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RESEARCH ARTICLE

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# A comparison of extraction systems for plant water stable isotope analysis

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**Rationale:** The stable isotope ratios of water ($\delta^2H$ and $\delta^{18}O$ values) have been widely used to trace water in plants in a variety of physiological, ecohydrological, biogeochemical and hydrological studies. In such work, the analyte must first be extracted from samples, prior to isotopic analysis. While cryogenic vacuum distillation is currently the most widely used method reported in the literature, a variety of extraction-collection-analysis methods exist. A formal inter-method comparison on plant tissues has yet to be carried out.

**Methods:** We performed an inter-method comparison of six plant water extraction techniques: direct vapour equilibration, microwave extraction, two unique versions of cryogenic vacuum distillation, centrifugation, and high-pressure mechanical squeezing. These methods were applied to four isotopically unique plant portions (head, stem, leaf, and root crown) of spring wheat (*Triticum aestivum* L.). Extracted plant water was analyzed via spectrometric (OA-ICOS) and mass-based (IRMS) analysis systems when possible. Spring wheat was grown under controlled conditions with irrigation inputs of a known isotopic composition.

**Results:** The tested methods of extraction yielded markedly different isotopic signatures. Centrifugation, microwave extraction, direct vapour equilibration, and high-pressure mechanical squeezing produced water more enriched in $^2H$ and $^{18}O$ content. Both cryogenic vacuum distillation systems and the high-pressure mechanical squeezing method produced water more depleted in $^2H$ and $^{18}O$ content, depending upon the plant portion extracted. The various methods also produced differing concentrations of co-extracted organic compounds, depending on the mode of extraction. Overall, the direct vapor equilibration method outperformed all other methods.

**Conclusions:** Despite its popularity, cryogenic vacuum distillation was outperformed by the direct vapor equilibration method in terms of limited co-extraction of volatile organic compounds, rapid sample throughput, and near instantaneous returned stable isotope results. More research is now needed with other plant species, especially woody plants, to see how far the findings from this study could be extended.

## 1 | INTRODUCTION

The stable isotope ratios of water ($^2H/^1H$ and $^{18}O/^{16}O$ ratios) have been widely used to trace water in a variety of ecohydrological, biogeochemical and climatological studies.[1-4] While many of these applications sample liquid water from streams and lakes, and relate it to precipitation, an increasing number of studies trace plant water sources. In such work, analytes must first be extracted from the plant tissue. Cryogenic vacuum distillation (CVD) is considered the standard method used to selectively extract water from plant tissues for stable isotope analysis.[3,5] However, since the first use of CVD and its subsequent wide-spread adoption, a variety of other methods have

  

been developed to access plant and soil-pore water for stable isotope analysis.[3,4,6] Recently, there have been calls for inter-comparison of these techniques.[4,7] Comparisons of soil water extraction methods have shown significant differences in the stable isotope ratios of extracted water, depending on the extraction method used.[3,8] To date, however, no formal inter-method comparison of the systems used to extract water samples from plants for the measurement of $^{2}H/^{1}H$ ($\delta^{2}H$ values) and $^{18}O/^{16}O$ ($\delta^{18}O$ values) ratios has been carried out.

Here we compare six available extraction systems used to selectively extract water from plant tissues for the measurement of $\delta^{2}H$ and $\delta^{18}O$ values. Our null hypothesis is that all extraction methods will return the same water isotopic composition. Our work thus focuses on extraction system accuracy, repeatability, and sample purity. The plant used in our study is spring wheat (*Triticum aestivum* L.). We compare the following extraction methods: direct vapour equilibration (DVE);[9,10] high-pressure mechanical squeezing (HPMS);[11] centrifugation;[2] cryogenic vacuum distillation in two forms based on: (a) Orlowski et al[12] (hereafter CVD-1) and (b) Koeniger et al[13] (hereafter CVD-2); and microwave extraction off-axis integrated cavity output spectroscopy (ME-OA-ICOS) analysis, based on Munksgaard et al.[14]

Our specific questions were:

i. How do the stable isotope results compare between the different systems of extraction?
ii. Do the co-extracted volatile organic compounds methanol and ethanol affect the isotopic results?
iii. Which extraction method has the best sample purity, repeatability, and throughput?

## 2 | METHODOLOGY

Spring wheat (CDC-Utmost cultivar) was grown in a 0.6 $m^3$ container with a 0.50 m potential rooting depth, outdoors in a small greenhouse in Saskatoon, Saskatchewan, Canada. The soil used for the trial (medium-fine sandy topsoil) was thoroughly mixed and characterized (Table S1, supporting information) prior to being placed in the trial container. To determine the initial soil water isotopic conditions, a total of six soil samples were collected prior to seeding. Wheat was planted in four rows lengthwise down the container with 10 cm spacing between rows and 2.5 cm seed spacing in the row. The wheat was irrigated every 2–3 days over the course of its growth, with a minimum of 10 L of water used per irrigation period. Irrigation water samples were collected at each irrigation period and their $\delta^{2}H$ and $\delta^{18}O$ values were measured (Table S2, supporting information). Wheat samples were collected at 56 days post-seeding near the end of anthesis, prior to hardening. Each plant was collected whole and split into four portions: head, stems, leaves and root crown. Our previous pilot testing with spring wheat and the CVD-1 system had shown that the various plant portions gave markedly different stable isotope results from one another. Each portion was weighed prior to being sealed into their appropriate extraction method containers. Volumes of plant matter appropriate to each method of extraction were collected. Five replicates per plant portion, per method of extraction, were collected for a total of 30 samples per extraction method. During plant sampling, soil samples were collected in two locations bounding either side of the collected wheat samples. Soil samples were collected with five replicates per 10 cm depth to the bottom of the container for a total of 50 soil samples. Soil pore water was extracted using HPMS as per Orlowski et al.[3]

The plant samples were stored in glass vials for CVD, ME-OA-ICOS and centrifugation. For HPMS, the samples were stored in 250 mL Nalgene™ HDPE bottles. Care was taken to minimize the headspace in the sealed containers to limit mixing with atmospheric water vapour internal to the container over the storage period. The glass vials and Nalgene™ bottles were tightly sealed by lids, wrapped with Parafilm®, and stored in a cooler at 4°C to minimize potential evaporation during storage, prior to extraction. The DVE samples were stored in Ziploc® double zipper freezer bags and analyzed within 48 h of sampling. The extraction/equilibration protocols and method-specific preparations are described in detail below. To determine the extraction efficiency, prior to and after extraction, the initial plant and soil sample weights were compared with weights taken after extraction and again after a 24-h drying period at 105°C. All the extracted liquid samples were filtered with 0.45 μm disk filters and transferred to 2 mL glass vials, capped, and sealed with Parafilm® prior to isotopic analysis.

### 2.1 | Plant water extraction techniques and parameters

#### 2.1.1 | Direct vapour equilibration

The plant samples were macerated prior to being placed inside 945 mL Ziploc® double zipper freezer bags. The plant material quantities used per replicate were: 8.0 g of head material, 7.0 g of leaf material, 14.0 g of stem material, and 5.0 g of root material. We followed protocols developed by Wassenaar et al.[10] The bags were inflated with dry air immediately after sampling and allowed to equilibrate for 24 h at room temperature (~22°C) prior to analysis. Isotopic analysis of the bag headspace was carried out on a IWA-45EP OA-ICOS analyzer (Los Gatos Research Inc., San Jose, CA, USA) in vapour mode, which allows for continuous flow headspace sampling. In vapour mode this instrument measured $H_2O$ concentration and the $H_2O$ isotopologues at 5-s integration intervals. The headspace sampling apparatus was a 21 G stainless steel needle connected to a impermeable plastic line (1 m × 0.95 mm) that was attached to the input port of the LGR IWA-45EP analyzer. Prior to, and in between sampling of the headspace, the needle was connected to a Drierite laboratory gas drying unit (W.A. Hammond Drierite Co. Ltd, Xenia, OH, USA) until the internal water content of the IWA-45EP analyzer was below 2000 ppmV $H_2O$. The headspace of the plant and water standard bags was sampled by piercing the sample bag with the needle. Sampling took on average 2 min. The water standard bags were re-sealed with tape between analyses. The $\delta^{2}H$ and $\delta^{18}O$ values were noted when the measured headspace water content stabilized at ~28,000 ppmV $H_2O$ for at least 1 min. Two lab water standards of known isotopic composition were used to correct for drift. Standard one had isotopic composition −201‰ ($\delta^{2}H$ value) and −26.17‰ ($\delta^{18}O$ value), and standard two had isotopic composition 2.5‰ ($\delta^{2}H$ value) and − 0.1‰ ($\delta^{18}O$ value). A volume of 20 mL of the water standard was placed into a

945 mL Ziploc® bag, filled with dry air and allowed to equilibrate for 1 h prior to analysis. Water standards were alternated and analyzed every four samples. A third, known, control water sample was analyzed every eight samples as a means of checking our drift corrections. The control water isotopic composition was: −137.5‰ ($\delta^2$H value) and − 17.45‰ ($\delta^{18}$O value).

