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Spectral analysis software improves confidence in plant and soil water stable isotope analyses performed by isotope ratio infrared spectroscopy (IRIS)

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Previous studies have demonstrated the potential for large errors to occur when analyzing waters containing organic contaminants using isotope ratio infrared spectroscopy (IRIS). In an attempt to address this problem, IRIS manufacturers now provide post-processing spectral analysis software capable of identifying samples with the types of spectral interference that compromises their stable isotope analysis. Here we report two independent tests of this post-processing spectral analysis software on two IRIS systems, OA-ICOS (Los Gatos Research Inc.) and WS-CRDS (Picarro Inc.). Following a similar methodology to a previous study, we cryogenically extracted plant leaf water and soil water and measured the $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of identical samples by isotope ratio mass spectrometry (IRMS) and IRIS. As an additional test, we analyzed plant stem waters and tap waters by IRMS and IRIS in an independent laboratory. For all tests we assumed that the IRMS value represented the “true” value against which we could compare the stable isotope results from the IRIS methods. Samples showing significant deviations from the IRMS value ($>2\sigma$) were considered to be contaminated and representative of spectral interference in the IRIS measurement. Over the two studies, 83% of plant species were considered contaminated on OA-ICOS and 58% on WS-CRDS. Post-analysis, spectra were analyzed using the manufacturer’s spectral analysis software, in order to see if the software correctly identified contaminated samples. In our tests the software performed well, identifying all the samples with major errors. However, some false negatives indicate that user evaluation and testing of the software are necessary. Repeat sampling of plants showed considerable variation in the discrepancies between IRIS and IRMS. As such, we recommend that spectral analysis of IRIS data must be incorporated into standard post-processing routines. Furthermore, we suggest that the results from spectral analysis be included when reporting stable isotope data from IRIS. Copyright © 2011 John Wiley & Sons, Ltd.

Isotope ratio infrared spectroscopy (IRIS) has emerged as a rapid, cost-effective and field-deployable means of stable isotope analysis of water. The stable isotope composition of pure water analyzed by IRIS is comparable in accuracy and precision with that obtained by isotope ratio mass spectrometry (IRMS),^[1–3] and IRIS avoids the chemical conversion steps required by IRMS. However, previous work has demonstrated the potential for large errors to occur when analyzing waters containing certain organic contaminants using IRIS.^[1,4] Spectral interference from these organic contaminants causes the estimated values of $\delta^2\text{H}$ and $\delta^{18}\text{O}$ from IRIS to deviate considerably from those obtained by IRMS, potentially rendering the analysis of many environmentally derived waters problematic.^[1,4] Ideally, methods leading to the removal or destruction of contaminants in the sample prior to injection into the analyzer need to be developed and validated, as this would prevent several downstream

consequences of contaminated injections.^[5] However, independent of progress in this area, there remains an immediate need for the reliable detection of contaminated water samples analyzed by IRIS. Without this, researchers are unable to verify the accuracy of their IRIS data without time-consuming and expensive cross-validation by IRMS. An important step in resolving the aforementioned issues is the development of post-analysis spectral analysis algorithms and user-friendly software that flags potentially contaminated samples.^[4] This flagging software has recently been developed by IRIS manufacturers in order to provide a tool for researchers to evaluate the quality of the data produced by IRIS. Here, we report a test of this flagging software on two commercially available IRIS systems, off-axis integrated cavity output spectroscopy (OA-ICOS, Los Gatos Research Inc.) and wavelength-scanned cavity ring-down spectroscopy (WS-CRDS, Picarro Inc.), with a focus on analyzing plant water extracts known to create large errors on IRIS.^[4] We also present data on the extent and variation of sample contamination over time and the detection of this contamination by IRIS post-processing spectral analysis software.

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EXPERIMENTAL

We conducted two independent tests of the post-processing spectral analysis software. Both experiments tested the ability of the software to correctly flag samples that showed significant discrepancies between IRMS- and IRIS-derived isotope ratios. The first experiment, designed to be comparable with a previous study,^[4] analyzed leaf water from multiple species and soil water at the University of California Berkeley (Berkeley, CA, USA) (Table 1). A second, completely independent experiment analyzed stem waters and tap waters at the University of Cape Town (Rondebosch, South Africa). These are described in sequence.

