different water pools within the plant, and that these compartments may contain unique sets of soluble organic compounds that can contribute to the measured isotopic signal, or result in spectral interference during isotopic analysis. Depending on the plant portion and method of extraction, certain water pools will dominate the extracted analyte's isotopic composition.[1] In this case we believe that the CVD-2 results are dominated by water and soluble organic compounds from non-transport-related plant water pools, whereas we think that the DVE results are more indicative of the transpiration stream. Wu et al[24] used advanced visualization techniques and reconstructive modeling to show the water transport structures in the roots of wheat. They showed that the total area, and therefore the water holding volume of the transport structures, is relatively small compared with the area of all other cellular structures.[24] Cellular structures around the transport vessels also contain extractable liquid, composed of water and other soluble organic compounds. As such, the transpiration stream isotopic signal only makes up a small portion of the total available liquid in a given plant portion. We therefore posit that any method that has a high extraction efficiency (such as CVD-2) will produce an analyte that will be strongly influenced by the isotopic signature of the water and the soluble content of the non-transport-related cells.

In this study, we expect the MeOH and EtOH concentrations in the extracted CVD-2 analytes to be similar to what was seen in our previous work for those plant portions (root crown and stem).[1] We predict that the concentrations of these organic compounds will vary throughout the growth stages of spring wheat, increasing as plant energetic organic compounds (carbohydrates, etc.) are created during photosynthesis and stored in the LGDZ for later mobilization during anthesis. During the production of organic compounds plants may be preferentially choosing ^1H over ^2H.[25] Were this the case in our wheat samples, the analyte produced by CVD-2, containing higher volumes of ^1H-dominated soluble organic compounds, could serve to drive the IRMS results in a more negative direction for δ^2H values (depleted in ^2H and enriched in ^1H). As we have not quantified the organic compound content for each sampling point we will not carry this assumption too far. Over the course of the wheat's growth, rising concentrations of these compounds in the LGDZ could result in increased co-extraction by CVD-2 and may explain the relatively more negative CVD-2 isotopic results and variations in the CVD-2 standard deviation over the trial. Similarly, variations in the concentration of these organic compounds during the wheat's growth could result in varying spectral interference levels during DVE analysis. Furthermore, we note that the plant analyte pools targeted by DVE may be less well defined than the bulk water pool targeted by CVD-2. This may explain the relatively higher standard deviations seen in the DVE results.

We posit that the CVD-2 isotopic results are accurate values for the analyte extracted by that system, due to samples having been

analyzed by IRMS. However, these results represent the bulk liquid pool of the wheat, previously defined as all extractable water and any water-soluble organic compounds present therein.[1] For DVE OA-ICOS results, if there are co-extracted organic contaminants sharing spectral features with water present in the analysis headspace, it may be that these OA-ICOS results are prone to some level of interference-related error. However, since our plant portions were chosen for their lower MeOH and EtOH content, we expected there to be limited spectral interference issues. When these analyses were undertaken, a tool for contaminant detection, such as LGR's Spectral Contamination Identifier (LWIA-SCI) software,[10,11] was not available for use during DVE analysis. This software is currently only available to use in liquid analysis modes. However, new work utilizing ^{17}O-excess as a contaminant detection tool shows promise for indicating organic interference during DVE analysis.[12] Unfortunately, this technique was not available during collection of this data set. We did, however, utilize the Spectra Fit Residuals plot[12] during DVE analysis and this tool indicated limited evidence of spectral interference. Since this approach is subjective and requires visual assessment by the researcher, we cannot entirely rule out the possibility of interference-related errors. Interference-related error could also explain the differences in the DVE and CVD-2 results.

Since water potential (in plant biology terms) is higher in the transpiration stream, this water pool should more rapidly equilibrate with the dry headspace air of the DVE sampling container. Thus, we believe that the DVE isotopic results are more representative of the transpiration stream water than the bulk extractable liquid pool. Water contained in leaves is known to become evaporatively enriched in ^{2}H during transpiration.[26] Water from sites of transpiration in the leaf has also been found to move back into the plant, in the case of wheat, carrying soluble photosynthates to the LGDZ.[13,27-29] Since we believe that DVE is targeting the transpiration stream, it may be that evaporatively enriched (in ^{2}H and ^{18}O) water from sites of transpiration is mixing with source water signals in the LGDZ and thus driving the DVE results in a relatively more positive direction (enriched in heavier isotopes). This could be an alternative explanation to the organic compound-driven differences suggested above, or perhaps both effects are contributing to differences in results between DVE and CVD-2. Finally, it is also possible that the equilibration times used during sample preparation for DVE analysis may have an effect on which water pools within the plant are being targeted, and thus may affect the isotopic results obtained. This proposal requires further investigation.

With a higher number of samples available for comparison in this study, we determined that there are significant differences in the stable isotope composition of analytes produced by the CVD-2 and DVE extraction–analysis approaches. We believe that these differences arise from the unique water pools targeted by each extraction–analysis approach. For the DVE approach, we cannot entirely rule out organic contamination interference effects, although we believe this problem to be limited with spring wheat samples. We note that further work is needed to determine what water pools within the plant samples are targeted by DVE analysis. Our findings further indicate the importance of choosing the extraction–analysis approach best suited to accessing specific water pools of interest for a given research campaign. For studies related to plant water sourcing, DVE may be a useful tool for accessing a plant's transpiration stream, provided that spectral interference can be detected and controlled for.

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