Isotopes in Environmental and Health Studies
Vol. 46, No. 4, December 2010, 476–483

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Accuracy and precision of a laser-spectroscopy approach to the analysis of δ^2H and δ^{18}O in human urine

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(*Received 19 July 2010; final version received 27 October 2010*)

The isotope ratio analysis of body water often involves large sample numbers and lengthy sample processing. Here we demonstrate the ability of isotope ratio infrared spectroscopy (IRIS) to rapidly and accurately analyse the isotope ratios of water in urine. We analysed water extracted from human urine using traditional isotope ratio mass spectrometry (IRMS) and compared those values with IRIS-analysed extracted water and un-extracted urine. Regression analyses for δ^2H and δ^{18}O values between (1) extracted water analysed via IRMS and IRIS and (2) urine and extracted water analysed via IRIS were significant ($R^2 = 0.99$). These results indicate that cryogenic distillation of urine was not required for an accurate estimate of the isotopic composition of urine when using IRIS.

Keywords: cavity ring-down spectroscopy; body water; urine; hydrogen-2; isotope measurements, methods and equipment; oxygen-18; infrared spectroscopy; isotope application in medicine

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1. Introduction

Analyses of the stable isotope ratios of hydrogen (δ^2H) and oxygen (δ^{18}O) in urine water, using isotope ratio mass spectrometry (IRMS), have been used to determine total energy expenditure (TEE) in both humans [1–8] and animals [9–11] and have future human clinical applications, including monitoring of water homeostasis [12]. Methods currently used for δ^2H and δ^{18}O analyses of water in urine are either time-consuming or require multiple isolation and purification steps; specifically, cryogenic distillation of water from a complex sample that includes water, acids, proteins and sugars [9–11] or a combination of CO_2 equilibration (^{18}O analysis) and zinc [13] or chromium [14] reduction (^{2}H analysis). For many medical and physiology-related applications to be feasible, the rapid and accurate measurement of a large number of urine samples is required. The time required for the isolation and purification of the water contained in a urine sample adds significantly to analysis time, limiting productivity. Isotope ratio infrared spectroscopy (IRIS) offers an opportunity to analyse complex fluids [15,16] such as blood, saliva and urine 'as is', without requiring cryogenic distillation in advance of isotopic analysis. Direct analysis of the

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ISSN 1025-6016 print/ISSN 1477-2639 online
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DOI: 10.1080/10256016.2010.536229
http://www.informaworld.com

isotopes of water within complex fluids would allow for a faster and more reasonable processing time, suitable to many physiological applications.

A previous study of the accuracy and precision of IRIS for the measurement of δ^2H and δ^{18}O values of alcohol/water mixtures [15] revealed that alcohol contamination resulted in a linear departure of apparent results from true values, as measured by the IRMS. Additionally, organic contaminants contained in leaf water extracts have been shown to interfere with water isotope analyses performed by both the commercially available IRIS analysers, wavelength-scanned cavity ring-down spectroscopy (CRDS) and off-axis integrated cavity output spectroscopy (OA-ICOS), even after treatment of the water extracts with activated charcoal [16]. However, Chesson *et al.* [17] have shown that it is possible to use IRIS-based approaches to measure the δ^2H and δ^{18}O values of water in other fluids including milk and fruit juices. Given these research results, our objective was to examine the accuracy of IRIS for measuring the isotopic composition of urine, the most direct means of estimating body water isotopes in humans and other animal systems. Specifically, we first analysed the δ^2H and δ^{18}O values of water extracted from urine samples using IRMS and then compared these values with both water extracted from urine and un-extracted urine analysed on an IRIS analyzer (CRDS, Picarro model L1102-i; Sunnyvale, CA, USA).