Experiment 1: Plant leaf waters and soil (University of California Berkeley)

Sample collection and water extraction were performed as described in West *et al.*^[4] Briefly, leaf samples were taken from a variety of species growing on and around the University of California Berkeley campus. The species (and individuals, where possible) were the same as for West *et al.*,^[4] allowing for a direct comparison of the temporal variation in contaminants in these species. Soil samples were taken from below the litter layer in the first 10 cm of the organic horizon. All samples were transferred immediately to the laboratory, where water was extracted by cryogenic vacuum distillation.^[6] The extracted samples were treated with activated

charcoal to remove organics so that the samples were clear and odourless. This step has previously been shown to reduce IRIS error and thus represented the best test for the flagging software.^[4] Once treated, samples were split into 12 replicates and divided into four identical sets (N=3 in each set) for analysis by two IRMS and two IRIS methods. Included in the sets were two calibration standards and two quality control standards.

The four identical sets were analyzed using four different isotopic analysis methods (Methods 1–4) at the Center for Stable Isotope Biogeochemistry at the University of California Berkeley as described below. A more detailed description of these methods can be found in West *et al.*^[4]

Method 1 – $\delta^2\text{H}$ measurement (IRMS)

Microliter quantities of water were injected into an H/Device coupled to a Delta Plus mass spectrometer (ThermoFinnigan, Bremen, Germany). Injected H_2O was reduced to H_2 gas in a hot chromium reactor and the $^2\text{H}/\text{H}$ ratio of this gas was then measured by mass spectrometry.^[7,8]

Method 2 – $\delta^{18}\text{O}$ measurement (IRMS)

Water samples were left to equilibrate with a 0.2% CO_2 headspace for 48 h at 21–23 °C.^[9] Following equilibration, the vials were inserted into a GasBench II interface connected to a Delta Plus XL mass spectrometer (ThermoFinnigan). The GasBench II was modified with a ten-port injection valve, allowing a 0.2% CO_2 reference injection to follow each sample CO_2 injection.

Method 3 – Off-axis integrated cavity output spectroscopy (OA-ICOS)

Analyses were performed on a liquid water isotope analyzer (DLT-100) from Los Gatos Research (Mountain View, CA, USA). Microliter quantities of water were injected into a vaporization chamber and then passed into an infrared absorbance cavity. The isotope ratios ($\delta^2\text{H}$ and $\delta^{18}\text{O}$) are calculated from the spectral absorbance at specific wavelengths using OA-ICOS.^[3]

Method 4 – Wavelength-scanned cavity ring-down spectroscopy (WS-CRDS)

Analyses were performed on a water isotope analyzer (L1102-i) from Picarro Inc. (Santa Clara, CA, USA). Microliter quantities of water were injected into a vaporization chamber and then passed into an infrared absorbance cavity. The isotope ratios ($\delta^2\text{H}$ and $\delta^{18}\text{O}$) are calculated from the ring-down time at specific wavelengths using WS-CRDS.^[2]

Experiment 2: Plant stem and tap water (University of Cape Town)

Water samples were obtained from stems of Rooibos (*Aspalathus linearis*) from the Nieuwoudtville Region, Western Cape, South Africa. Samples were collected on the same day from eight individuals in close proximity. These stems were kept frozen until reaching the laboratory. In the laboratory, water was extracted from these stems using cryogenic vacuum distillation.^[6] In addition, eight tap water samples were obtained from several locations around South Africa.

Table 1. Description of samples analyzed in this study. Sample locations indicated by superscript: ^a Berkeley, CA, USA; ^b South Africa