### 2.1.2 | Cryogenic vacuum distillation

The CVD-1 system comprised six separate extraction units, each with four extraction-collection lines. This allowed for the extraction of up to 24 samples at a time. Our modification to the original CVD-1[12] system included the addition of one extra extraction-collection line per extraction unit, and the removal of the nitrogen purging system. System protocols for sample extraction included heating the sample material to 100°C for 210 min under a baseline vacuum pressure of 0.1 Pa. The total volatile fraction in the plant samples was vaporized and collected in a liquid nitrogen cold trap. Once extractions were completed, the collected analyte was defrosted at room temperature, in sealed conditions, and the liquid was sampled for subsequent isotopic analysis. To ensure appropriate analytical volumes of extracted water, 6.0 g of head material, 6.0 g of leaf material, 11.0 g of stem material, and 5.0 g of root material were used per replicate.

The CVD-2 system used in this study was based on the design detailed in Koeniger et al.[13] This system was composed of independent extraction-collection units made up of two Exetainer® vials (Labco Ltd, Lampeter, UK) connected by a stainless steel capillary (2.00 × 0.95 mm). The samples were heated to 200°C for 15 min under a baseline vacuum pressure of 87.0 Pa. The volatile fraction in the plant sample was vaporized and collected in the second Exetainer vial, set in a liquid nitrogen cold trap. The samples were defrosted at room temperature in sealed conditions and the collected liquid was sampled for isotopic analysis. To ensure appropriate analytical volumes of extracted liquid 5.0 g of head material, 5.0 g of leaf material, 10.0 g of stem material, and 4.0 g of root material were used per replicate.

### 2.1.3 | Microwave extraction

For each plant portion extracted (head, leaf, stem, and root) a 5.0 g subsample of each replicate was macerated and distributed evenly on the base of the extraction vessel. Our ME-OA-ICOS system was constructed based on Munksgaard et al.[14] A model NN-ST6615 household microwave (Panasonic Corp., Kadoma, Japan) and extraction system was connected to an IWA-45EP OA-ICOS analyzer (Los Gatos Research). The system utilized microwave irradiation at 300 W for 15 min (equivalent to ~60–80°C); the volatile components were evaporated from the samples in a sealed container and passed into a dry air stream. This air stream moved through a cooled condensation chamber that controlled the vapor concentration and flow rate into the analyzer. To calibrate and correct for drift, liquid water standards were run for every fourth sample. The same water standards as were used in the DVE extractions were used in the ME-OA-ICOS extractions. For this step a piece of Whatman® 541 filter paper was placed inside the extraction vessel and 0.3 mL of standard water was added to the filter paper as per Munksgaard et al.[14] The same extraction procedure was used on the isotope standards. The $\delta^{18}$O and $\delta^2$H values were calculated by applying machine-specific corrections to the sample dataset, based on the protocols in Schmidt et al.[15]

### 2.1.4 | Centrifugation

We followed the centrifugation method of Peters and Yakir,[2] and the centrifugation vials were a modification of the vials that they used. The sampled plant material was placed in 15-mL centrifugation vials, which were then frozen with liquid nitrogen to limit evaporation from the plant matter during a subsequent 20-s maceration process.[2] After the maceration process the samples were re-warmed to room temperature under sealed conditions prior to centrifugation. A cap with a centrally located 3.0 mm hole, layered fine stainless-steel mesh, and two pieces of Whatman® grade 1 filter paper, was placed on the 15 mL centrifugation vials. These vials were then inverted and placed into a larger 50 mL centrifugation vial, thus allowing liquid to move from the sample and collect in the larger centrifugation vial. All plant samples were spun at 10,000 rpm (16,000 *g*) for 30 min at 4°C in an Avanti™ JXN-26 centrifuge (Beckman-Coulter Inc., Brea, CA, USA). Previous pilot testing had shown that for times less than 30 min, the extraction efficiencies were low, and for times beyond 30 min no more liquid could be extracted from the samples. To ensure appropriate analytical volumes of extracted liquid, 9.0 g of head material, 7.0 g of leaf material, 9.0 g of stem material, and 14.0 g of root material were used per replicate. The samples collected after initial centrifugation contained high amounts of co-extracted organic compounds as noted by a dark discoloration (Figure 1). Particulate matter was removed from the analyte by a second centrifugation step in an model 5804 centrifuge (Eppendorf, Hamburg, Germany) at 11,000 rpm (~8000 *g*) for 1 h. The samples were then filtered with 0.45-µm disk filters into 2-mL glass vials prior to isotopic analysis. The samples retained the noted color after the second centrifugation and filtering.

### 2.1.5 | High-pressure mechanical squeezing

Our HPMS approach followed Bottcher et al.[11] The squeezers were composed of a stainless-steel chamber, a porous titanium filter disk overlaying the exit port, and a stainless-steel piston, which applied compression to the top of the sample. Both plant and soil samples that underwent HPMS extraction were placed in the chamber and squeezed at a pressure of 10,000 psi (69.8 MPa) for 24 h. Liquid expelled by the plants and soils was collected via a syringe attached to the output port, filtered through a 0.45 µm syringe filter and stored in 2 mL glass vials prior to isotopic analysis. Care was taken to prevent the samples from contacting the atmosphere during the extraction process. To ensure appropriate sample volumes, 6.0 g of head material, 5.0 g of leaf material, and 10.0 g of stem material were used per replicate. The root sample replicates did not produce enough liquid with this method of extraction and they were combined into a single composite sample.

## 2.2 | Water analyses

### 2.2.1 | Isotope analyses

To rule out any potential analytical differences, when possible the extracted liquid samples were analysed by both OA-ICOS and IRMS. It has been noted in previous studies that liquid samples extracted

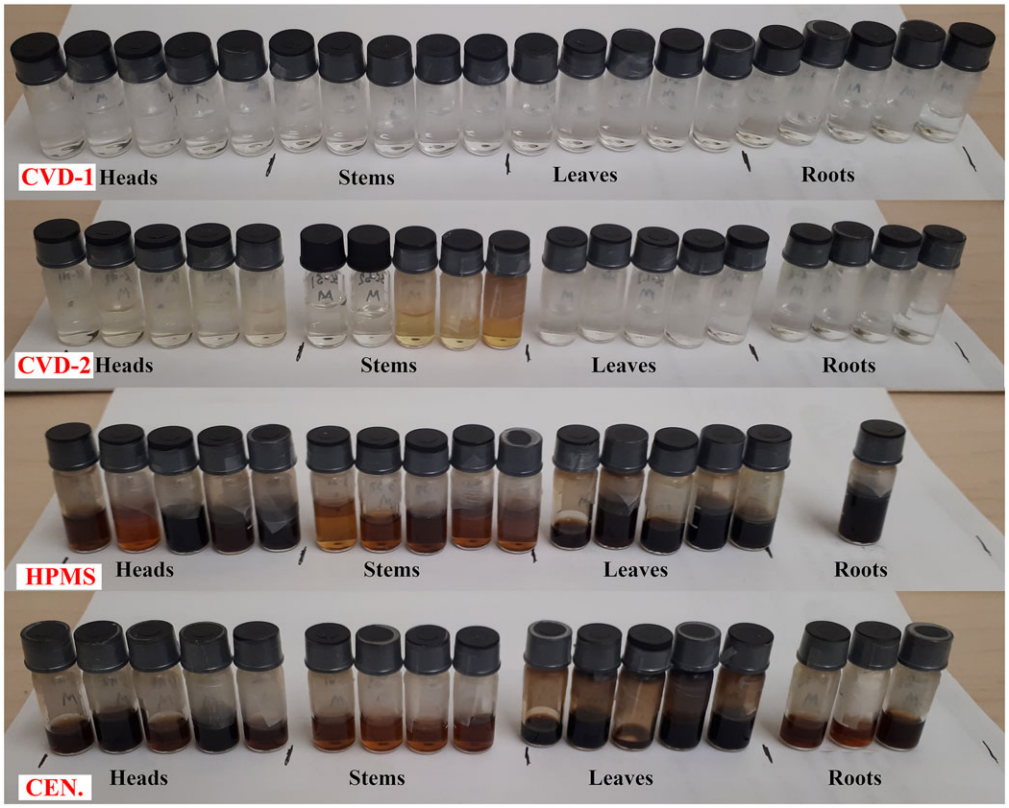


**FIGURE 1** Photographic compilation of liquid extracted from cryogenic vacuum distillation (CVD-1 and CVD-2), high-pressure mechanical squeezing (HPMS), and centrifugation (CEN), showing colour variations linked to co-extracted organics. Note: photos were taken after subsampling for analysis occurred, so volumes are not representative of total collected analyte [Color figure can be viewed at wileyonlinelibrary.com]

from plants often contain co-analytes such as methanol, ethanol, phenolics, acids, terpenes, sugars, proteins, and glycols.[16-18] These co-analytes can interfere with spectrometric measurements but are believed to be in low enough concentrations that they do not affect mass-based analysis systems. We note that previous work has shown that the two major isotope ratio infrared spectroscopy (IRIS) analysis systems, cavity ring down spectroscopy (CRDS) (Picarro, Santa Clara, CA, USA) and OA-ICOS (LGR), yield significantly different results when exposed to the same amounts of methanol and ethanol contamination.[14,17,18] The centrifugation and HPMS samples could not be analyzed by OA-ICOS due to the high content of co-extracted compounds. The highly viscous nature of the centrifuged and squeezed samples prevented proper injection into the sampling chamber of the OA-ICOS analyzer. Extracts from soil samples were analyzed only on the OA-ICOS system as they were not flagged for organic contamination during OA-ICOS analysis.