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2. Methods

To maximise the range of stable isotope ratio values within the sample set, urine was obtained from five US cities with isotopically distinct drinking water. The isotopic range for drinking waters from these cities was −155 to 0‰ for δ^2H values and −20 to 1‰ for δ^{18}O values, and a similar range was expected for urine samples. Urine samples were frozen until processing. Urine was aliquoted into two 2 ml samples for 'extracted water' and 'un-processed' analysis. For extracted water samples, water was cryogenically extracted from urine samples using a vacuum distillation line (see West *et al.* [18] for a schematic of the distillation line). Both extracted water and un-processed samples were refrigerated and treated with activated charcoal for 24 h. Previous unpublished research in our laboratory indicated that water extracted from urine contained organic compounds (as evidenced by a strong odour) and that the presence of these compounds impacted the measured isotope ratios of body water; treatment with activated charcoal removed these compounds. Similar results have been found with organic compounds in leaf water extracts [16]. All samples were then filtered to remove charcoal, using an Acrodisc® 13 mm syringe filter with a 0.2 μm GH Polypro hydrophilic polypropylene membrane (Pall Corporation, Port Washington, NY, USA). Aliquots of 0.4 ml each of the urine and water-extracted-from-urine samples were then transferred to 1.8 ml crimp-top gas chromatography (GC) vials and sealed for subsequent isotope ratio analyses.

Samples were analysed at the Stable Isotope Ratio Facility for Environmental Research (SIRFER; http://sirfer.utah.edu) at the University of Utah in Salt Lake City, UT. Stable isotope ratios of extracted water were independently measured using IRMS and IRIS methods. In order to maintain sample integrity, extracted water samples and reference materials were first measured on the temperature conversion elemental analyser (TCEA)-IRMS, re-capped and then re-analysed on the IRIS with sequence order preserved. For IRMS analyses, we used a ThermoFinnigan-MAT Delta Plus XL isotope ratio mass spectrometer (Breman, Germany) with a high TCEA attached. Water was introduced to the TCEA using a PAL autosampler (LEAP Technologies, Carrboro, NC, USA) where samples were pyrolysed at 1400 °C to produce H_2 and CO gases. Gases were separated using a 1 m, 0.25″ molecular sieve 5 Å GC column (Costech Analytical, Valencia, CA, USA), thus producing both hydrogen and oxygen peaks from a single injection of water. The δ^2H and δ^{18}O values of water in urine were measured using a Picarro liquid water isotope analyser, model L1102-i (see Gupta *et al.* [19] for a complete description of the system). Urine and

extracted water samples were analysed along with three laboratory pure-water reference materials that had been previously calibrated against international standards on the Vienna Standard Mean Ocean Water (VSMOW) scale. Reference materials were interspersed among samples throughout the analysis sequence. For IRIS analyses, three sequential replicate injections of samples and reference materials were analysed, with results of the first injection eliminated to reduce sample-to-sample memory. For the TCEA-IRMS, two replicates were analysed for each sample and reference material. The analytical precision for δ^2H and δ^{18}O values, as measured by the standard deviation around the reference material mean (1σ), was ±0.72 and ±0.04‰ for the TCEA-IRMS and ±0.47 and ±0.07‰ for the IRIS, respectively.

Stable isotope abundances are reported in δ-notation as parts per thousand (‰), where

$$\delta = \left(\frac{R_A}{R_S} - 1\right) \times 1000$$

and R_A and R_S are the molar ratios of the rare-to-abundant isotope (e.g. ^{2}H/^{1}H) in the sample of interest and a standard, respectively. The international standard for both hydrogen and oxygen stable isotope analysis is VSMOW.

To describe the properties of urine, we measured the following characteristics in both urine and extracted water samples: pH, specific gravity, salinity, protein content, glucose content and ketone content. The pH was measured using a Chekmite® pH-20 sensor (Nova Analytics Corporation, Woburn, MA, USA). Specific gravity was measured using a Reichert® r^2 mini refractometer (Depew, NY, USA). Protein, glucose and ketone contents were assessed using Siemens Multistix® reagent strips for urinalysis (New York, NY, USA).

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2.1. *Memory correction calculations*

To account for sample-to-sample memory in both IRIS and IRMS, a memory correction was applied [19]. The memory coefficient (X) is calculated as

$$X = \frac{M - P}{T - P},$$

where M is the measured value of the current injection, T the true value of the current injection and P the true value of the immediately previous injection. Data were corrected using a template built in Microsoft® Excel® 2004 for Mac. Fractional memory correction values were manually entered into the data template until the combined average standard deviation of the three reference materials was minimised.