Name	Species/Description	Family
Alder ^a	<i>Alnus rhombifolia</i> Nutt.	Betulaceae
Baccharis ^a	<i>Baccharis pilularis</i> DC.	Asteraceae
Bay ^a	<i>Umbellularia californica</i> (Hook. & Arn.) Nutt.	Lauraceae
Brugmansia ^a	<i>Brugmansia</i> sp. (cultivated hybrid, formerly <i>Datura</i>)	Solanaceae
Citrus ^a	<i>Citrus</i> sp. (cultivated hybrid, likely <i>C. reticulata</i>)	Rutaceae
Eucalyptus ^a	<i>Eucalyptus globulus</i> Labill.	Myrtaceae
Ginkgo ^a	<i>Ginkgo biloba</i> L.	Ginkgoaceae
Grass ^a	<i>Poa</i> sp. (cultivated hybrid)	Poaceae
Maple ^a	<i>Acer macrophyllum</i> Pursh	Aceraceae
Pine ^a	<i>Pinus radiata</i> D. Don	Pinaceae
Redwood ^a	<i>Sequoia sempervirens</i> (D. Don) Endl.	Cupressaceae
Soil ^a	From organic A horizon	
BSMOW ^a , BWW ^a , SPW3 ^a and DI ^a	Pure water standards used to assess the error of the IRMS – IRIS comparison	
Rooibos ^b	<i>Aspalathus linearis</i> (Burm.f.) R.Dahlgren	Fabaceae
Tap water ^b	Eight samples from various locations around South Africa	

The stem and tap water samples were divided into three aliquots and were analyzed using three different isotopic methods (Methods 5–7) as described below.

Method 5 – Wavelength-scanned cavity ring-down spectroscopy (WS-CRDS)

Analyses were performed on a water isotope analyzer (L2120-i) from Picarro Inc. using the same protocol as for Method 4.

Method 6 – $\delta^2\text{H}$ measurement (IRMS)

Analyses were performed using the closed-tube zinc reduction method.^[10] Between 100 and 105 mg of zinc were loaded into 6 mm borosilicate break-seal vials, which were connected to a vacuum line. The vials were heated at 450 °C for 5 min with an HE2300 Metabo heat gun (Nürtingen, Germany), while evacuating the loaded zinc to 10^{-4} Torr. Each vial was then loaded with a 2 μL water sample in a Hirschmann micro-capillary pipette (Eberstadt, Germany), returned to the vacuum line and frozen with liquid nitrogen for 5 min. After evacuating the frozen samples to 10^{-4} Torr, the glass tubes were flame-sealed. The vials were then combusted in a furnace at 450 °C for 1 h to evolve H_2 gas. The isotopic ratio of the H_2 gas was measured via dual-inlet on a MAT 252 mass spectrometer (ThermoFinnigan).

Method 7 – $\delta^{18}\text{O}$ measurement (IRMS)

Analyses were performed using the CO_2 equilibrium method of Socki *et al.*^[11] Pre-evacuated, 11 mL Kimax vacutainers (Laboratory and Scientific Equipment Ltd., Cape Town, South Africa) were filled with 0.5 bar of 100% CO_2 and a 1.5 mL water sample. The vacutainers were agitated in a water-bath at 25 °C for 48 h for headspace equilibration. Subsequent to equilibration, the vacutainers were attached to a vacuum line, frozen with liquid nitrogen, and then evacuated to 10^{-4} Torr. CO_2 was sublimed with an ethanol/dry-ice slurry and then trapped in 6 mm break-seal vials and flame-sealed. The vial contents were analyzed via dual-inlet on a MAT 252 mass spectrometer.

For both experiments and all methods, isotope ratios are expressed in ‰ as.^[12]

$$\delta^N E = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \quad (1)$$

where N represents the heavy isotope of element E and R is the ratio of the heavy to light isotopes ($^2\text{H}/\text{H}$ or $^{18}\text{O}/^{16}\text{O}$). For all methods, two calibration standards were used to adjust the delta values relative to the international standard Vienna Standard Mean Ocean Water (VSMOW). The long-term precision obtained via working standards with identical isotope ratios introduced into every run over time was no greater than 1.2‰ for $\delta^2\text{H}$ and 0.15‰ for $\delta^{18}\text{O}$ (1σ) and for Methods 1–5, and 2‰ for $\delta^2\text{H}$ and 0.2‰ for $\delta^{18}\text{O}$ for Methods 6 and 7.

