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# Technical note: Method effects on isotope-based inference of apple tree water sources

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## Abstract

Stable hydrogen and oxygen isotopes are widely used to trace plant water sources, but extraction and analytical choices can bias interpretation. Using an apple orchard on the Shandong Peninsula as a field example, we compared cryogenic vacuum extraction (CVE) with centrifugation, laser spectroscopy with isotope-ratio mass spectrometry (IRMS), and tested how apparent xylem $\delta^2H$ offsets affect MixSIAR source apportionment. Across plant organs, water became progressively enriched from roots and branches to leaves and fruit. In paired branch samples measured by IRMS, centrifugation yielded $\delta^2H$ values about 10‰ higher than CVE, whereas $\delta^{18}O$ differences were small (≈1–2‰). Laser spectroscopy produced systematically higher $\delta^{18}O$ than IRMS for plant extracts (typically 1–3‰), while non-plant source waters agreed closely between instruments. A $\delta^2H$ offset correction shifted inferred uptake from shallow to deeper soil water. These results show that methodological choices can alter isotope-based inference of plant water sources and should be explicitly evaluated and reported.

## Keywords

stable isotopes; apple tree; xylem water; cryogenic vacuum extraction; centrifugation; laser spectroscopy; IRMS; MixSIAR; deuterium offset

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## 1. Introduction

Hydrogen and oxygen stable isotopes in water molecules are widely used to infer plant water uptake patterns by comparing the isotopic composition of plant xylem water with that of potential source waters such as precipitation, soil water, irrigation water, and groundwater (Dawson and Ehleringer, 1991, 1993; Ehleringer and Dawson, 1992; Sprenger et al., 2016; Penna et al., 2020). Under the classical assumption of no fractionation during root uptake, xylem water should represent a conservative mixture of source waters (Zimmermann et al., 1967). Yet many studies report systematic offsets between bulk stem water and candidate sources - most notably in $\delta^2H$ - across a range of climates and species (Ellsworth and Williams, 2007; Brooks et al., 2010; Geris et al., 2015; Evaristo et al., 2017; Vargas et al., 2017; Barbeta et al., 2019, 2020, 2022; Dubbert et al., 2019; De la Casa et al., 2022; Bowers and Williams, 2022; Ceperley et al., 2024). Proposed explanations span biological and physical processes (e.g., membrane transport effects and within-stem water heterogeneity) (Knighton et al., 2020; Barbeta et al., 2022) and methodological artifacts arising from how water is extracted and analyzed (Orlowski et al., 2016b; Penna et al., 2018; Chen et al., 2020; Allen and Kirchner, 2022; Younger et al., 2024).

Methodological uncertainty has two main components. First, the water extraction method can influence which water pools are recovered (mobile sap vs. tissue/bound water), and may therefore shift measured isotopic values (West et al., 2006; Koeniger et al., 2011; Orlowski et al., 2016a, 2016b; Millar et al., 2018; Thielemann et al., 2019; Adams et al., 2020; Orlowski and Breuer, 2020; Wen et al., 2021, 2022). Second, the analytical technique matters: laser spectroscopy can be affected by organic contaminants in plant extracts, whereas isotope-ratio mass spectrometry (IRMS) is comparatively robust because water is converted to gases before isotope measurement (West et al., 2011; Penna et al., 2012; Cui et al., 2021). In practice, extraction and analysis biases can be comparable in magnitude to the ecohydrological signals that mixing models are trying to interpret.

Apple orchards provide a useful model system because they are intensively managed yet often water-limited, and root water uptake patterns vary with climate, irrigation, and soil depth (Ma and Song, 2016, 2019; Cao et al., 2018; Mahindawansha et al., 2018; Wu et al., 2018; Wang et al., 2018; Li et al., 2019; Liu et al., 2023; Wang et al., 2023; Pang et al., 2025). Orchard studies that rely on stable isotopes can be particularly sensitive to methodological biases because soil evaporation and irrigation both shape the isotope depth profile.

The objective of this technical note is to show how extraction and analytical choices affect plant-water isotope interpretation and source apportionment in an apple orchard system. Specifically, we (1) quantify isotopic differences between cryogenic vacuum extraction (CVE) and centrifugal extraction for apple branch xylem water; (2) evaluate laser spectroscopy and isotope-ratio mass spectrometry (IRMS) for plant and source waters, identifying sample types most prone to analytical bias; and (3) test, using MixSIAR, how plausible $\delta^2H$ offsets alter inferred water-source contributions.