2.2. *Statistics*

A reduced major axis regression was used to determine, for both δ^2H and δ^{18}O values, the relationship between (1) extracted water analysed via IRMS and IRIS and (2) urine and extracted water analysed via IRIS. The slopes and the y-intercepts of regression lines were compared with a line with a slope = 1 and an intercept = 0 using one-way analysis of variance to identify differences at $\alpha = 0.01$. Statistical analyses were completed using Prism 5 for Mac OS X (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

The pH and specific gravity of urine samples ranged from 5.5 to 7.5 and 1.033 to 1.034, respectively (Table 1). While the specific gravity remained constant, the pH of water extracted from the urine

Table 1. Characteristics of urine and extracted water.

	pH range	Specific gravity range	Protein content	Glucose content	Ketone content
Urine	5.5–7.5	1.033–1.034	Negative	Negative	Negative
Extracted water	6.5–9.0	1.033	Negative	Negative	Negative

samples became more basic after extraction and had a range of 6.5–9.0. Both urine and extracted water samples were negative for abnormal levels of protein, glucose and ketones (Table 1). The isotopic range for urine samples was −115 to −1‰ for δ^2H values and −12 to 0‰ for δ^{18}O values.

The δ^2H and δ^{18}O values for extracted water samples analysed via IRMS were compared to those same samples analysed using IRIS (Figures 1a and b, respectively). Best-fit lines for the data sets were described by the equations:

$$\delta^2\mathrm{H}_{\mathrm{TC/EA-ext.water}} = 1.03^{*}\delta^2\mathrm{H}_{\mathrm{IRIS-ext.water}} + 2.37‰(R^2 = 0.99,\ P < 0.0001)$$

$$\delta^{18}\mathrm{O}_{\mathrm{TC/EA-ext.water}} = 0.99^{*}\delta^{18}\mathrm{O}_{\mathrm{IRIS-ext.water}} - 0.11‰(R^2 = 0.99,\ P < 0.0001).$$

We can compare the slope and intercept of these lines with a slope of 1 and intercept of 0 to determine deviation from a perfect relationship. For H, the slope of the best-fit line (1.03) was not significantly different from 1, whereas the y-intercept (2.37) was significantly different from 0 (slope: $F = 2.03$, $P = 0.06$; y-intercept: $F = 19.34$, $P < 0.0001$). Both the slope (0.99) and the y-intercept (−0.11) of the O line were statistically indistinguishable from 1 and 0, respectively (slope: $F = 2.98$, $P = 0.09$; y-intercept: $F = 2.89$, $P = 0.09$).

Next, the δ^2H and δ^{18}O values for extracted water samples run on the IRIS were compared with the original un-extracted urine samples run on the IRIS (Figures 2a and b, respectively). Best-fit lines for the data sets were described by the equations:

$$\delta^2\mathrm{H}_{\mathrm{IRIS-ext.water}} = 0.99^{*}\delta^2\mathrm{H}_{\mathrm{IRIS-urine}} + 2.50‰(R^2 = 0.99,\ P < 0.0001)$$

$$\delta^{18}\mathrm{O}_{\mathrm{IRIS-ext.water}} = 1.0^{*}\delta^{18}\mathrm{O}_{\mathrm{IRIS-urine}} + 0.33‰(R^2 = 0.99,\ P < 0.0001).$$

In comparing the slope and intercept of the H best-fit line with those of a 1:1 line, we found that while the slope (0.99) was not significantly different from 1 ($F = 2.25$, $P = 0.1$), the y-intercept (2.5) was significantly different from 0 ($F = 53.3$, $P < 0.0001$). Both the slope (1.0) and the y-intercept (0.33) of the O best-fit line were statistically indistinguishable from 1 and 0, respectively (slope: $F = 0.19$, $P = 0.7$; y-intercept: $F = 19.34$, $P < 0.0001$).