For each plant species, the mean δ -value obtained by IRMS was subtracted from the mean δ -value for each IRIS method. This difference was compared with the convolved error of the methods, using the long-term precision data, calculated as:

$$\varepsilon_{(A-B)} = \sqrt{(\varepsilon_A^2) + (\varepsilon_B^2)} \quad (2)$$

where A and B are the two means being subtracted and ε is the error associated with those means. The difference between the IRIS and IRMS results was only considered

significant if it was greater than or less than twice the convolved standard deviation (2σ) of the instruments. This was 3.1‰ ($\delta^2\text{H}$) and 0.42‰ ($\delta^{18}\text{O}$) for Experiment 1, and 4.6‰ ($\delta^2\text{H}$) and 0.5‰ ($\delta^{18}\text{O}$) for Experiment 2.

Spectral analysis

Post-processing spectral analysis for IRIS data was performed using the manufacturers' software with all default settings. For the OA-ICOS data, we used the Spectral Contaminant Identifier (SCI) software, version 1.0.0 (Los Gatos Research). The SCI software analyzes the recorded spectra to produce an interference metric indicating the likelihood of contamination from narrow-band (e.g. methanol) and broad-band (e.g. ethanol) absorbers. This metric is compared with that of known clean samples in the run (the standards) and a good/bad flag is assigned to indicate the reliability of the isotopic measurement. A more complete description of this approach is available from the manufacturer.^[13]

For the WS-CRDS data, we used the Picarro post-processing software ChemcorrectTM, version 1.0.0, with the analysis file 'chemcorrect_inst_avg_orgeval_06.csv' (contained in the Supporting Information). This software attempts to identify contamination in the water samples both through fitting to a known library of spectral features, and by examining changes in baseline, slope, line-broadening and residual noise of the spectra. Depending on a set of user-definable parameters contained in an analysis file (we used the manufacturer's defaults for this study, see Supporting Information), samples are assigned metrics describing the magnitude of contamination as well as the potential source, together with a flag representing good (green), possibly contaminated (yellow) and bad (red). Further description of this approach is available from the manufacturer.^[14]

RESULTS

Experiment 1

Several species showed considerable differences between δ -values measured by IRIS and by IRMS (Fig. 1). The largest range in reported values for a given sample (Bay) was 31‰ ($\delta^2\text{H}$) and 14‰ ($\delta^{18}\text{O}$) (Fig. 1). OA-ICOS typically returned values more positive than IRMS, whereas WS-CRDS values were more negative (Fig. 1).

The spectral analysis software correctly identified contaminated samples in the majority of cases. The OA-ICOS analysis software correctly identified 7 of the 9 contaminated samples (Fig. 1). The two samples that were false negatives (*Eucalyptus* and pine) were very close to the 2σ threshold, exceeding it only in $\delta^{18}\text{O}$, by 0.24‰ and 0.22‰, respectively. However, the SCI interference metrics were not able to distinguish between these samples and others that showed no contamination (Fig. 1(E)). The WS-CRDS software correctly identified 4 of the 5 contaminated samples (Fig. 1). The one sample that was a false negative (*Citrus*) did not exceed the $\delta^2\text{H}$ threshold, and exceeded the $\delta^{18}\text{O}$ threshold by 0.28‰ (Fig. 1(D)).

A comparison of the discrepancies between IRIS and IRMS from this study with the data presented by West *et al.*^[4] revealed that the discrepancies may not be consistent across time (Fig. 2). Species sampled a year later did not necessarily