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## 2. Materials and methods

### 2.1 Study site and sampling

The field study was conducted in Qixia City (37°13′N, 120°38′E) on the Shandong Peninsula of China, which has a temperate monsoon climate with hot, humid summers (Wang et al., 2023). Three orchard plots with differing geology and soil conditions were selected, and in each plot one mature apple tree was sampled together with surrounding soils and available water sources (precipitation, irrigation reservoir water, and groundwater) (Pang et al., 2025).

Intensive sampling was conducted over five days (8–12 July 2021), twice daily (approximately 08:00 and 15:00 local time), except during storm conditions, yielding 283 samples for isotope analysis. Precipitation collected throughout the 2021 growing season was used to define the local meteoric water line (LMWL).

Soil and root samples were collected using a hand auger at depth intervals of 0–20, 20–40, 40–60, 60–100, and 100–140 cm (triplicate). Tree tissues (branches/twigs, leaves, and fruits) were sampled with pruning shears. In this manuscript, “trunk” refers to outer trunk xylem tissue (sapwood beneath the cambium, with bark and phloem removed) to avoid confusion with tree cores. Fresh samples were sealed immediately and transported in coolers to minimize post-cut evaporation.

### 2.2 Water extraction

Cryogenic vacuum extraction (CVE) followed Koeniger et al. (2011). Samples were frozen and connected to a vacuum line (~10 Pa). Soil samples were heated to 205 °C and plant tissues to 100 °C, and extracted water was trapped in a collection vial immersed in liquid nitrogen (Wen et al., 2022). Extraction time was approximately 1–2 h depending on sample type. Extraction efficiency was assessed gravimetrically, and only samples meeting the recovery criterion (99–101 %) were retained.

Centrifugal extraction was applied to a subset of concurrently collected samples for comparison, using a high-speed centrifuge (Cavitron-type setup) to expel sap water from branch xylem. Because the protocol requires long branch segments (~27–28 cm), centrifugation was only feasible for branch xylem in our sampling design. Branch segments were prepared by removing bark and phloem prior to extraction, and extracted sap was immediately sealed to prevent evaporation. We use this method here as a branch-level comparison with CVE rather than as a universal extraction approach for all sample types.

### 2.3 Isotope analysis and quality control

Hydrogen and oxygen isotope ratios are reported in delta notation ($\delta^2H$ and $\delta^{18}O$, ‰) relative to VSMOW. Laser spectroscopy measurements were performed using a Picarro L2140-i analyzer. Each sample was injected five times and the last three injections were averaged to reduce memory effects. Spectral screening software was used to flag potential organic interference. Laser analytical precision was approximately ±0.2‰ for $\delta^2H$ and ±0.05‰ for $\delta^{18}O$.

To assess analytical bias for plant-derived waters, a subset of samples was additionally measured by isotope-ratio mass spectrometry (IRMS) (Thermo Scientific 253 Plus). IRMS converts water to H2 and CO2 for isotope analysis, reducing the influence of dissolved/volatile organics (West et al., 2011; Cui et al., 2021). IRMS precision was approximately ±0.1‰ for $\delta^2H$ and ±0.02‰ for $\delta^{18}O$.

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Root water was extracted by CVE and analyzed by laser spectroscopy, with a subset of plant waters also analyzed by IRMS. Branch xylem water was extracted by both CVE and centrifugation, and the extraction-method comparison (CVE vs. centrifugation) is based on branch samples analyzed by IRMS for consistency. Soil and external source waters were primarily analyzed by laser spectroscopy, with selected checks by IRMS for quality control.

### 2.4 Data analysis and source apportionment

Dual-isotope plots ($\delta^2H$ vs $\delta^{18}O$) were used to compare sources and plant waters. Line-conditioned excess (LC-excess = $\delta^2H - a \cdot \delta^{18}O - b$, with a and b from the LMWL) quantified hydrogen offsets.

Source contributions were estimated with the MixSIAR Bayesian mixing model framework (Bowen et al., 2018). Sources included soil water grouped by depth (shallow 0–40 cm, intermediate 40–80 cm, and deep 80–140 cm, representing the deepest sampled interval), plus precipitation, irrigation water, and groundwater.

