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ABSTRACT

Background: Given the utility of the doubly labeled water (DLW) method for determination of energy expenditure, additional techniques for isotope analysis of the samples are welcome. Laser-based instruments are one such new analytical tool, but their accuracy and feasibility for DLW studies are grossly understudied.

Objectives: We assessed the accuracy of laser-based isotope ratio measurements as part of the DLW method for estimation of carbon dioxide production rate (rCO_2) and total energy expenditure (TEE), in between-group comparison study designs.

Methods: Urine samples from a previous study were analyzed with a laser-based instrument [off-axis integrated cavity output spectroscopy (OA-ICOS)]. In that study, participants consumed a high-, moderate-, or low-carbohydrate diet for 20 wk; urine samples were obtained in weeks 18–20 before and after a ^{2}H- and ^{18}O-enriched water dose. Isotope ratios (δ^2H and δ^{18}O), rCO_2, and TEE calculated by standard methods were compared to results previously obtained with the standard technique of isotope ratio mass spectrometry (IRMS). Bias, SD, and bias ± 1.96SD bands between IRMS and OA-ICOS were computed.

Results: The between OA-ICOS and IRMS rCO_2 and TEE trends were equivalent (within 1.2% and 4.1%, respectively), in spite of the differences in measured δ^{18}O values at high enrichment levels. The OA-ICOS δ^{18}O values displayed an increasing offset from the IRMS results as the ^{18}O enrichment increased (mean ± SD 4.6–5.7‰ ± 2‰ offset at the time point with highest ^{18}O enrichment, ∼135‰), whereas the hydrogen isotope ratio (δ^2H) differed only slightly between the methods (mean offset −4.9‰ for all time points). The between-diet differences in TEE from the previous study were recapitulated with a smaller subset of participants and time points.

Conclusions: OA-ICOS analysis is an accurate and feasible technique for the DLW method. Given the δ^{18}O offset observed at high enrichment, validation of each OA-ICOS instrumental setup against established methods (e.g., IRMS) is recommended. *J Nutr* 2022;152:78–85.

Keywords: doubly labeled water, total energy expenditure, stable isotopes, OA-ICOS, isotope ratio mass spectrometry

Introduction

The amount of energy that an animal expends is a combination of the basal or resting metabolic rate, the thermic effect of food, and the amount of activity undertaken. None of these 3 parameters are easy to measure in free-ranging individuals, and determining the total amount of energy expended can require the use of indirect calorimetry with the concomitant loss of free movement. A technique of long standing called the doubly labeled water (DLW) method is a powerful tool for nutrition and metabolic studies, giving an estimate of total energy expenditure (TEE) in free-ranging subjects (1–5). The DLW technique involves measuring the decline of ^{2}H and ^{18}O in the subject's body water after administration of an isotopically enriched water dose; in human studies, urine is often sampled. Three major challenges await researchers in DLW studies: the procurement and cost of the ^{18}O-labeled water

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(^{2}H-labeled water is relatively easy to obtain and inexpensive); administration of the labeled water and collection of samples to be measured; and, finally, the measurement of the amount of isotopes both in the dose and in the samples collected. This article focuses on the last of the challenges: the instrumentation used to measure the isotopic composition of the fluid collected over time and the associated costs involved.

Traditionally, isotope ratio mass spectrometry (IRMS) has been used for δ^2H and δ^{18}O determination, whereby the amount of ^{2}H and ^{18}O in the sample relative to a standard is measured. Commonly, δ^2H is measured on H_2 gas generated by reduction of the aqueous sample with a metal catalyst (e.g., zinc, chromium) or on H_2 gas which has been equilibrated with the aqueous sample and a catalyst (6). Separately, δ^{18}O is determined on carbon dioxide gas equilibrated with the water in the sample (2–4, 7, 8). An alternative IRMS method uses a Thermal Conversion Elemental Analyzer, using high temperature to convert water to H_2 and carbon monoxide gas to determine δ^2H and δ^{18}O on the same aliquot of sample (9). Relatively recent alternative instrumentation for δ^2H and δ^{18}O measurement has become commercially available. Two different types of laser-based instruments—cavity ring-down spectroscopy (CRDS), manufactured by Picarro, Inc., or off-axis integrated cavity output spectroscopy (OA-ICOS), manufactured by Los Gatos Research, Inc.—use infrared spectroscopy to determine the amounts of ^{2}H and ^{18}O in the sample. These laser-based instruments measure the intensity of absorption of infrared light passing through the vaporized water sample. Both instruments are far less expensive and simpler to house and operate than IRMS instruments. Currently, the cost differential to purchase an IRMS with the necessary peripherals compared with a laser-based instrument is ∼5-fold, not counting infrastructure remodeling costs. A study that utilized a CRDS (10) detailed some generalized facts regarding laser-based instruments: the benefit of a smaller footprint, less or no demand for compressed specialty gases, a substantially reduced training time, and reduced operating expertise. The laser-based instruments have been enthusiastically adopted for isotopic measurements of nonsaline water, but the use of these instruments with biological samples is less widespread. At this juncture, without further research, the 2 laser-based instruments cannot be assumed to be analytically equivalent to IRMS in DLW studies.