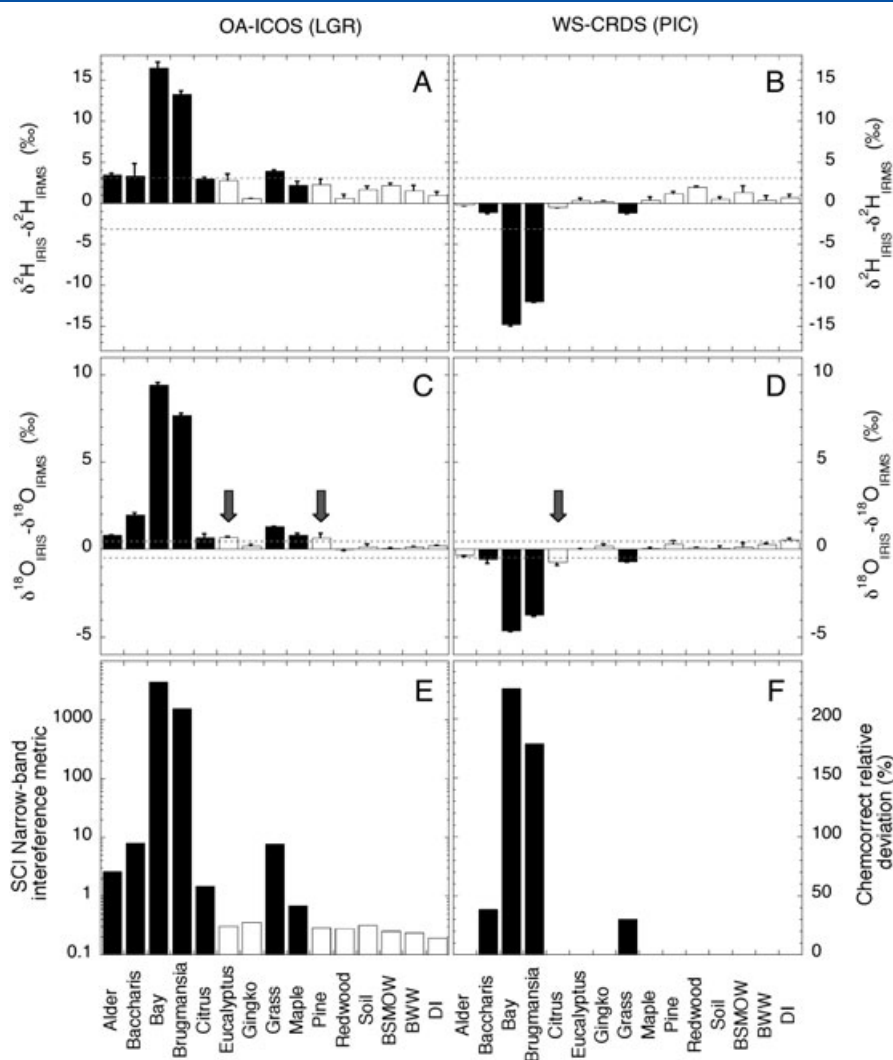


Figure 1. (A–D) Deviation from IRMS value for samples analyzed by two IRIS methods. Black bars represent samples flagged as potentially contaminated by spectral analysis software. Dashed lines represent $\pm 2\sigma$ for convolved instrument error. Samples falling above or below these lines are considered significant deviations from the “true” value obtained via IRMS. (E, F) Flagging metrics from the two spectral analysis software packages, indicating the relative degree of interference from contaminants in the sample. Dark arrows in (C) and (D) identify false negatives. Note the log scale in (E). Samples are described in Table 1.

show the same discrepancy between IRIS and IRMS and there was no consistent trend in the discrepancies; some species showed increased discrepancies from IRMS, whereas others showed decreases (Fig. 2).

Experiment 2

All stem water samples from *Aspalathus linearis* were correctly flagged as contaminated by ChemcorrectTM (Fig. 3). There was considerable variation in the degree of deviation from IRMS for the eight samples, ranging between -38 and -4% for $\delta^2\text{H}$, and between -6.6 and -1.5% for $\delta^{18}\text{O}$, suggestive of variation in individual plants' contaminant levels. All tap waters were correctly identified as uncontaminated by ChemcorrectTM. The largest discrepancies between IRIS and IRMS for these samples approached, but did not exceed, 2σ of the convolved instrument precision.

When combining the results from a previous study^[4] with those presented in this study, it can be seen that the majority of plant water samples showed signs of significant contamination when measured by IRIS. For OA-ICOS, 10 of the 12 plant species (83%) exceeded the 2σ threshold from the IRMS value, indicative of significant contamination. For WS-CRDS, 7 of the 12 species (58%) exceeded the 2σ threshold.