To demonstrate sensitivity to $\delta^2H$ offsets, we ran MixSIAR for (i) uncorrected xylem isotopes and (ii) a $\delta^2H$-corrected scenario. The correction was implemented as an empirical offset that removes the mean deuterium deficit of branch xylem relative to the LMWL ($\delta^2H_{corr} = \delta^2H + \Delta$, where $\Delta$ is the mean observed offset; approximately +8‰ in our dataset). This correction is used here as a sensitivity test rather than as a definitive mechanistic adjustment (Bowen et al., 2018; Chen et al., 2020; Allen and Kirchner, 2022).

## 3. Results

### 3.1 Method comparisons

Branch xylem water extracted by CVE and centrifugation showed systematic isotopic differences when measured by IRMS. Centrifugation-extracted xylem water was enriched in $\delta^2H$ by roughly 10‰ relative to CVE-extracted water from the same branches, whereas $\delta^{18}O$ differences were smaller (typically ~1–2‰). Thus, CVE tended to yield more $\delta^2H$-depleted branch water than centrifugation, while $\delta^{18}O$ was comparatively less sensitive to extraction choice.

Across plant-derived waters, laser spectroscopy tended to report slightly enriched $\delta^{18}O$ relative to IRMS. For trunk, branch, and leaf waters, $\delta^{18}O$ measured by laser spectroscopy was commonly ~1–3‰ higher than IRMS, while $\delta^2H$ differences were generally within ~1–2‰ (near combined analytical precision). Soil water and non-plant source waters showed minimal differences between laser spectroscopy and IRMS. This pattern is consistent with sample-dependent spectral interference in laser analyzers from plant-derived organic compounds, affecting $\delta^{18}O$ more strongly than $\delta^2H$.

### 3.2 Isotopic patterns across sources and plant organs

Rainfall during the study period spanned approximately $\delta^{18}O$ = -9‰ to -1‰ and $\delta^2H$ = -60‰ to 0‰, consistent with the LMWL. Soil water, groundwater, and irrigation water were isotopically more depleted than plant waters. Within trees, water became progressively enriched from roots and branches to leaves and fruits. Leaf water plotted well below the LMWL with strongly negative LC-excess, indicating strong

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evaporative fractionation at the leaf surface. Fruit water was enriched but generally closer to the soil evaporation trend.

A key observation was the apparent $\delta^2H$ depletion of root and branch xylem water relative to soil water sampled in the same plots. Root xylem water was ~5–10‰ lower in $\delta^2H$ than expected from the soil water line given its $\delta^{18}O$, whereas $\delta^{18}O$ differences were comparatively small. Because both soil and root waters were extracted by CVE, this pattern is interpreted here as an apparent $\delta^2H$ offset that may reflect both process signals and method effects (see Sect. 4.1).

### 3.3 Source apportionment sensitivity to $\delta^2H$ offsets

MixSIAR results with uncorrected isotope data suggested dominant use of shallow soil water (0–40 cm), with smaller contributions from intermediate soil (40–80 cm) and minor contributions from deep soil (80–140 cm). Groundwater, precipitation, and irrigation water each contributed only small fractions, indicating that tree water originated mainly from soil moisture rather than direct uptake of groundwater or recent rainfall.

When the $\delta^2H$ offset correction was applied ($\delta^2H$ increased by ~8‰ as a sensitivity test), modeled source contributions shifted markedly toward deep soil water. This demonstrates that $\delta^2H$ offsets of only several per mil—whether arising from fractionation, internal heterogeneity, or method bias—can significantly change mixing-model inference of uptake depth.

## 4. Discussion

### 4.1 Process signals versus methodological artifacts

Many studies interpret $\delta^2H$ depletion of xylem water relative to source waters as evidence of fractionation during uptake or within-plant redistribution (Ellsworth and Williams, 2007; Evaristo et al., 2017; Vargas et al., 2017; Barbeta et al., 2019, 2020; Poca et al., 2019; De la Casa et al., 2022). At the same time, methodological studies show that CVE can yield $\delta^2H$-depleted extracts relative to sap-targeted methods, potentially because CVE mobilizes additional tissue or bound water pools and/or promotes exchange processes during heating (Orlowski et al., 2016b; Penna et al., 2018; Chen et al., 2020; Allen and Kirchner, 2022; Younger et al., 2024). Our branch comparison indicates a ~10‰ $\delta^2H$ difference between CVE and centrifugation, which is comparable to the magnitude of many reported stem-source offsets. We therefore interpret the observed deuterium depletion conservatively as an apparent $\delta^2H$ offset rather than attributing it exclusively to biological fractionation.