For OA-ICOS analyses, a IWA-45EP OA-ICOS analyzer (Los Gatos Research) was used with an accuracy of ±1.0‰ for $\delta^2H$ values and ± 0.2‰ for $\delta^{18}O$ values. For water vapour measurements, the ME-OA-ICOS and DVE samples were analyzed on the IWA-45EP system. In vapour mode, for a 30 s reading period, the results were accurate to ±1.8 for $\delta^2H$ values and ± 0.3 for $\delta^{18}O$ values. The isotopic data for soil and plant extracts from the OA-ICOS system were checked for spectral contamination using the Spectral Contamination Identifier (LWIA-SCI) post-processing software (Los Gatos Research Inc.) All the plant samples extracted with the CVD-1 and CVD-2 systems, analyzed on the IWA-45EP, were flagged for narrow band and broad band spectral contamination, noting the presence of organic contaminants.

For IRMS analyses, an Isoprime isotope ratio mass spectrometer (Elementar UK Ltd, Cheadle Hulme, UK) was used. The hydrogen isotopic compositions were determined by reduction of water to hydrogen by reaction with elemental chromium, following the method of Morrison et al.[19] The system protocol follows: 0.8 µL of water was injected into a quartz reactor containing elemental chromium at 1030°C, and the resultant $H_2$ gas was separated on a 1-mmol sieve gas chromatography column and introduced into the Isoprime isotope ratio mass spectrometer. For the CVD-1 samples, the memory effects were reduced by injecting two replicates of each sample and discarding the first measurement. The CVD-2, centrifuge and HPMS samples were injected between two and six times, due to the more viscous nature of the liquid and the high content of co-extracted organic content. In this case, we then discarded all but the last two injection results. The resultant raw delta values of the measured hydrogen were normalized to the VSMOW-SLAP[20] scale by analyses of two calibrated waters, INV1 and ROD3, with $\delta^2H$ values of −218‰ and − 4‰, respectively. For oxygen isotopes, we used the $CO_2$-$H_2O$ equilibration technique of Epstein and Mayeda.[21] A Micromass multi-flow device (Waters Corp., Milford, MA, USA) was connected to the Isoprime isotope ratio mass spectrometer at 25°C for all $CO_2$-$H_2O$ equilibrations. The results are reported relative to the VSMOW-SLAP scale by normalizing to INV1 and ROD3, with $\delta^{18}O$ = −28.5‰ and − 1.0‰, respectively, as above. The mass

spectrometer used in this study had a precision of ±1.5‰ for $\delta^2H$ values and ± 0.3‰ for $\delta^{18}O$ values.

The isotope ratios (R) for $\delta^2H$ ($^2H/^1H$) and $\delta^{18}O$ ($^{18}O/^{16}O$) are expressed in per mil (‰) relative to Vienna Standard Mean Ocean Water (VSMOW), defined as follows:

$$\delta^2H \text{ or } \delta^{18}O = (R_{SAMPLE}/R_{STANDARD} - 1) \quad (1)$$

where $R_{SAMPLE}$ is the $^2H/^1H$ or $^{18}O/^{16}O$ ratio of the sample, and $R_{STANDARD}$ is the $^2H/^1H$ or $^{18}O/^{16}O$ ratio of the VSMOW standard.[20,22]

### 2.2.2 | Quantifying methanol and ethanol content

As there is currently no appropriate method, such as the z-scores used by Wassenaar et al.,[23] for gauging extraction system accuracy when applied to plant water extractions, we discuss accuracy in terms of the purity of water extracted by that system. The inability to utilize a z-score type method comes from the difficulty in defining a reference water value in studies of this type. Since co-extracted organic compounds are known to cause errors in stable isotope results when extracted liquids are analyzed by spectrometric methods (CRDS/OA-ICOS),[17,18] any extraction system that produces a low-purity analyte has the potential to be less accurate than those that produce extracted water of a higher purity. Liquid samples from CVD-1, CVD-2, HPMS and centrifugation systems were analyzed for methanol and ethanol content, as these were the two most often noted co-extracted compounds causing spectral contamination during analysis on laser-based (CRDS and OA-ICOS) spectrometers.[17,18] During the analysis of the DVE and ME-OA-ICOS samples, we closely monitored the absorption plots produced by the LGR IWA-45EP OA-ICOS analyzers. This plot, produced in real time during analysis, will show fluctuations between absorption peaks when spectral contaminants are present in the analysis stream. A further tool for assessing the presence of spectral contaminants during analysis on LGR systems is the 'Spectra Fit Residuals plot' (SFR), which has higher sensitivity to the presence of contaminants than the absorption plots, although this plot was not available for use during this study. The Saskatchewan Research Council-Environmental Analytics Laboratory analyzed liquid water samples, via a gas chromatography/flame ionization detector (GC/FID) method for methanol and ethanol content. The results are reported in µg/mL.

### 2.2.3 | Statistical analyses

Analysis was carried out with the statistical software R 3.3.2.[24] To quantify consistency and repeatability, the mean and standard deviation (sd) were calculated for the replicates of the various plant portions across all methods of extraction, and for both systems of analysis. Standard dual isotope plots [$\delta^{18}O$, $\delta^2H$] were used to discern general trends.

Plant and soil water stable isotope data was tested for normality using histograms, quantile-quantile plots, and the Shapiro–Wilk normality test.[25,26] The plant water data, grouped by extraction method, was found to be normally and non-normally distributed. Thus, a non-parametric Kruskal-Wallis (one-way analysis of variance (ANOVA) on ranks) test[27,28] was used to determine if the population medians, separated by plant portion extracted (head, leaf, stem, and root crown), were statistically similar across the extraction methods used in this study. Subsequently, Dunn's test[29] was used to determine which of the extraction methods produced significantly different results for the plant portion in question ($p \leq 0.05$). To control false discovery rates and adjust p-values, the Benjamini-Hochberg adjustment was used.[30] The soil water data was found to be normally distributed by the Shapiro–Wilk test. However, since normality tests do not have the power to reject Ho for small sample sizes, these small samples will often pass normality tests.[26] The Q-Q normal plots of the soil data implied non-normality. Thus, the non-parametric Mann–Whitney-Wilcox test[31,32] was used to determine which of the soil layers were significantly different from one another at a 95% confidence interval ($p \leq 0.05$).

## 3 | RESULTS

Table 1 summarizes the descriptive statistics for the extracted isotopic ratios of all plant portions, for all methods of extraction, and for each method of isotopic analysis (OA-ICOS and IRMS). The stable isotope signatures of irrigation water used in this study were − 128.25‰ (sd: 0.65, $n$ = 18) for $\delta^2H$ values and − 15.80‰ (sd: 0.11, n = 18) for $\delta^{18}O$ values. For the $\delta^2H$ results, the CVD-1 and CVD-2 systems were the most repeatable across the various plant portions, except for the stem results for CVD-2. The DVE, centrifugation, and HPMS methods were moderately reproducible, decreasing in reproducibility in that order. The ME-OA-ICOS system was the least reproducible with the highest and most consistently unacceptable standard deviations. For the $\delta^{18}O$ results, CVD-1 had the most reproducible results, followed by centrifugation, HPMS, DVE, CVD-2 and ME-OA-ICOS. Across all plant portions, CVD-1 had an sd range of ±0.86 to 2.87 for $\delta^2H$ values and ±0.34 to 1.22 for $\delta^{18}O$ values. CVD-2 had an sd range of ±1.50 to 15.70 for $\delta^2H$ values and ±1.52 to 8.75 for $\delta^{18}O$ values. DVE had sd ranges of ±1.48 to 6.92 for $\delta^2H$ values and ±0.64 to 3.41 for $\delta^{18}O$ values. The centrifugation sd ranges were ±0.88 to 6.78 and ±0.30 to 1.45 for $\delta^2H$ and $\delta^{18}O$ values, respectively. HPMS had sd ranges of ±4.23 to 6.92 and ±0.58 to 0.95 for $\delta^2H$ and $\delta^{18}O$ values, respectively. Finally, ME-OA-ICOS had sd ranges of ±5.50 to 13.70 for $\delta^2H$ values and ±1.27 to 2.91 for $\delta^{18}O$ values.

Figure 2 summarizes the stable isotope results for the extracted soil profile water. Analysis by the Mann–Whitney-Wilcox test showed that the soil was split into significantly different horizons. The $\delta^2H$ data shows the 0–10 cm, 10–40 cm, and 40–50 cm layers to be significantly different from one another. The $\delta^{18}O$ data shows the 0–10 cm and 10–50 cm layers to be significantly different from one another. All the layers were more enriched in heavier isotopes than the irrigation water and were progressively enriched in the heavier isotopes towards the soil surface with the 0–10 cm layer being most enriched for both $^2H$ and $^{18}O$.