DISCUSSION

In our two independent experiments, the spectral analysis software supplied by the manufacturers performed well and successfully identified all samples that showed major discrepancies from the IRMS value (Figs. 1 and 3). This represents an important step forward for increasing user confidence when measuring the isotope composition of potentially

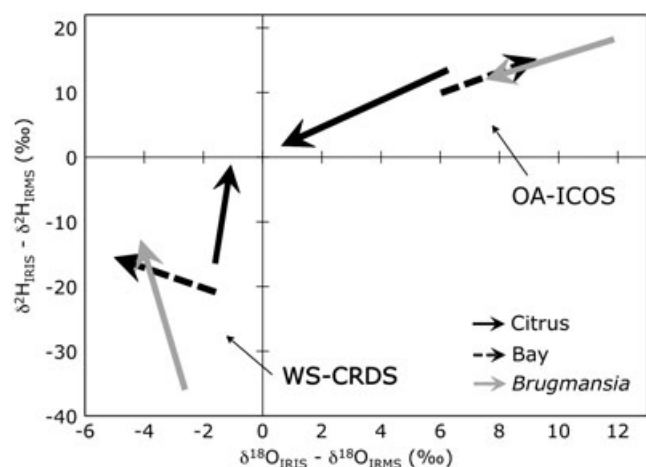


Figure 2. Changes in discrepancies between IRIS value and IRMS value for three representative species sampled a year apart. Arrows show the direction and magnitude of change from first sampling period (data from West *et al.*^[41]) to second sampling period (this study). The magnitude of the discrepancy from the IRMS value in $\delta^2\text{H}-\delta^{18}\text{O}$ space does not appear to be consistent through time, suggestive of varying contaminant concentrations and composition.

contaminated water samples by IRIS. Previously, IRIS users had no way of determining whether the analyses were being compromised by spectral interference other than through direct comparison with IRMS, a tedious and expensive practise that eliminated the benefits of utilizing IRIS. The spectral analysis software provides researchers with a tool for quantitatively identifying samples that have been compromised by spectral interference from organic compounds. However, a few important caveats remain, which we expand upon below.

The spectral interference software does not solve the problem of contamination, it only identifies where potential problematic samples may exist. 83% (OA-ICOS) and 58% (WS-CRDS) of tested plant species were identified as having significant contamination problems, resulting in erroneous measurements by IRIS. Thus, at present IRIS is still not an appropriate means of analysis for samples containing certain organic contaminants. In order for IRIS to be adopted more widely, additional chemical procedures must be developed to remove all interference contaminants so that this problem is eventually solved,^[5] thus rendering IRIS measurements independent from direct comparisons with IRMS results.

While the spectral analysis software identified all of the major discrepancies in this study, not all samples that contained low levels of contaminants were correctly identified. Some false negatives were detected in $\delta^{18}\text{O}$ (Fig. 1). False negatives are of concern as they may lead to contaminated samples escaping detection and thus compromising the quality of the IRIS results. In our first experiment, three false negatives occurred for $\delta^{18}\text{O}$ and these reached a magnitude of 0.28‰ outside the 2σ detection limit. For our study, this meant that identical samples analyzed by IRMS and IRIS could differ by as much as 0.7‰ in $\delta^{18}\text{O}$ without being flagged as problematic by the spectral analysis software. Users need to evaluate whether this magnitude of error is acceptable for their study system.

The comparison of paired samples taken a year apart (Fig. 2) and the analysis of multiple individuals of *A. linearis* (stem waters, Fig. 3) illustrate the need for the constant