### 4.2 Implications of extraction method choice

CVE is widely used because it can be applied to many sample types and can achieve high recovery (Koeniger et al., 2011; Wen et al., 2022). However, CVE may extract mixtures of mobile and immobile water pools, which can shift measured isotope values depending on tissue composition and soil properties (Orlowski et al., 2016a, 2016b; Thielemann et al., 2019; Adams et al., 2020). Centrifuge/Cavitron approaches are designed to target mobile sap water and have been used to address stem–source offsets (Barbeta et al., 2022; Duvert et al., 2024), but their applicability is constrained by sample size and the

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requirement for long stem segments. In our dataset, this limitation is important: centrifugation could only be applied to branch segments, whereas CVE could be used consistently across tissues and soils.

We do not conclude that CVE is inherently more reliable than centrifugation. Rather, CVE is more broadly applicable across sample types, but may introduce $\delta^2H$ bias; centrifugation is more sap-targeted but more constrained and may co-extract organics that complicate laser analysis. When direct method matching across all sample types is not possible, authors should explicitly report extraction protocols, intercompare methods where feasible, and propagate plausible method-driven isotope uncertainty into source apportionment.

### 4.3 Implications of analytical method choice

Our data show a systematic enrichment of plant-water $\delta^{18}O$ measured by laser spectroscopy relative to IRMS by approximately 1-3‰, whereas $\delta^2H$ was comparatively stable between methods. This pattern is consistent with previous evaluations showing that organic contaminants can bias laser spectra, particularly for plant waters, and that post-processing alone may not fully remove the effect (West et al., 2011; Penna et al., 2012; Cui et al., 2021). Because a $\delta^{18}O$ bias of this magnitude can affect inferred evaporative enrichment and alter dual-isotope mixing spaces, at least a subset of extracted plant waters should be cross-checked against IRMS when possible.

Laser spectroscopy is well suited for high-throughput characterization of source waters (precipitation, groundwater, irrigation water, and many soil waters), but plant waters should ideally be cross-checked against IRMS, or processed with robust contamination-mitigation routines, when $\delta^{18}O$ differences of a few per mil are ecohydrologically meaningful.

### 4.4 Consequences for mixing-model inference

The MixSIAR sensitivity test illustrates that modest $\delta^2H$ offsets can substantially affect inferred uptake depth, consistent with prior methodological analyses of source partitioning (Bowen et al., 2018; Allen and Kirchner, 2022). We therefore recommend that isotope-based source-apportionment studies routinely include offset sensitivity analyses (e.g., ±5–10‰ in $\delta^2H$), especially when they rely on CVE-extracted xylem water or laser-measured plant waters. While $\delta^{18}O$-only approaches can reduce $\delta^2H$-specific ambiguity, they may also reduce mixing discrimination where sources overlap in $\delta^{18}O$. Using both isotopes remains informative, but only when methodological and process-based uncertainties are made explicit.

## 5. Conclusions

Overall, this technical note shows that methodological choices in both extraction and analysis can introduce isotope shifts of the same order as commonly interpreted ecohydrological signals. The main implications are:

1. Extraction matters: CVE produced branch xylem $\delta^2H$ values ~10‰ lower than centrifugation, while $\delta^{18}O$ differences were smaller (~1–2‰).

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2. Analysis matters: laser spectroscopy produced a systematic enrichment of plant-water $\delta^{18}O$ (~1–3‰) relative to IRMS, consistent with organic interference, whereas source waters showed minimal inter-instrument differences.
3. Source attribution is sensitive: a plausible $\delta^{2}H$ offset correction (approximately +8‰) shifted modeled uptake from shallow soil dominance to deeper soil dominance, illustrating strong sensitivity of mixing-model inference to small $\delta^{2}H$ biases.
4. Best-practice guidance: (i) clearly state study objectives and define which water pools are extracted, (ii) report extraction techniques and key parameters and validate recovery where possible, (iii) cross-check plant waters with IRMS or robustly address organic interference in laser spectroscopy, and (iv) conduct offset sensitivity analyses in mixing models.