There are few, and industry-associated, published studies comparing laser-based instruments to IRMS measurements for urine samples. Specifically, there was a comparison of DLW TEE with an early instrument design against IRMS [CRDS, 14 subjects (10)]; a second comparison of isotope ratios obtained by IRMS and an OA-ICOS instrument (9); and a third comparison of carbon dioxide production rate (rCO_2) from a DLW study obtained via IRMS and OA-ICOS, and rCO_2 from whole-room indirect calorimetry [17 subjects (8, 11)].

Here we focus on several issues in the use of laser-based spectroscopy for human DLW studies. We assess the impact the urine samples have on the vaporization chamber in terms of cleaning protocols and time costs. Second, we assess the differences and variability between IRMS and laser-based data at the natural abundance level and with increasing ^{2}H and ^{18}O amounts. Third, we compare the industry-supplied and -certified standard isotopic values as they relate to DLW studies. In a blind test, we determine whether the laser-based instrument data, based on only 29 subjects, can replicate the previous published trends of a large-scale nutritional study (12).

Methods

We obtained bio-banked (−80°C) urine samples from 30 individuals, 10 from each of 3 diet treatments from the study of Ebbeling et al. (12). That study protocol was approved by the Institutional Review Board at Boston Children's Hospital and registered at clinicaltrials.gov (NCT02068885). Briefly, after a 9- to 10-wk energy-restricted run-in phase, participants consumed a high-, moderate-, or low-carbohydrate diet for a 20-wk weight-maintenance phase. Urine samples used in this current study were obtained in weeks 18–20 before and after a ^{2}H- and ^{18}O-enriched water dose for DLW measurement of energy expenditure (12). For each individual, we used 4 precisely known collection time points for analysis: predose, day 1, day 7–8, and day 14–15. The urine samples were sent to us with deidentified codes but identified collection time points. We were blinded to the diet group assignment and the IRMS isotopic values until the data from the laser-based study were supplied to collaborators. The original study used 10 time points; however, for comparison with OA-ICOS we recalculated rCO_2 and TEE using the original IRMS data from the same 4 time points. The original IRMS data were generated between January 2015 and March 2017, and the OA-ICOS data between September 2019 and December 2019; validation work shows that results are reproducible over 4.4 y (13).

Before analysis the urine samples were thawed, centrifuged at 10,000 × *g* for 10 min at 4°C, and 1–2 mL of supernatant were transferred to analysis vials. Samples were analyzed on a Los Gatos Liquid Water Isotope Analyzer, an OA-ICOS instrument, model T-LWIA-45-EP (hereafter named "OA-ICOS").

Following a previously published study (9), isotopically enriched urine samples were injected 12 times; the first 9 injections were ignored and the mean of the last 3 injections was taken. For the baseline (predose) samples, 8 injections were made and the mean of the last 4 was taken. Three to 5 waters that were standardized to the Vienna Standard Mean Ocean Water (VSMOW)-Standard Light Antarctic Precipitation (SLAP) scale and spanned and bracketed the samples' isotopic composition (**Table 1**), as well as 1 of 2 in-house control waters, were analyzed periodically throughout the run (1 previously published; 14). Standards used with the sample isotopic range were as follows: δ^2H −36‰ to −2‰ and δ^{18}O −5‰ to −1‰: 3C, 4C, 5C; δ^2H 104‰–537‰ and δ^{18}O 10‰–47‰: 5C, ER1A, ER2, ER3, WA103; δ^2H 323‰–834‰ and δ^{18}O 42‰–90‰: ER1A, ER2, ER3, WA103; δ^2H 736‰–1313‰: ER3, International Atomic Energy Agency (IAEA) 302a, WA103, IAEA 302b, WA104; δ^{18}O 110‰–197‰: ER3, WA103, IAEA 304a, WA104 (Table 1). Isotopic values relative to the international reference water, VSMOW, were computed by generating a linear regression of the nominal isotopic ratios of the standard waters (reported by the supplier) on the measured (raw) values from the instrument. The regression slope and intercept were used to calculate the VSMOW-normalized sample δ^2H and δ^{18}O values from the raw values (yielding results we name A). The Los Gatos postprocessing software was used to average the multiple injections per sample, generate the regression lines, and compute the isotopic values. Each standard water has supplier-provided reference values relative to the internationally agreed VSMOW-SLAP

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Supplemental Discussion, Supplemental Figures 1–3, and Supplemental Tables 1–5 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.
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Abbreviations used: CRDS, cavity ring-down spectroscopy; DLW, doubly labeled water; IAEA, International Atomic Energy Agency; IRMS, isotope ratio mass spectrometry; OA-ICOS, off-axis integrated cavity output spectroscopy; ppm, parts per million; rCO_2, carbon dioxide production rate; RQ, respiratory quotient; SLAP, Standard Light Antarctic Precipitation; TEE, total energy expenditure; USGS, United States Geological Survey; VSMOW, Vienna Standard Mean Ocean Water.