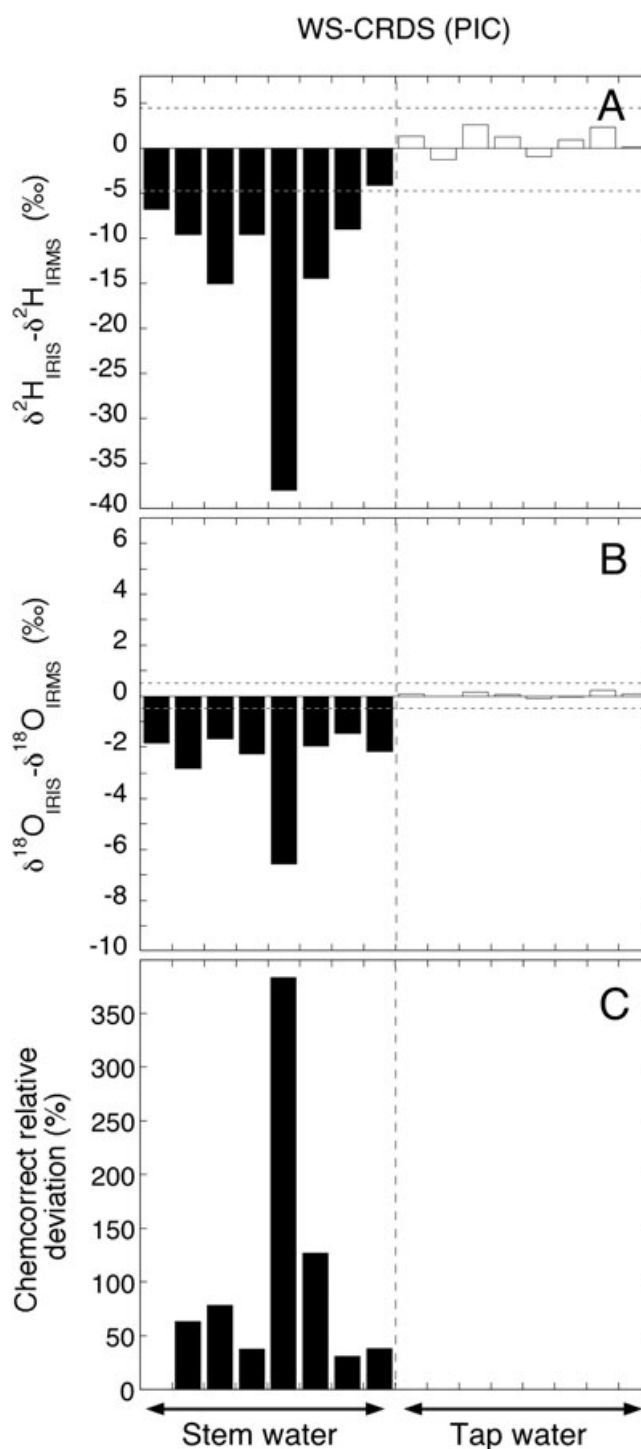


Figure 3. (A, B) Deviation from IRMS value for eight plant stem water samples (*Aspalathus linearis*) and eight tap water samples from various locations across South Africa. Black bars represent samples flagged as potentially contaminated by spectral analysis software. Horizontal dashed lines represent $\pm 2\sigma$ for convolved instrument error. Samples falling above or below these lines are considered significant deviations from the “true” value obtained via IRMS. (C) Flagging metric from Chemcorrect™ spectral analysis package indicating the relative degree of interference from contaminants in the sample. The absence of a bar indicates that the sample was not flagged as contaminated. Note that all contaminated samples were correctly flagged.

application of post-processing spectral analysis software to IRIS data. While our current study has confirmed previous work,^[1,4] indicating that large errors are possible when analyzing plant waters by IRIS, it has also indicated that these errors are not necessarily constant over time or between conspecific individuals in close proximity. This is probably due to variations in the concentration and/or composition of organic compounds in the plant at the time of sampling. Thus, applying a species-specific correction factor based on a single assessment of the IRIS-IRMS discrepancy is not recommended. Rather, we recommend that all samples must be routinely assessed by post-processing spectral analysis software and, in the case of finding contamination, the sample must be analyzed by IRMS.

It is important to note that our study represents only two independent tests of the currently available software on a limited range of sample types (leaf, stem, soil and tap water). As such, it is premature to conclude that the software will function effectively for all sample types. In this regard, we recommend testing the efficacy of the spectral analysis software by direct comparisons with IRMS data whenever water samples from new species or materials are analyzed. Once the software has been shown to be reliable in identifying all problematic samples, it seems reasonable that the direct comparison with IRMS could be reduced, or possibly eliminated, for further analysis of similar samples.

For the reasons outlined above, we recommend that post-processing spectral analysis of IRIS data must become incorporated into standard data-processing protocols. We therefore suggest the following steps as a possible foundation for the establishment of high-quality IRIS data (Fig. 4):