4. Discussion

The range of δ^2H and δ^{18}O values of water in urine samples collected and analysed in this study spans the expected range of values across the USA. Within the range examined, −115 to −1‰ for δ^2H and −12 to 0‰ for δ^{18}O, the values for δ^2H and δ^{18}O obtained from IRIS analysis for both un-extracted urine and extracted water from urine were similar to those obtained for extracted water via IRMS analysis. The largest isotopic differences between two consecutive samples or 'jumps' were 115‰ for ^{2}H and 12‰ for ^{18}O. To reduce sample-to-sample memory within runs on the IRIS, Picarro guidelines for use of their CRDS system suggest bracketing samples so that the change in δ-value between adjacent samples is less than 100 and 10‰ for ^{2}H and ^{18}O, respectively. It is important to note that the isotopic jumps measured by the IRIS system in this

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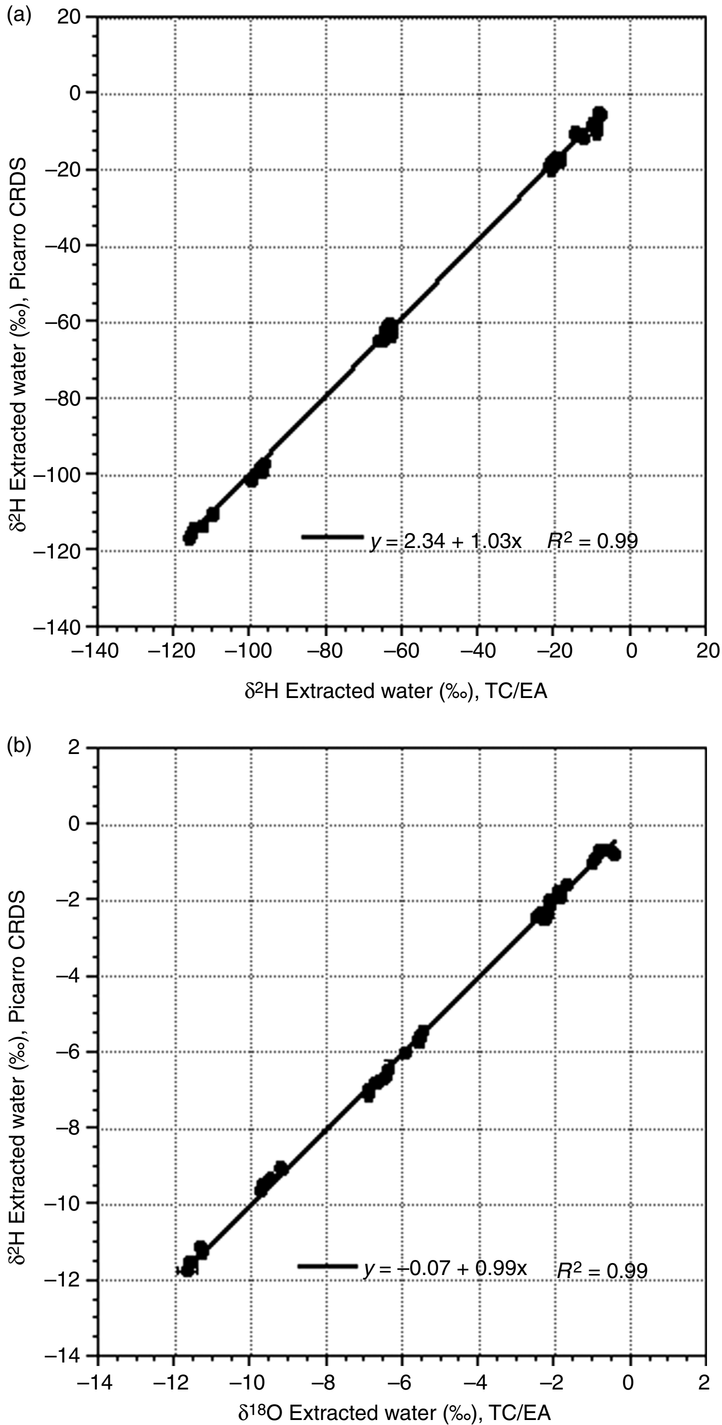

Figure 1. Isotope ratios of water extracted from urine and measured using both IRMS and IRIS. (a) For δ^2H values, the slope and y-intercept of the regression are significantly different from those of a 1:1 line, whereas for (b) δ^{18}O values, the slope and y-intercept of the regression are statistically indistinguishable from those of a 1:1 line.