### 3.1 | Plant portion and extraction method differences

The plant portions sampled followed a similar pattern of enrichment/depletion of measured isotopes through all systems of extraction. Figures 3 and 4 show the irrigation, plant, and soil water results in

**TABLE 1** Means and standard deviations (sd) of extracted $\delta^2H$ and $\delta^{18}O$ values from spring wheat samples for four different plant portions: head, leaf, stem, and root crown, measured via OA-ICOS and IRMS

| | OA-ICOS | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Head | | | | Leaf | | | | Stem | | | | Root Crown | | | |
| | $\delta^2H$ [‰] | | $\delta^{18}O$ [‰] | | $\delta^2H$ [‰] | | $\delta^{18}O$ [‰] | | $\delta^2H$ [‰] | | $\delta^{18}O$ [‰] | | $\delta^2H$ [‰] | | $\delta^{18}O$ [‰] | |
| | Mean | sd | Mean | sd | Mean | sd | Mean | sd | Mean | sd | Mean | sd | Mean | sd | Mean | sd |
| CVD-1 | −83.12 | 2.19 | −0.09 | 0.31 | −80.11 | 2.81 | −1.12 | 1.02 | −125.60 | 1.01 | −12.59 | 0.34 | −115.40 | 0.93 | −11.95 | 0.34 |
| CVD-2 | −74.90 | 2.83 | 0.02 | 0.82 | −74.41 | 1.50 | 1.36 | 1.75 | −112.99 | 15.70 | −5.15 | 8.53 | −107.73 | 2.61 | −9.45 | 1.25 |
| DVE | −64.19 | 6.92 | 3.83 | 3.41 | −64.96 | 1.48 | 0.98 | 0.65 | −113.82 | 4.33 | −11.32 | 0.81 | −99.39 | 3.72 | −9.20 | 0.64 |
| ME-OA-ICOS | −37.32 | 5.50 | 5.78 | 1.27 | −33.86 | 13.70 | 1.99 | 2.91 | −95.93 | 6.72 | −11.68 | 1.70 | −91.14 | 5.92 | −6.97 | 1.74 |
| | IRMS | | | | | | | | | | | | | | | |
| | Head | | | | Leaf | | | | Stem | | | | Root Crown | | | |
| | $\delta^2H$ [‰] | | $\delta^{18}O$ [‰] | | $\delta^2H$ [‰] | | $\delta^{18}O$ [‰] | | $\delta^2H$ [‰] | | $\delta^{18}O$ [‰] | | $\delta^2H$ [‰] | | $\delta^{18}O$ [‰] | |
| | Mean | sd | Mean | sd | Mean | sd | Mean | sd | Mean | sd | Mean | sd | Mean | sd | Mean | sd |
| CVD-1 | −81.79 | 1.28 | −1.10 | 0.41 | −82.08 | 2.87 | −1.88 | 1.05 | −127.28 | 2.35 | −13.03 | 0.34 | −115.58 | 0.86 | −12.26 | 0.35 |
| CVD-2 | −77.20 | 3.51 | 3.59 | 4.39 | −80.85 | 2.22 | −1.14 | 1.44 | −120.95 | 3.24 | −9.72 | 3.04 | −107.57 | 1.56 | −5.16 | 4.36 |
| HPMS | −67.40 | 4.24 | −1.34 | 0.80 | −76.36 | 6.92 | −3.91 | 0.58 | −107.69 | 4.23 | −14.95 | 0.95 | −101.32 | n.v | −12.69 | n.v |
| Centrifugation | −79.12 | 0.88 | −2.38 | 0.53 | −66.31 | 5.89 | −0.07 | 0.30 | −99.89 | 3.15 | −11.53 | 0.51 | −93.17 | 6.78 | −8.99 | 1.45 |

n.v.: indicates no data for this sample.

| Depth (cm) | Soil Water Stable Isotope Values | | | |
|---|---|---|---|---|
| | δ2H [‰] | | δ18O [‰] | |
| | Mean | sd | Mean | sd |
| 0 -10 | -101.97 | 3.37 | -10.12 | 0.75 |
| 10-20 | -110.38 | 1.02 | -11.99 | 0.30 |
| 20-30 | -110.37 | 1.10 | -12.11 | 0.35 |
| 30-40 | -111.27 | 0.61 | -12.53 | 0.22 |
| 40-50 | -113.43 | 1.21 | -12.92 | 0.19 |

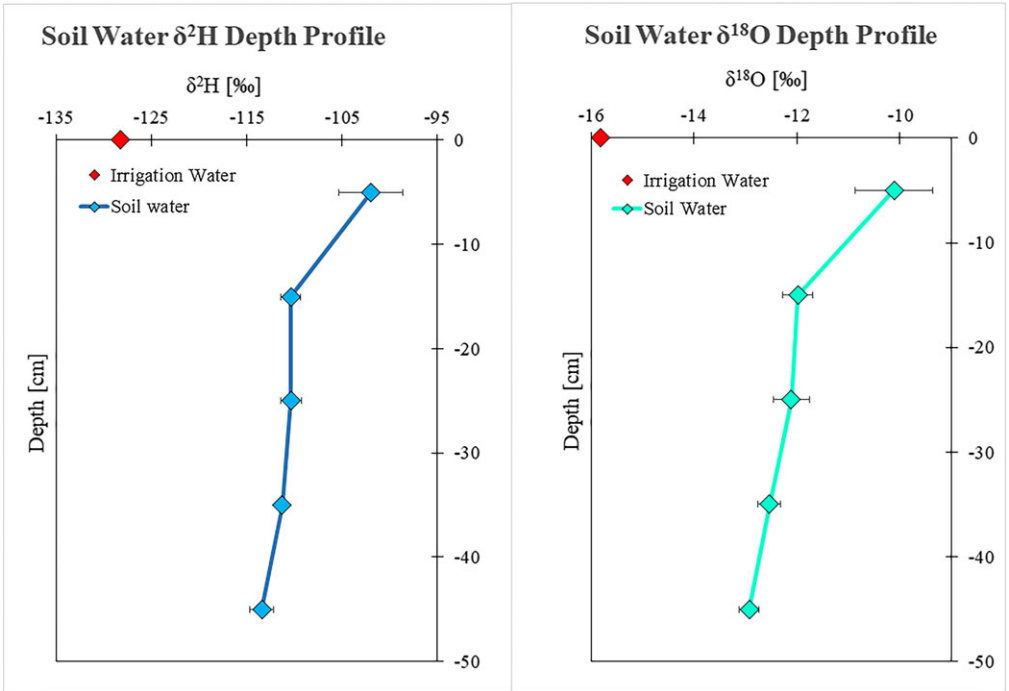


**FIGURE 2** Stable isotope means and standard deviations (sd) of extracted soil pore water ($n$ = 10/depth), grouped by significantly different layers ($p \leq 0.05$), with associated soil water stable isotope depth profiles. Water extracted via HPMS and analyzed via OA-ICOS [Color figure can be viewed at wileyonlinelibrary.com]

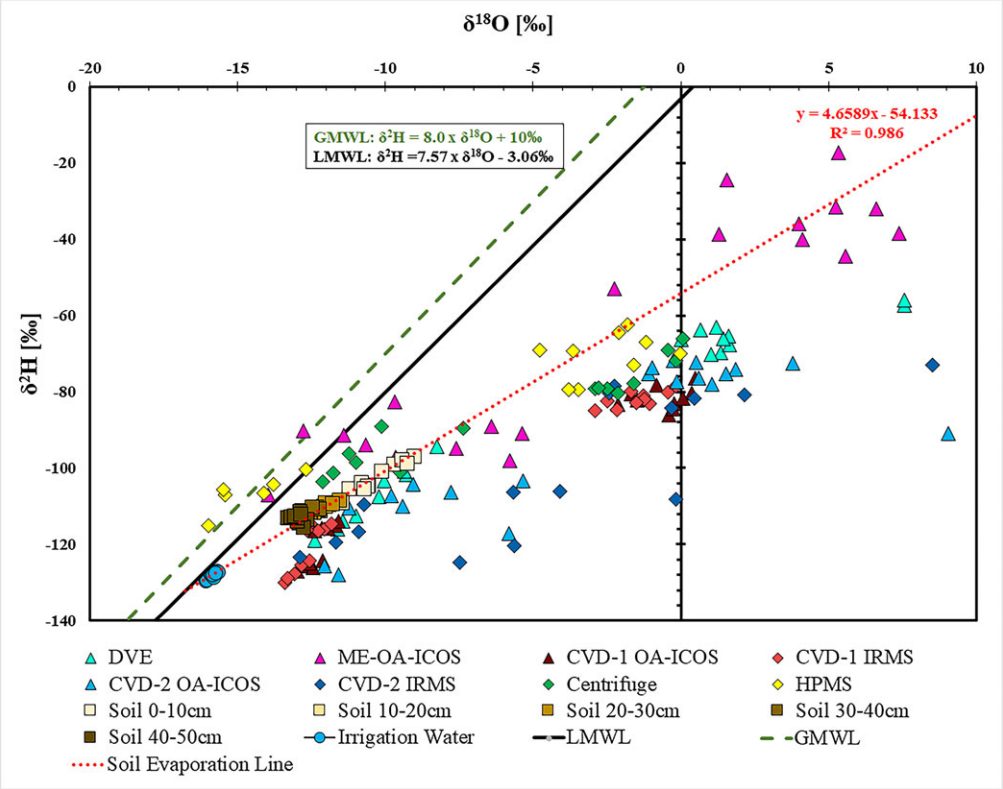


**FIGURE 3** Dual isotope plot of all results: irrigation water, extracted soil water (via HPMS), and liquid analyte extracted from plant samples for all methods of extraction-analysis. Cryogenic vacuum distillation system results (CVD-1 and CVD-2) are grouped by analysis system used (OA-ICOS and IRMS) [Color figure can be viewed at wileyonlinelibrary.com]

standard dual isotope space. For the plant water samples, the stem results were the most depleted in heavier isotopes ($^{2}H$, $^{18}O$) over all extraction systems. The root crown results were more enriched in $^{2}H$ and $^{18}O$ than the stems and tended to closely resemble the 0–10 cm soil layer $\delta^{2}H$ and $\delta^{18}O$ values, except for the CVD-1 system root crown results, which more closely resembled the 20–50 cm soil layers. The leaf and head plant water were the most enriched in $^{2}H$ and $^{18}O$ for all extraction systems. The leaf and head results were similar to each other for all extraction systems, except in the case of the HPMS and centrifuge results. For HPMS, the heads were more enriched in $^{2}H$ and $^{18}O$ than the leaves. For centrifugation the opposite was true, with the leaf water results being more enriched in $^{2}H$ and $^{18}O$ than the heads.