## Data availability

All isotope data and MixSIAR input files will be made available in a public repository upon acceptance.

## Author contributions

YZ and TP designed the study; TP and HY conducted field sampling and laboratory work; MP and QH contributed to conceptual framing and manuscript revision; TP and YZ performed data analysis; YZ led writing with contributions from all authors.

## Competing interests

The authors declare no competing interests.

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## Figure captions

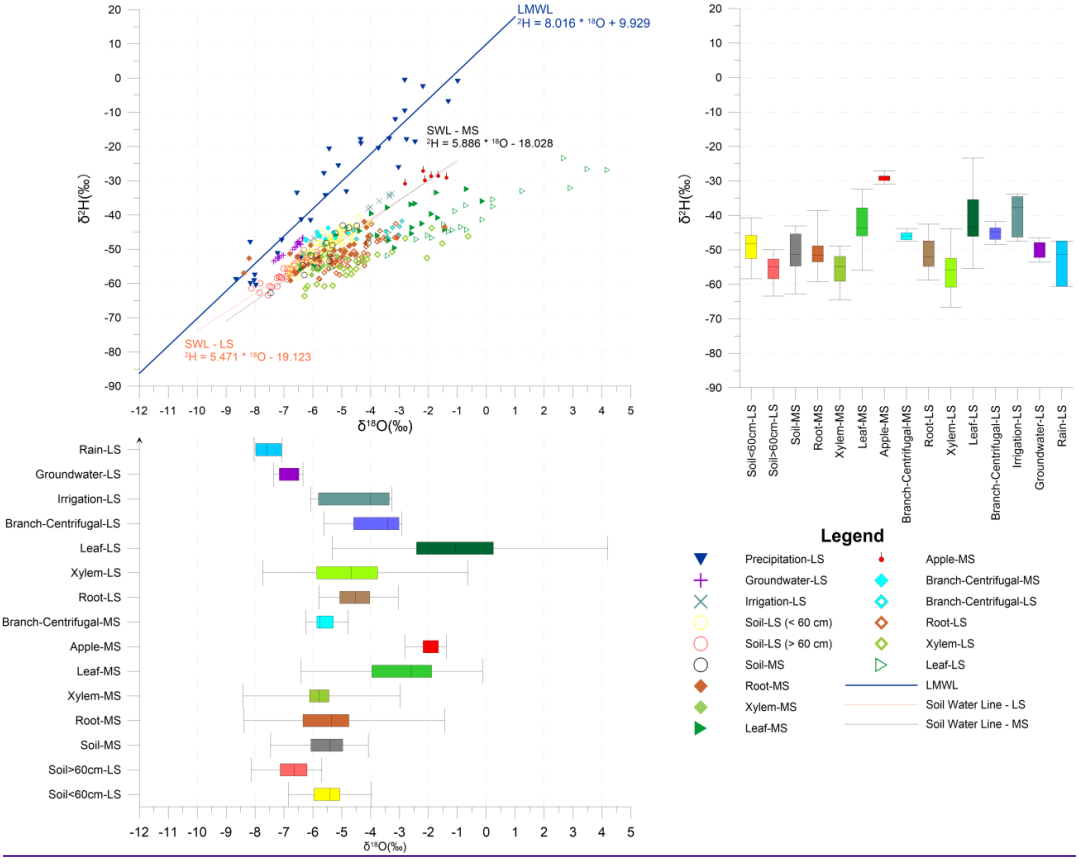


Fig. 1. Dual-isotope plot of all sample types analyzed using both extraction techniques and both analytical methods, with regression lines (upper panel), and box-and-whisker plots for $\delta^{18}O$ (lower panel) and $\delta^{2}H$ (right panel). Samples measured by laser spectroscopy are marked as LS, and those measured by mass spectrometry as MS.