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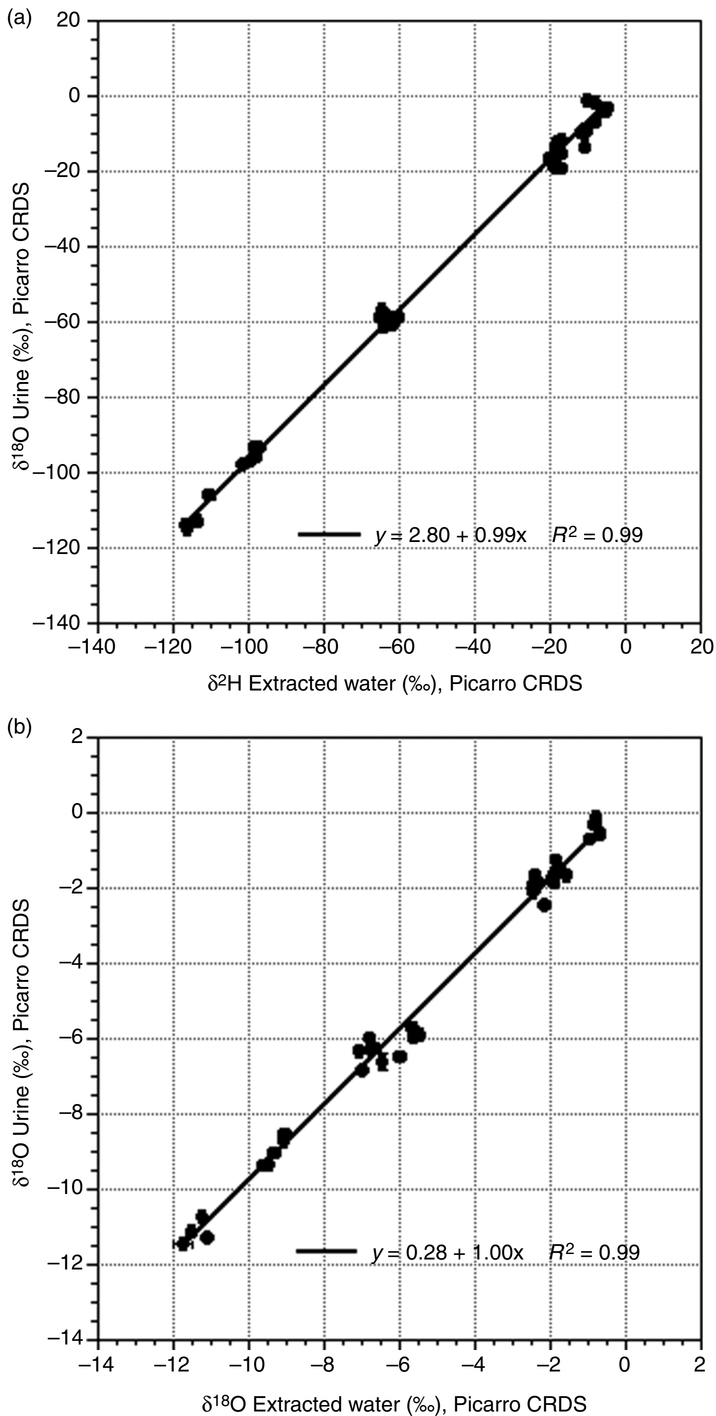

Figure 2. Isotope ratios of water extracted from urine and un-processed urine analysed via IRIS. For both (a) δ^2H values and (b) δ^{18}O values, the slope of each regression line is not significantly different from that of a 1:1 line, but the y-intercept is significantly different from zero.