## 3.2 | Co-extracted organics and resultant differences between OA-ICOS and IRMS analyses

Only the CVD-1 and CVD-2 methods produced a liquid analyte that could be run on the two methods of liquid analysis: OA-ICOS and IRMS. The other methods of extraction either did not produce liquid, as in the case of DVE and ME-OA-ICOS, or produced a liquid that was too viscous, due to co-extracted compounds, to be run on the OA-ICOS system. The methanol and ethanol content of water from all extraction systems that provided liquid analyte is summarized by plant portion in Table 2. For the co-extracted ethanol content, HPMS and centrifugation produced the highest concentrations, followed by CVD-2 and then CVD-1. For the co-extracted methanol content, HPMS and centrifugation again produced the highest concentrations, followed by CVD-2. CVD-1 produced one to two orders of magnitude less methanol than the other systems.

For results from the CVD-1 extraction system, stable isotope differences between methods of analysis (IRMS vs OA-ICOS) for all plant portions ranged from 0.03‰ to 5.10‰ (average: 1.54‰, sd: 1.23) for $\delta^{2}H$ values and from 0.21‰ to 1.66‰ (average: 0.78‰, sd: 0.44) for $\delta^{18}O$ values. For the CVD-1 system, the methanol and ethanol content was highest in the heads, followed by stems, leaves, and then root crowns. While the CVD-1 system results were flagged for narrow and broad band contamination on the OA-ICOS systems, the presence of co-extracted methanol and ethanol did not appear to be large enough to significantly skew the results between the two analysis systems (IRMS vs OA-ICOS). Although the boiling points of methanol and ethanol are 64.7°C and 78.2°C, respectively, the extraction temperature of 100°C used by the CVD-1 system did not appear to be sufficient to co-extract enough methanol/ethanol to negatively affect the results of OA-ICOS analysis for spring wheat. For the CVD-2 extraction system, the differences between methods of analysis (IRMS vs OA-ICOS) for all plant portions ranged from 0.61‰ to 28.61‰ (average: 6.26‰, sd: 5.95) for $\delta^{2}H$ values, and from 0.24‰ to 20.73‰ (average: 6.13‰, sd: 5.62) for $\delta^{18}O$ values. The methanol and ethanol content extracted by the CVD-2 system was nearly an order of magnitude higher for all plant portions than that extracted by the CVD-1 system, and this is probably due to the higher extraction temperature of 200°C. For the CVD-2 system, the ethanol content was highest in the heads, followed by stems, leaves, and then root crowns. However, the methanol content was highest in stems, followed by heads, leaves, and root crowns. The results of the CVD-2 system were also flagged for

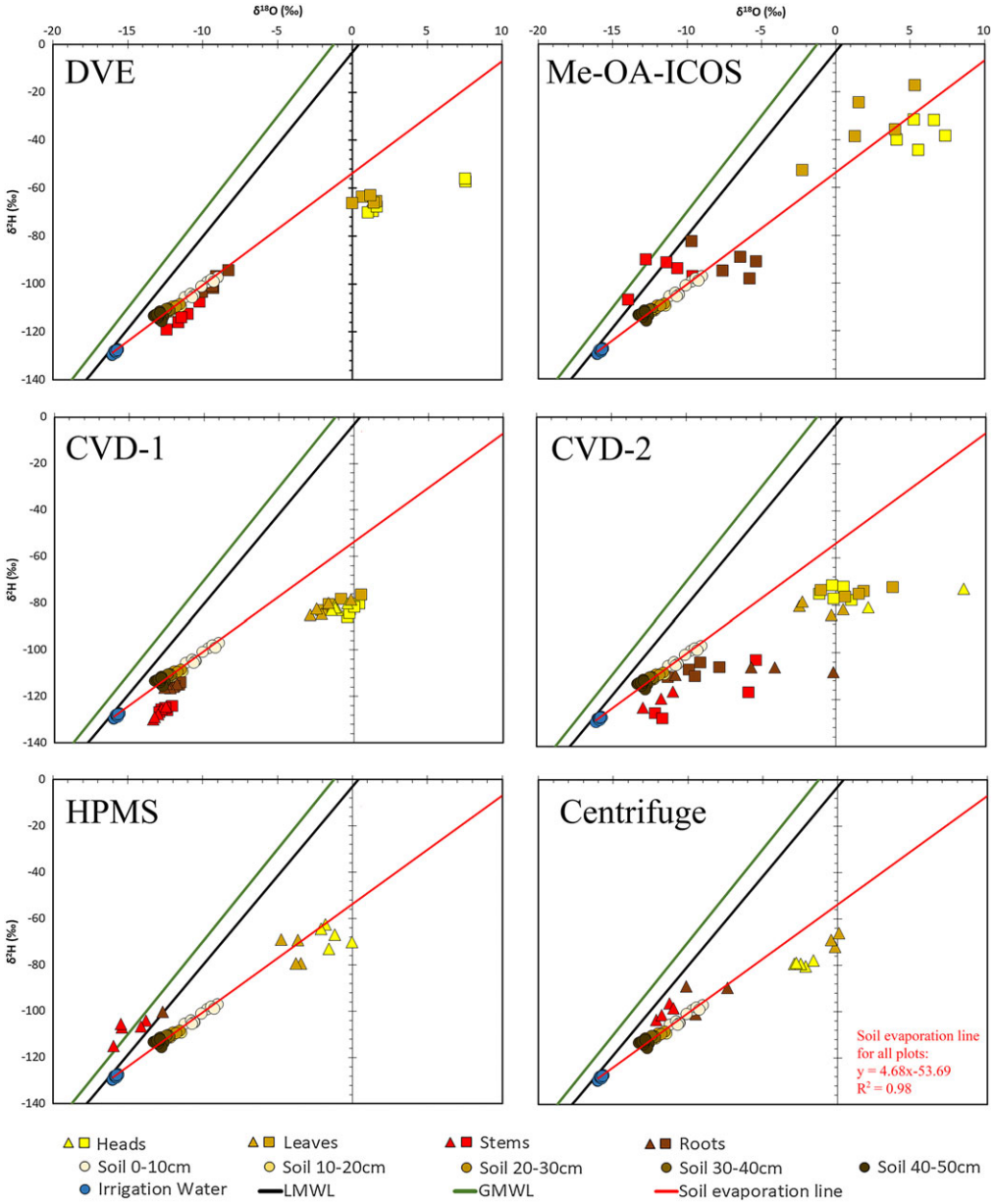


**FIGURE 4** Dual isotope plots of extracted plant analyte for all methods of extraction. DVE, ME-OA-ICOS, CVD-1, and CVD-2 analytes were analyzed via OA-ICOS (square markers). HPMS, centrifuge, CVD-1 and CVD-2 analytes were analyzed via IRMS (triangle markers). Extreme outliers were removed from the centrifugation results (n = 1, leaf), the CVD-2-IRMS results (*n* = 2, 1, 2: head, leaf, stem, respectively) and the CVD-2-OA-ICOS results (n = 1, stem) [Color figure can be viewed at wileyonlinelibrary.com]

narrow and broad band contamination on the OA-ICOS analysis system and we believe that the larger differences between the CVD-2 IRMS and OA-ICOS results are probably a byproduct of these co-extracted volatile organic compounds (VOCs). Centrifugation and HPMS co-extracted methanol and ethanol in quantities one to two orders of magnitude higher than the CVD-1 method. In addition, depending on plant portion, the centrifuge and HPMS systems typically produced more methanol and ethanol than the CVD-2 system. We expected minimal amounts of methanol and ethanol to be present in the headspace of DVE samples as the DVE extraction-analysis step takes place at room temperature (~22°C). At this temperature, based on equilibrium thermodynamics of partial pressures, minimal volumes of methanol and ethanol would be volatized during the equilibration of plant water with the dry headspace of the extraction vessel. To this point, the DVE samples did not show evidence of spectral contamination on the LGR IWA-45EP OA-ICOS analyzer absorption plot during analysis. However, this is not conclusive evidence for the lack of contaminants in the headspace of plant samples analyzed via DVE. Further research is needed to quantify volumes of organic contaminants potentially present in the headspace of DVE samples.