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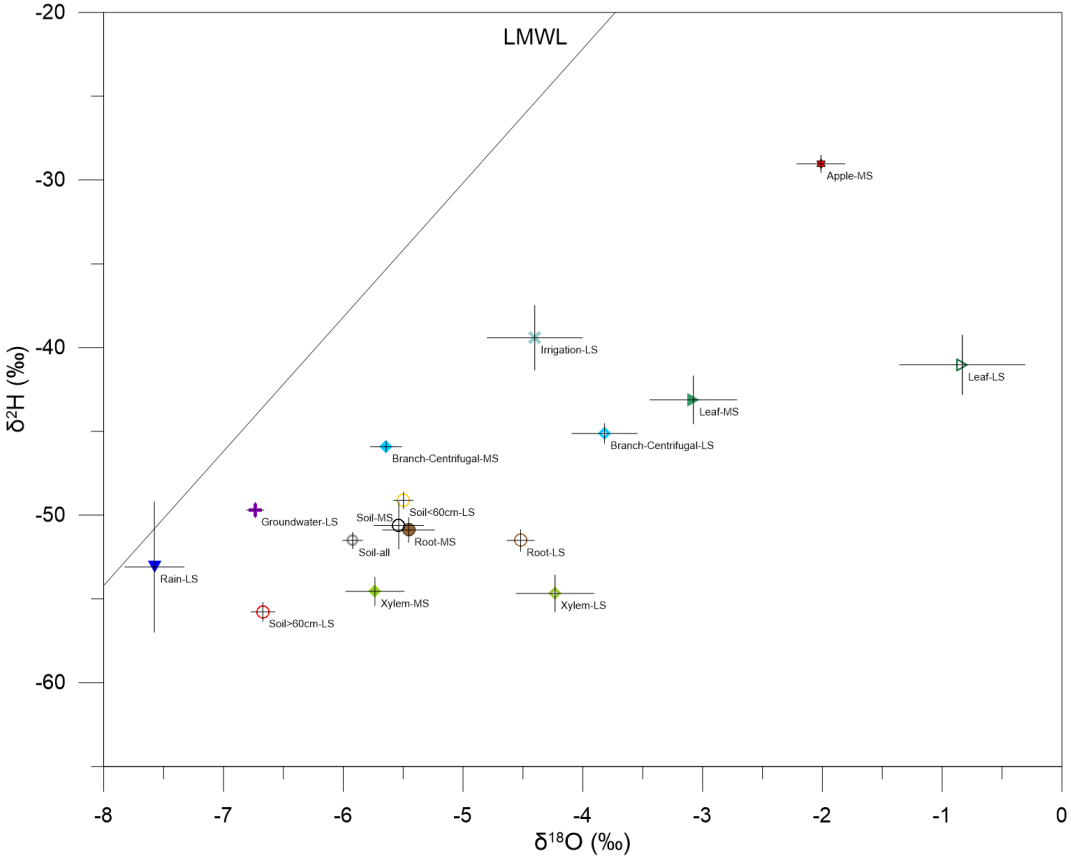


Fig. 2. Means and standard errors of dual-isotope values for the main components of the apple orchard system, including samples analyzed by both extraction methods (CVE and centrifugation) and both analytical methods (LS and MS).

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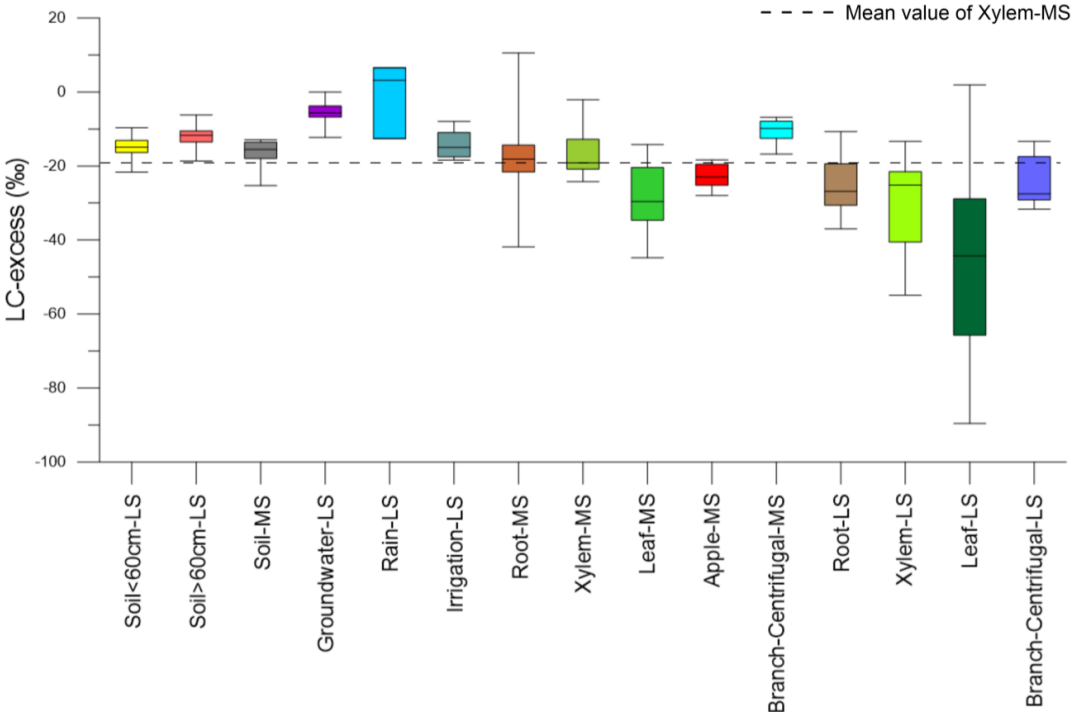