- 1) All samples analyzed by IRIS must be routinely run through the most current spectral analysis software.
- 2) If samples are flagged as BAD, the IRIS data should be discarded and analyses should be conducted using IRMS.
- 3) If samples are flagged as GOOD and this is a novel sample (e.g. water from a new species, or a different organic matrix), the samples should be analyzed by both IRIS and IRMS to detect discrepancies between the two technologies. Different laboratories will need to design suitable methods for detecting statistical discrepancies between IRIS and IRMS methods based on the precision necessary to address their research questions and the long-term precision of their individual instruments. Herein, we present only one possible statistical approach to the problem.
- 4) If the discrepancies between IRMS and IRIS data fall within acceptable precision, the IRIS data may be used. If the discrepancies fall outside the acceptable precision, the IRIS data should be discarded and the IRMS data should be used. Furthermore, this material should be regarded as unsuitable for future IRIS analysis.
- 5) If the samples are flagged as GOOD and there is no potential for organic contamination (e.g. pure waters), or the material has already been successfully validated before (as per step (3) above), the IRIS data may be used. It is important to bear in mind that contamination within a plant species may not be consistent over time (Fig. 2). If in doubt, samples should be cross-validated with IRMS until the user is confident that there is no contamination.
- 6) When reporting stable isotope data from IRIS, details must be provided on the application of post-processing software to check for contamination, including the software

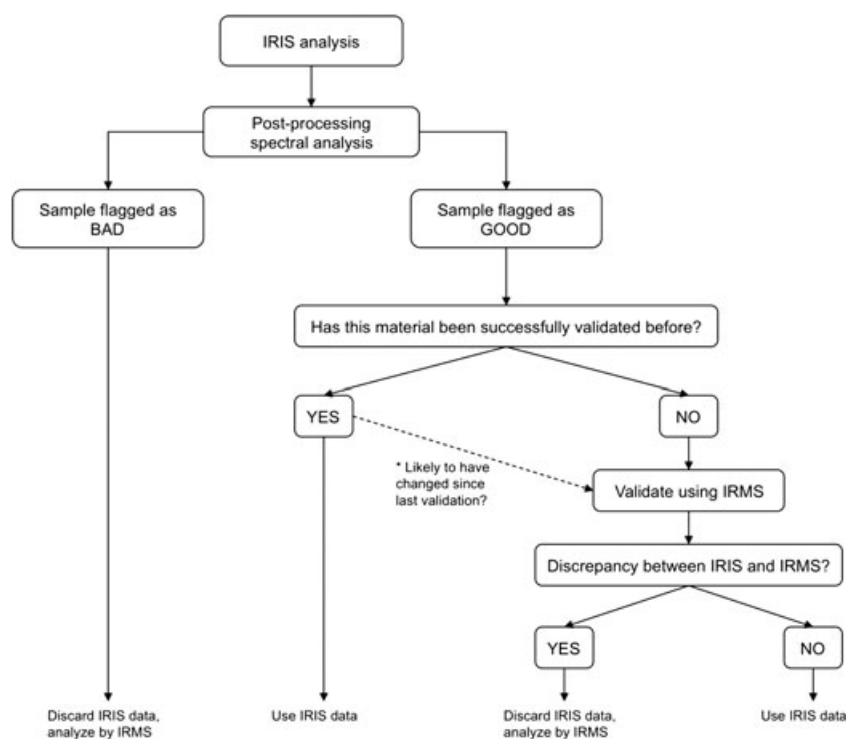


Figure 4. Proposed decision-making flowchart for stable isotope analysis of waters using IRIS. *As contaminants can vary over time (Fig. 2), multiple validations may be required to increase confidence.

version and any user-specific or custom settings used. Wherever possible, research reports that use IRIS should also indicate how they verified that the isotope ratios obtained from at least a subset of their samples were checked against accepted IRMS methods, the standards used to perform these checks, and any statistical procedures used to determine the reliability of the data shown and used.

CONCLUSIONS

The development of spectral analysis software represents an important advance for the stable isotope analysis of water using IRIS. The manufacturers' software appears capable of identifying problematic samples (an essential for data quality control); however, it does not remove the underlying problem of contamination affecting the IRIS measurement. This means that currently IRIS is not a suitable, independent method for water isotope investigations where the potential for spectral interference by organic contaminants exists. This is particularly the case for plant water extracts. However, in certain cases, careful validation may permit the use of IRIS for such studies. In order to solve this problem, and to render IRIS measurements independent from direct comparisons with IRMS results, additional chemical procedures must be developed to remove all potential contaminants from the water sample.^[5] Even in the event of such an analytical solution, post-processing spectral analysis will still be needed for quality control assurance on any IRIS measurement.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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