study were only slightly above those recommended by the manufacturer relative to bracketing design, and larger jumps, greater than 100 and 10‰ for ^{2}H and ^{18}O, respectively, may introduce larger memory issues into sample analysis. TEE measurements that use the doubly labelled water method and dose participants with ^{2}H and ^{18}O are likely to result in isotopic values of body water (e.g. collected urine) outside of this range and may require additional sample replicates and/or washing steps to reduce sample-to sample memory between large isotopic jumps. A previous study of inter-sample memory effects, using IRIS analytical methods, has shown that isotopic jumps of 223 and 28‰, for ^{2}H and ^{18}O, benefit from five sequential injections to reduce memory effects, discarding the first two injection values and accepting the mean of the last three observations [20]. For this study, three sequential replicate injections were analysed because of the relatively small magnitude of isotopic differences between samples. This is acceptable, as Lis *et al.* [20] showed that for a 60‰ difference in δ^2H values between consecutive water samples, the mean of the second and third injections was within an acceptable analytical error of the correct value.

Specific to the urine samples analysed here, Picarro warns of the potential build-up of salts in the vaporiser and suggests vaporiser cleaning if memory increases and this becomes an issue. During a run of more than 1000 consecutive samples, it was determined that given a 4% (by weight) saltwater solution, salt concentration did not impact precision memory or concentration measurements by IRIS [21]. We have not experienced deterioration in the performance of our IRIS system due to the analysis of un-processed urine, as measured by secondary reference materials run both before, alongside, and since urine samples were processed. While our laboratory has continued to analyse both un-processed urine and water samples on our IRIS system, without encountering substantial memory problems, the total number of urine samples examined is in the hundreds and not thousands.

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The IRIS method for analysing water in urine provided the same accurate result as obtained with the IRMS method. It is important to note that the slopes of each of the δ^2H and δ^{18}O regression lines were not significantly different from 1. Additionally, the δ^{18}O y-intercepts were not significantly different from a value of 0. However, both of the y-intercepts for the relationship of the δ^2H values were significantly different from 0 (Figures 1a and 2a). Since the y-intercepts of 2.4 and 2.5‰ for extracted water analysed via IRMS and IRIS and for urine and extracted water analysed via IRIS, respectively, were indistinguishable (Figures 1a and 2a), it is most likely that this offset was associated with a contaminant not absorbed by the activated charcoal. Although both urine and extracted water were treated with activated charcoal, it is possible that contaminants remained in both sample types and that these contaminants may cause optical interference in IRIS analyses [22]. Organic contaminants contained in leaf water extracts have been shown to interfere with both IRIS and OA-ICOS, even after the treatment of water extracts with activated charcoal [16]. As pointed out by Brand *et al.* [22], in a recent letter to the editor discussing the measurement of extracted leaf water, 'activated carbon is less efficient in removing components such as alcohols, glycols, strong acids and bases', and thus treatment of either urine or extracted water with charcoal may not fully resolve the error associated with the δ^2H measurement of these samples. While further analytical research to identify the compounds present in urine and extracted water is necessary, the magnitude of the offset associated with δ^2H measurements in this study ($\sim$ 2‰) is close to machine precision for δ^2H analysis (2.0‰) and would not impact practical analysis of the data.

The analysis of the stable isotope ratios of water in urine with IRIS will be extremely useful for assessing population-level variation of body water, as well as in physiological and clinical applications in which fast and efficient processing of a large number of samples is necessary. Additional studies investigating the long-term impact of running urine and other high salt solutions through the IRIS system are required, but here we demonstrate the ability of IRIS to rapidly and accurately analyse the isotope ratios of water in urine within a normal biological isotopic range. The accuracy

of the IRIS measurement of urine is promising in terms of its further applications to other complex fluids, including additional biological fluids (i.e. blood and saliva) and beverages [17].

Acknowledgements

We would like to thank the IC Postdoctoral Fellowship Program for funding this research and Lesley Chesson, Libby Stern and Luciano Valenzuela for their comments on previous versions of this manuscript.

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