Fig. 3. LC-excess (‰) of the main sample types in the apple orchard system. Data from all plots are pooled. Boxes indicate the 25th and 75th percentiles, whiskers indicate the 10th and 90th percentiles, and horizontal lines indicate the medians.

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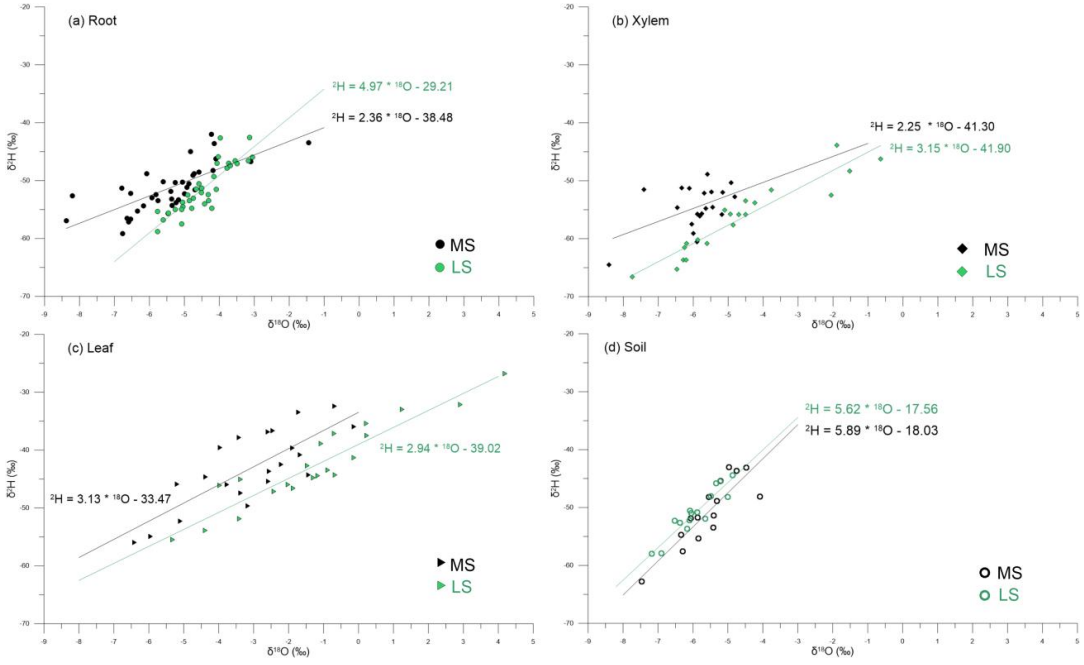


Fig. 4. Comparison of MS and LS analytical methods for root (a), branch and trunk (b), leaf (c), and soil (d) samples, all extracted by CVE.