## 3.3 | ANOVA

The Kruskal-Wallis one-way ANOVA by ranks test demonstrated significant differences in the isotopic results between the methods of extraction for each plant portion. The results of this test are summarized in Figure 5. Overall, for all plant portions (PP) and isotope ratios, the ME-OA-ICOS system had the greatest number of

**TABLE 2** Results of methanol and ethanol content analysis by GC-FID of liquid analyte from all extraction systems that produced liquid analyte

| Extraction method and plant portion | Ethanol (μg/mL) | Methanol (μg/mL) |
|---|---|---|
| CVD-1 – Head | 4700 | 56 |
| CVD-2 – Head | 22000 | 690 |
| HPMS – Head | 22000 | 1900 |
| Centrifuge – Head | 30000 | 1100 |
| CVD-1 – Leaf | 150 | 44 |
| CVD-2 – Leaf | 7400 | 530 |
| HPMS – Leaf | 8700 | 870 |
| Centrifuge – Leaf | 1700 | 690 |
| CVD-1 – Stem | 1700 | 30 |
| CVD-2 – Stem | 8200 | 1100 |
| HPMS – Stem | 16000 | 1290 |
| Centrifuge – Stem | 15000 | 1400 |
| CVD-1 – Root | <5 | <5 |
| CVD-2 – Root | 670 | 190 |
| HPMS – Root | 2800 | 120 |
| Centrifuge – Root | n.v. | n.v. |

A result of <5 signifies that methanol or ethanol content was below detection level.

n.v.: signifies no sample was supplied for analysis.

significantly different results, followed by CVD-1, HPMS, centrifuge, DVE, and finally the CVD-2 system. Generally, for $\delta^2H$ and $\delta^{18}O$ values, the CVD systems produced results that were similar to each other for all PP. The CVD-2 system (OA-ICOS and IRMS results) produced the least number of significantly different results across all PP, but the CVD-2 OA-ICOS results had fewer significant differences than the CVD-2 IRMS results for all PP. Again, we note the relatively higher amount of co-extracted VOCs in the CVD-2 samples, which may account for the differences between the OA-ICOS and IRMS results. The CVD-1 OA-ICOS and IRMS results had more significant differences for $\delta^2H$ than $\delta^{18}O$ results across all PP and were inconsistently different depending on the plant portion of interest. The mechanical methods HPMS and centrifugation produced results which were not significantly different for $\delta^2H$ values. However, for leaf and stem portions, the $\delta^{18}O$ results were significantly different between these two methods. HPMS results had far more significant differences for $\delta^{18}O$ than for $\delta^2H$ values. The opposite was true for centrifugation which provided a greater number of significantly different results for $\delta^2H$ than for $\delta^{18}O$ values. Although the ME-OA-ICOS and CVD systems are based on similar mechanisms, the ME-OA-ICOS results tended to be consistently different from the CVD-1 and CVD-2 results. The ME-OA-ICOS system produced more significantly different results for $\delta^2H$ than for $\delta^{18}O$ values. Overall, the DVE method produced the second lowest number of significantly different results. DVE had a similar number of significant differences for both $\delta^2H$ and $\delta^{18}O$ results and it produced results that were inconsistently different for both isotope ratios depending on the plant portion sampled. When DVE was compared with the standard method, cryogenic vacuum distillation, it produced results that were not significantly different from those obtained by the CVD-2 system for all PP, except for $\delta^2H$ results from leaf tissue. Consistent differences between DVE and CVD-1 $\delta^2H$ values were noted, but the $\delta^{18}O$ results were consistently similar for these methods.

### 3.4 | Extraction efficiencies and sample throughput

The CVD-1 and CVD-2 systems had extraction efficiencies of 99.8% (sd: 1.26) and 98.1% (sd: 0.34), respectively. The ME-OA-ICOS method had an average extraction efficiency of 98.2% (sd: 2.27). Thus, for methods utilizing heat to extract analyte from samples, we are confident that no Rayleigh fractionation processes would have affected our results. The HPMS system had an average extraction efficiency of 82.1% (sd: 9.24). Increasing the pressure beyond 69.8 MPa was not possible with our system and extracting samples for longer than the 24 h noted in the HPMS protocol did not increase the extraction efficiency. Centrifugation had the lowest extraction efficiencies at 48.9% (sd: 9.60) on average. We found that increasing the rotational speed and duration beyond 10,000 rpm (16,000 *g*) and 30 min, respectively, did not further improve extraction efficiencies.

The sample throughput rates for the extraction/analysis methods are calculated per 8-h period. DVE had the highest sample throughput with 74 samples followed by the CVD-2 system with 60 samples. We note that the CVD-2 throughput rate is a product of heating block specifications and could be improved with a larger heating apparatus. Centrifugation had a throughput of 38 samples. The CVD-1 system had a throughput of 24 samples, which is dependent upon the number of extraction-collection lines added into the system. We note that the addition of extra extraction-collection lines to this system results in diminishing returns on sample throughput, as additions increase the setup and post-extraction sampling time. The ME-OA-ICOS system requires a cool-down period after each sample resulting in a throughput of 16 samples. The HPMS system had the lowest sample throughput which is a byproduct of requiring 24 h to extract one sample. Sample throughputs with the HPMS system could be increased by adding more squeezing units. We had access to three squeezing units for this study and could process three samples every 24 h. At the end of the extraction period described here, only the DVE system immediately provides isotopic ratio results. The cryogenic systems, HPMS and centrifugation, provide a liquid that will then require another 24–48 h of analysis on either the IRMS or the OA-ICOS analyzer before isotopic values are available. Both IRMS and OA-ICOS analyzers also have sample throughput rates that limit the speed of data acquisition. The ME-OA-ICOS system also provides delta values and isotopic ratios during analysis. However, the raw ME-OA-ICOS data must first go through a post-processing integration and correction step, before it is useful.

## 4 | DISCUSSION

### 4.1 | Methodological controls on accessible water pools within the plant

The methods of extraction used in this study can be split into two groups, defined by the way that they extract water from a sample: (1) 'mechanical methods' such as HPMS and centrifugation that use

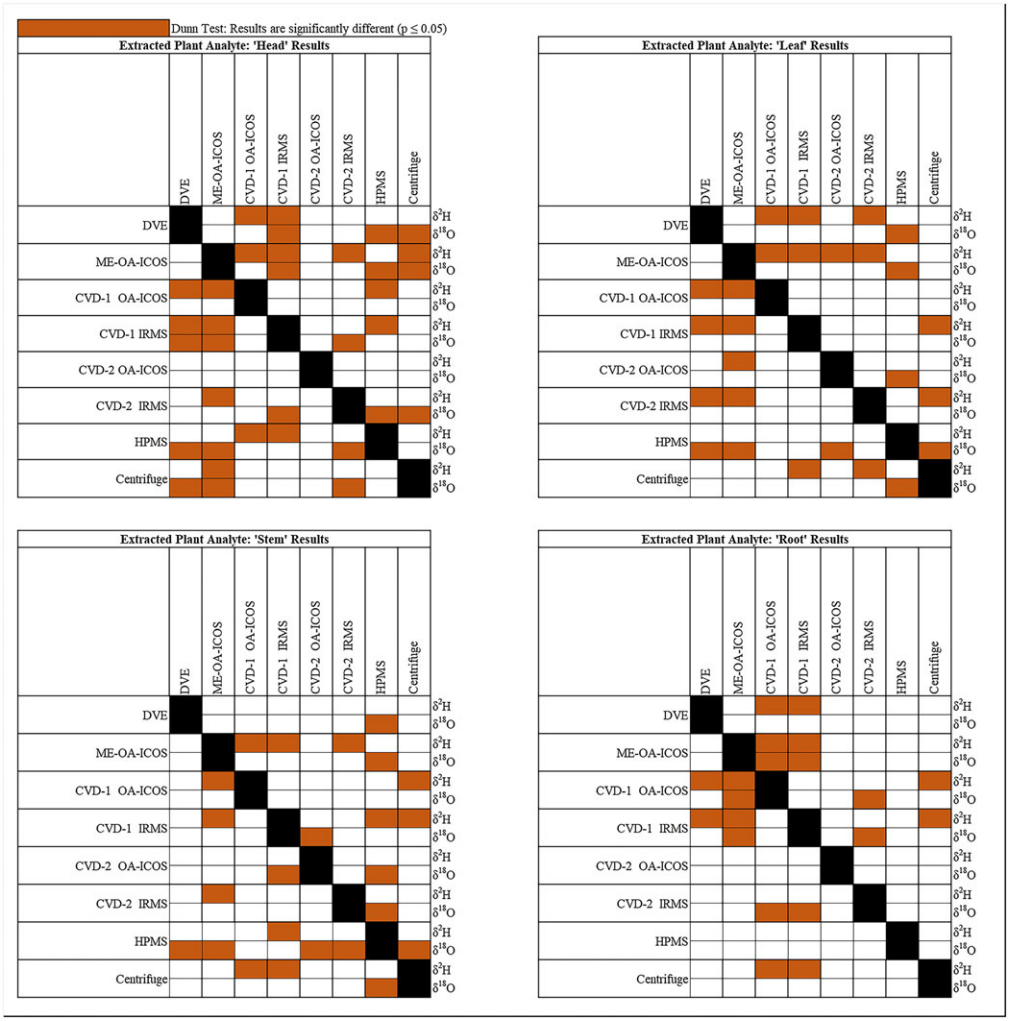


**FIGURE 5** Collected statistical results detailing significant differences ($p \leq 0.05$) between extraction methods per plant portion extracted for $\delta^2H$ and $\delta^{18}O$ values. Statistical results produced by the post-hoc Dunn test utilizing the Benjamini-Hochberg adjustment to prevent false discovery rates [Color figure can be viewed at wileyonlinelibrary.com]

physical force to push liquid out of the samples and (2) 'phase change methods' such as CVD-1, CVD-2, ME-OA-ICOS and DVE. Cryogenic and microwave methods rely on heat and pressure changes to volatize the liquid in the sample and pull it out of the sample for collection/analysis. The DVE system is unique in that while it relies on the phase change of water, no heat or pressure changes are used to induce this phase change, only equilibrium processes.

It is also possible to group the methods of extraction by the water pools and the hydrogen and oxygen pools within the plant that they are capable of accessing. Here we define the total water pool as all available water within a plant sample, that is: the relatively more mobile xylem and inter-cellular water; and the relatively less mobile intra-cellular, cell wall, and organelle constrained water. The mobile, transpiration water, is defined as that which is taken up by roots and rapidly moved through the xylem to sites of transpiration. Depending upon the path of water molecules through the plant, (diffusional vs bulk flow), it will have greatly different residence times. This is important as we know that precipitation isotope signals and available soil water isotope signals change seasonally. Thus, a mixture of stable isotope signals connected to different uptake periods in time will be present simultaneously in the total water isotope signal of a plant. For studies interested in investigating the sourcing of water by plants, it is critical to be able to connect plant water isotope signals with the slice of time in which the plant sampling occurred.

For hydrogen and oxygen pools within plants, it has been previously noted that isotopic fractionation of H and O occurs during primary production.[33] In the case of hydrogen, plants have been shown to preferentially use $^1H$ in the production of metabolic and other organic compounds. For oxygen, it is the isotopic composition of the available plant water at the time of its production that determines the $\delta^{18}O$ signature of organically bonded cellulose.[33] These isotopic signals are thus laid down in cellulose and other plant organic compounds during their production and may be accessible by virtue of the way that water is extracted by our tested extraction systems.

The CVD-2, CVD-1 and ME-OA-ICOS methods extract up to 99% of the water in a sample, and are thus accessing total plant water. This may serve to produce results that are more depleted in $^2H$ and $^{18}O$, due to extraction of the water and H/O pools that are more enriched in $^1H$ (depleted in $^2H$). Indeed, our results for both CVD-1 and CVD-2 extraction systems were depleted in $^2H$ and $^{18}O$ relative to the all systems average. HPMS and centrifugation are also capable of accessing

the total plant water pool; however, they do so through destruction of tissue, bursting cell walls and organelles and allowing the water contained therein to be extracted along with the more mobile xylem and inter-cellular water. This destruction of tissue does, however, mean that the HPMS and centrifugation systems are also accessing organic-compound-bound hydrogen and oxygen pools. These organic compound pools may not be the same as those extracted by the CVD and ME-OA-ICOS systems, and thus their effect on $\delta^2H$ and $\delta^{18}O$ values may also be different. The high contents of co-extracted compounds produced by HPMS and centrifugation are observed in Figure 1 and Table 2.

## 4.2 | On the accuracy of extraction methods based on co-extracted organic compound content

While we are primarily interested in the transpiration water within plants, co-extraction of organic compounds can occur simultaneously with water extraction, the amount of which appears to depend on the extraction method used. These co-extracted organic compounds such as methanol, ethanol, phenolics, terpenes, and other xylem sap constituents such as sugars and proteins, can influence the measured stable isotope signals during analysis.[3,16,34,35] We quantified only the methanol and ethanol content present in extracted liquid water. Typically, extracted plant water is analyzed via IRMS, as the co-extracted contaminants are often present in small enough volumes relative to the total mass of water H and O, that they will not affect the accuracy of results. The rise of low-cost, rapid analysis by refractive laser-based spectroscopy methods such as CRDS and OA-ICOS has led a number of groups to attempt to utilize this analysis method on plant water.[16] Unfortunately, the problematic co-extracted compounds have optical absorption characteristics similar to water and this can thus result in errors in the measurement of $\delta^2H$ and $\delta^{18}O$ values on laser-based (CRDS and OA-ICOS) spectrometers.[3,4,16] Some extraction systems may, as a by-product of the methods of extraction, produce greater amounts of co-extracted compounds, resulting in analytes of lower purity. However, little work has been done to quantify how much and what type of co-extracted compounds are produced by the various extraction systems. The various co-extracted compounds may each be contributing different $\delta^2H$ and $\delta^{18}O$ signals to the total water signal, based on the H and O originally used at the time of their synthesis. These various signals are currently difficult to disentangle from the total water isotope signal and may require modelling to fully understand.

Table 2 shows that the mechanical methods co-extracted methanol and ethanol in quantities one to two orders of magnitude greater than the CVD-1 method did. In addition to the visually observed contamination shown in Figure 1, HPMS and centrifugation produced a liquid that was far more viscous than that produced by the CVD-1 and CVD-2 methods. The co-extraction of organic compounds by centrifugation and HPMS makes us consider the final extracted analyte as more of a jelly, containing water and organic contamination at magnitudes large enough that the $\delta^2H$ and $\delta^{18}O$ results from these systems may be unrepresentative of the water contained within the plant, although they were analyzed via IRMS. We therefore conclude that the HPMS and centrifugation methods are producing less accurate results due to the relatively high content of co-extracted organic compounds and the low purity of the analyte obtained. Interestingly, the CVD-2 method yielded results that were not significantly different from those of the DVE system for all plant portions and isotope ratios of interest, except for the CVD-2-IRMS leaf water $\delta^2H$ results, although the CVD-2 leaf water contained substantial amounts of methanol and ethanol. As such, the CVD-2 system may also be an acceptable extraction system for plant water analysis. However, we would recommend that a lower extraction temperature of 100°C be used, and that analytes extracted from plant samples with this method should only be analyzed via mass-based spectrometry techniques such as IRMS. The similarity between the DVE and CVD-2 results could be a by-product of the low number of samples ($n$ = 5 per plant portion per method compared) analyzed with the non-parametric ANOVA. Further research with higher (n) per method of extraction should be undertaken.

The DVE system is the only method that is accessing the mobile xylem and inter-cellular water pools with limited co-extraction of organic compounds. This is because DVE relies upon evaporative equilibration of water within the plant sample with that of the dry air added to the sampling container as an equilibration medium. Water contained in the xylem and within inter-cellular spaces is more mobile than water contained in the intra-cellular spaces and than the H and O atoms contained within cellulose structures. This xylem and inter-cellular water will therefore more rapidly equilibrate with the air injected into the DVE sampling container. The more tightly held water must first diffuse through cell walls and will be slower to equilibrate with the dry air in the DVE analysis bag. We believe that the bulk of the water signal that we are seeing in DVE results is that of the more mobile water pool within the plants and it is therefore more representative of the water being taken up and transpired by the plant on a daily basis. For studies looking at the ecohydrological dynamics of plant water uptake, DVE could be the system that is most closely accessing the water of interest in these studies (the transpiration stream). We note a lack of fluctuations between peaks in the absorption plots of analyzed DVE samples to indicate low to no presence of organic contaminants in the headspace of those samples. However, as we did not quantify the presence of organic contaminants in the headspace of the DVE samples and instead relied on observation of the absorption plot for flagging of contamination, we cannot completely rule out organic contamination issues with the DVE results.

## 4.3 | Extraction methods and their effects on $\delta^{18}O$ and $\delta^2H$ values

The CVD-1 system across all plant portions consistently produced the results most depleted in $^2H$ and had the lowest standard deviation between replicates across all plant portions. The CVD-1 system is accessing total plant water by extracting ~99.8% of the water from a sample. It is likely that the measured relative depletion in $^2H$ is a result of the access of this system to the light hydrogen ($^1H$) that has been preferentially taken up into plant organic compounds. This light hydrogen pool will probably make up a greater proportion of the total water extracted, relative to the light hydrogen pools accessible to a system such as DVE. Similarly, the CVD-2 system also produced consistently depleted results for $^2H$ for all plant portions. Again, we attribute this to the access of these systems to the plant organic