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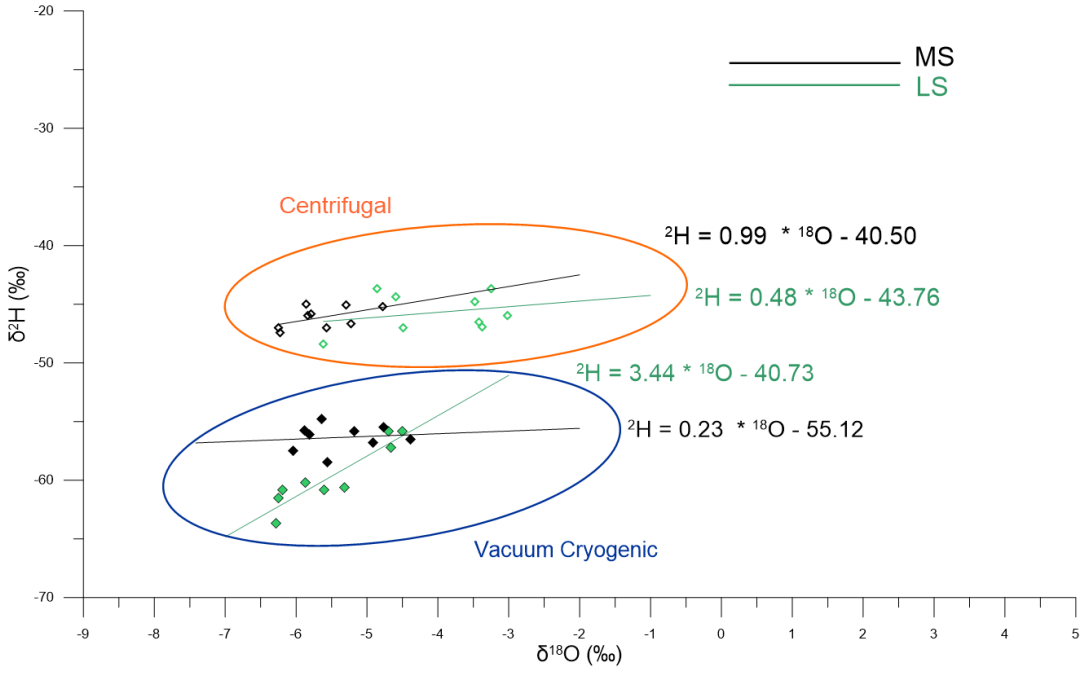


Fig. 5. Comparison of two water extraction methods (centrifugation and CVE) and two analytical determinations (LS and MS) for the same set of branch samples.

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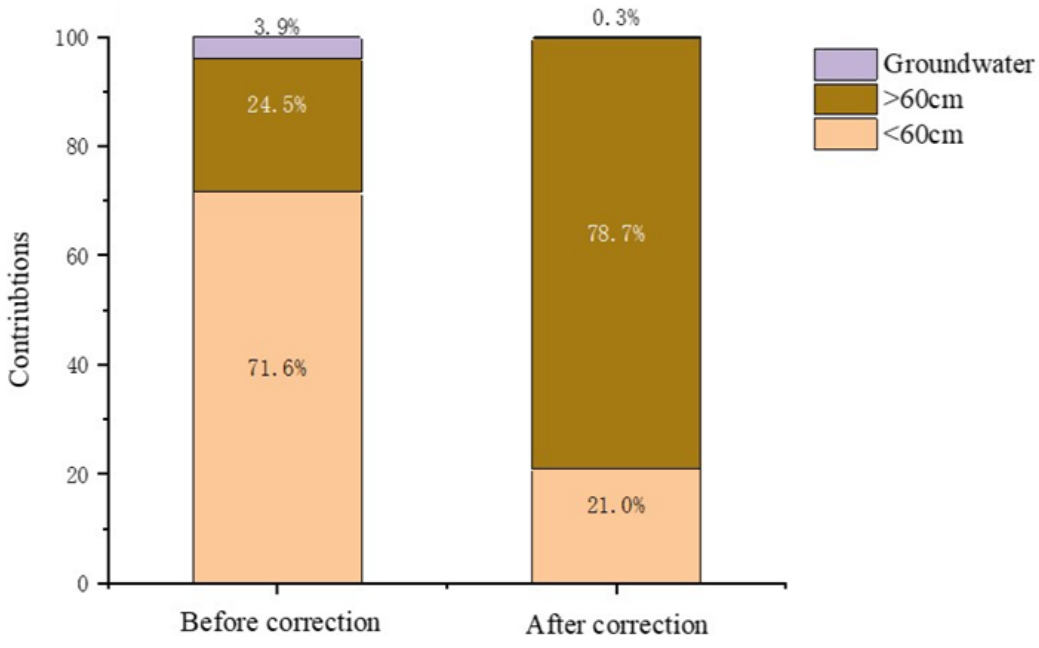


Fig. 6. Comparison of corrected and uncorrected datasets for water-source contributions to plant water uptake estimated with MixSIAR in the apple orchard system.