compound pools. It should be noted that the CVD-2-IRMS $\delta^{18}O$ results for the roots and heads had a much wider spread than both its $\delta^{2}H$ results and the CVD-2-OA-ICOS $\delta^{18}O$ results. We attribute the relatively enriched $^{2}H$ results of the CVD-2 system (compared with those of the CVD-1 system) to the higher amounts of co-extracted methanol, associated with higher extraction temperatures. The ME-OA-ICOS system consistently produced $^{2}H$ and $^{18}O$-enriched results across all plant portions. The spread of the ME-OA-ICOS results was also consistently the largest amongst all the extraction systems, although this was more pronounced for $\delta^{2}H$ values than for $\delta^{18}O$ values. Interestingly, the relatively $^{2}H$- and $^{18}O$-enriched results produced by the ME-OA-ICOS method in our study are consistent with the findings of Orlowski et al.,[3] implying that a problem with the method itself exists, wherein an operational effect results in an enrichment of the $^{2}H$ and $^{18}O$ isotopic signals. We note there was difficulty during the ME-OA-ICOS extractions in maintaining seals on the extraction vessel. Samples were re-run in cases where we noted damage to the extraction vessel seals, but these leaky seals may be another source of analytical error, and enrichment of $^{2}H$ and $^{18}O$ for the ME-OA-ICOS system. The HPMS system, across all plant portions, produced consistently depleted results for $^{18}O$, but its $\delta^{2}H$ results were generally close to the average of the all systems result. The centrifugation results were the least consistent of all extraction systems in terms of their depletion or enrichment of $^{2}H$ and $^{18}O$ relative to the other systems. For the heads, the centrifugation results for both isotope ratios were depleted in the heavier isotopes but, for the leaves, stems, and roots, the centrifugation results tended to be enriched in $^{2}H$ and relatively similar to the other extraction systems for $\delta^{18}O$ values. The spread of the $\delta^{2}H$ results for centrifugation was among the worst, while the spread of the $\delta^{18}O$ results was consistently amongst the best. We note that due to the relatively abundant content of co-extracted organic contaminants in the mechanically extracted (HPMS and centrifugation) water samples, it may be difficult to adequately explain our isotopic results as we have not determined the full extent of what organic contaminants are present, in what quantities, and what their $\delta^{2}H$ and $\delta^{18}O$ signals are. The DVE system produced results that were more enriched in $^{2}H$ and $^{18}O$ than those of the standard cryogenic extraction systems (CVD-1 and CVD-2). We believe that this is because DVE is accessing the rapidly mobilized xylem water pools in the plant and less so the intra-cellular and organic-compound-bonded H and O pools, meaning that we should expect to see more enriched $^{2}H$ and $^{18}O$ results. The spread of the DVE results was moderate relative to the other extraction systems with the $\delta^{2}H$ results having more acceptable standard deviations than the $\delta^{18}O$ results.

Generally, our results are comparable in principle with the soil-based work of Orlowski et al[3] for the extraction systems' apparent effect on $\delta^{2}H$ and $\delta^{18}O$ values. The inter-comparison of Orlowski et al[3] evaluated the methods of extraction: CVD-1, HPMS, DVE, ME-OA-ICOS, and centrifugation. In their study, the CVD-1 system tended to produce $^{2}H$ and $^{18}O$ depleted results relative to the spike water, the ME-OA-ICOS, and centrifuge systems produced results which were enriched relative to the spike signal, and DVE produced enriched results for $^{18}O$ only and closely matched the spike signal for $\delta^{2}H$ values. However, a noted difference between our results and those reported by Orlowski et al..[3] is that the HPMS system tended to produce results depleted in the heavier isotopes in our trial, whereas the HPMS system produced results enriched in $^{2}H$ and $^{18}O$ in their study.[3] With regard to significant differences between methods of extraction, their study found far more consistently different results across all systems of extraction, especially at lower soil water contents, than the less consistent number of significant differences found in our study. Care should be taken when attempting to compare the results of our study with those of the inter-comparison of Orlowski et al,[3] as the water-carrying media (soil vs plant matter) are highly different from one another with regard to relative inertness, internal chemistry, effects on water isotope ratios, and presence of co-extractable organic compounds.

### 4.4 | Inter-comparison of extraction methods

While a variety of extraction methods are available for plant and soil water extraction, little work has been done to inter-compare the extraction methods and their effects on the isotopic signal of the recovered water. Typically, previous method comparison studies would test a new method against one or two other established extraction methods to validate the new method.[2,14,36-39] The newly developed extraction method would commonly be compared with and validated against the previously established 'gold standard' of cryogenic vacuum distillation.[5] Orlowski et al[3] carried out the first formal inter-comparison of the major lab-based soil water extraction techniques. Their study involved the extraction of spiked soil water from two unique soil types at three water contents per soil type. They found that extraction methods returned results which were significantly different from one another depending upon soil type and water content. In fact, this work called into question the use of cryogenic extraction as the 'gold standard', especially when extracting water from soils with low water contents or with a highly clayey composition.[3,4,8] The CVD system was called into question as it produced water whose $\delta^{2}H$ and $\delta^{18}O$ values were significantly different from those of the water produced by all other tested methods. The CVD $\delta^{2}H$ and $\delta^{18}O$ results were also significantly different from the spiked reference water $\delta^{2}H$ and $\delta^{18}O$ values ($p \leq 0.05$).[3] A methodological comparison of plant water extraction techniques for stable isotope analysis was carried out by Yang et al.[6] They reviewed available methods of plant water extraction, but did not investigate method performance during the experiment. Ours is the first study to experimentally inter-compare the available methods of plant water extraction.

The results of the ANOVA above show that there are significant differences in the stable isotope ratios returned by the tested methods of extraction, depending upon the plant portion and isotope ratio of interest. However, the significant differences between the values measured by the extraction systems were not consistent across all plant portions tested nor for both $\delta^{2}H$ and $\delta^{18}O$ values. This implies that a variable beyond just the methodological effects of extraction may be responsible for causing significant differences between systems. We posit that the complex and varied chemistry of the different plant portions will allow for co-extraction of a unique set of compounds, depending upon the plant portion sampled, that could in turn be modifying the retuned stable isotope signals upon analysis

by IRMS and OA-ICOS, and contributing in part to the differences between the methods of extraction. Thus, the fact that DVE may not have issues with co-extracted compounds and is primarily accessing the more mobile plant water pools further cements its usefulness as an analytical method.

### 4.5 | Limitations of our study approach and recommendations

Our study is the first that we are aware of to inter-compare the methods of plant water extraction for potential differences in their measured stable isotope ratios. However, there are limitations to extending these results to other plant water extractions. While wheat is perhaps relatively similar to other herbaceous grass species or other soft tissue crops such as maize and rice, woody biomass water extractions may be more problematic with regards to co-extractable compounds. The relative volumes of co-extracted organic compounds present in the water pools of herbaceous grasses may be vastly different from the volumetric content of VOCs in larger tree species. This could modify the result of studies inter-comparing the methods of extraction on woody plant species. Further work is urgently needed to quantify types of co-extracted organics in woody plant material and their effects on the measured $\delta^2H$ and $\delta^{18}O$ signatures.

Special care should be taken when investigating inter-method differences for plant species that have historically proven problematic when analyzed on laser-based spectrometers due to their production of high volumes of co-extracted compounds. Further to this point, future work should investigate how these co-extracted compounds affect the $\delta^2H$ and $\delta^{18}O$ signals obtained when extracted water is analyzed via OA-ICOS. Quantification of the volumes and isotopic signatures of co-extracted compounds that will cause inaccurate results; and further development of filtration techniques and post-analysis correction techniques, should also be undertaken. Since we advocate that the DVE system is the most appropriate method for characterizing xylem and thus source water signals in plant water sourcing studies, more work is also needed to investigate the standard operating procedures of the DVE system. Specifically, studies should be carried out investigating: the effects of various equilibration times on the isotopic signals obtained, and the types of dry air that are most appropriate for generating an initial isotopically unbiased non-equilibrium condition in analysis containers. Based on intended storage length, users of the DVE system should choose the most appropriate containers for storage and analysis. Hendry et al[9] suggested that for storage under 10 days the Ziploc® double zipper freezer bag is acceptable, but for storage periods >30 days, Sprenger et al[4] recommended laminated coffee bags.

## 5 | CONCLUSIONS

We compared six different extraction/analysis systems used in determining the stable isotope composition of plant water, on four isotopically unique plant portions of spring wheat. Where possible we analyzed the resultant extracted liquid analyte on IRMS and OA-ICOS systems to determine if the presence of co-extracted organic compounds modified the obtained isotopic results. We reject our null hypothesis that all extraction methods will yield the same water isotopic composition. We found significant differences between the stable isotope results produced by the extraction/analysis methods, depending upon the plant portion analyzed.

Inter-comparisons between the methods of analysis (IRMS and OA-ICOS) showed that for a lower extraction temperature of 100°C, organic contamination was not significant, but that for the higher extraction temperature of 200°C, organic contamination appeared to have a greater effect on the results probably due to increasing methanol and ethanol co-extraction. Further to this, the mechanical methods of centrifugation and HPMS produced such large quantities of co-extracted compounds in their extracted analyte that it was physically not possible to analyze it on the OA-ICOS system. Thus, it may not be reasonable to compare the analytes produced by HPMS and centrifugation with those produced by the other extraction methods, due to the large volumes of undefined co-extracted compounds present. We recommend use of lower extraction temperatures over longer extraction periods for cryogenic methods, to limit issues with organic contamination as well as to limit potential burning or oxidation of plant tissues which may contribute to inaccurate stable isotope results.

While difficulty exists in defining a reference water value to use in quantifying the accuracy/precision in plant water extraction, we determined via investigation of the mechanisms of extraction, and by quantifying the co-extracted methanol and ethanol content in other extraction methods, that DVE is probably the most appropriate method to use when investigating plant water sourcing, at least for wheat. The rapid sample throughput, portability, and near instant analytical results of the DVE-OA-ICOS system will allow for much greater temporal resolution in studies utilizing plant water isotope ratios. However, more research is needed to confirm the utility and accuracy of DVE for other plant species.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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