TABLE 1 Water reference materials used[1]

Standards	δ^2H_{VSMOW}, ‰				$\delta^{18}O_{VSMOW}$, ‰				Supplier
	Reported by supplier[2]	Measured (USGS)[3]	Measured raw OA-ICOS[4]	OA-ICOS *n*	Reported by supplier[2]	Measured (USGS)[3]	Measured raw OA-ICOS[4]	OA-ICOS *n*	
3C	−97.3	—	−100 ± 2	30	−13.39	—	−12.4 ± 0.8	30	Los Gatos
4C	−51.6	—	−54 ± 1	30	−7.94	—	−6.9 ± 0.8	30	Los Gatos
5C	−9.2	—	−13 ± 3	39	−2.69	—	−1.9 ± 0.9	39	Los Gatos
ER1A	107.2	105.44	107 ± 4	16	12.25	12.21	13.1 ± 1.1	16	Los Gatos
ER2	191.40	186.17	190 ± 3	20	23.03	22.71	23.8 ± 1.1	22	Los Gatos
ER3	383.30	380.37	384 ± 4	28	47.88	47.39	48.7 ± 1.2	29	Los Gatos
IAEA 302a	506	—	514 ± 2	15					IAEA[5]
WA103	843.43	831.2	840 ± 3	41	108.63	107.43	109.0 ± 1.3	41	Elemental Microanalysis
IAEA 302b	992.3	—	1008 ± 2	12					IAEA[5]
IAEA 304a					252.9	251.01	253.8 ± 1.9	14	IAEA[5]
WA104	1701.83	1673.51	1693 ± 9	13	266.83	263.53	266.9 ± 1.7	14	Elemental Microanalysis

[1]IAEA, International Atomic Energy Agency; OA-ICOS, off-axis integrated cavity output spectroscopy; USGS, United States Geological Survey; VSMOW, Vienna Standard Mean Ocean Water.
[2]Standard values reported by supplier calibrated to the VSMOW-Standard Light Antarctic Precipitation scale.
[3]Measured at the USGS by isotope ratio mass spectrometry as an unknown sample.
[4]Values are mean ± 1SD.
[5]International standard from the IAEA (16); no longer available.

normalization scale; for example, reference water WA103 (Table 1) was calibrated against VSMOW2 and SLAP2 by IRMS, using a third reference GISP (Greenland Ice Sheet Precipitation) as a check (15). We were fortunate to have access to ^{2}H- and ^{18}O-enriched waters (IAEA 302a, 302b, 304a) with internationally agreed recommended values (16) as part of our working standard set (Table 1), providing a solid anchor to the VSMOW-SLAP scale.

Isotope data are reported in δ notation to indicate that they are relative to an international standard, according to the following equation:

$$\delta^2H\,(‰) = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 1000 \quad (1)$$

where R is the ratio ^{2}H:^{1}H (or ^{18}O:^{16}O for δ^{18}O). In the case of H and O, the standard is VSMOW, and δ^2H and δ^{18}O are normalized to the VSMOW-SLAP scale (17, 18). DLW calculations are made using parts per million (ppm) ^{2}H or ^{18}O above baseline, which can be obtained by transformation of the δ values (4, 19).

A subset of the most highly enriched reference waters was analyzed by IRMS at the United States Geological Survey (USGS) (Table 1). Subsequently, the urine samples' isotopic composition was recomputed using the newly obtained values from the USGS; our intent was to examine the effect of variation of the isotopic composition of the reference materials on sample isotopic composition (results B).

We followed the multipoint method calculation protocol as described by the IAEA (19) with the recently developed best practice equations (20). We used the slope and intercept of ln(ppm excess) as a function of time for each isotope, the diluted dose measured by IRMS, and the equations are as follows (20):

$$N(mol) = \frac{1}{2}(N_o/1.007 + N_d/1.043) \quad (2)$$

$$r_{CO_2}(L/d) = N\{0.45859(-k_o) - 0.47498(-k_d)\} \times 22.26 \quad (3)$$

$$TEE(MJ/d) = r_{CO_2}(1.106 + 3.94/RQ) \times 4.184/10^3 \quad (4)$$

where N is total body water; N_o and N_d are the oxygen and hydrogen dilution spaces, respectively (19, equation III.8); k_o and k_d are the slopes of the ln(ppm excess) regressions on time for oxygen and hydrogen, respectively; and RQ is the respiratory quotient.

The food quotient for each group (12) was substituted for RQ. One subject was excluded from further analysis owing to poor regression fit in all analyses, both with IRMS and with OA-ICOS data [$r^2 = 0.989$–0.991 for ln(ppm H excess) on time].

We assessed the option of using the isotopic offset between the 2 instruments to generate an OA-ICOS-instrument-specific correction factor; this test is detailed in the supplementary data (**Supplemental Tables 1–5, Supplemental Figures 1–3**). These calculation methods have therefore resulted in several computed data sets: A with the nominal standard values, B with the remeasured standard values, and 2 additional data sets considering an instrument-specific correction (supplementary data). We calculated the bias (mean difference), SD, and bias ± 1.96SD bands between IRMS and OA-ICOS for δ^2H, δ^{18}O, N_d, N_o, k_d, k_o, rCO_2, and TEE. We also computed the linear regression of the bias in TEE on mean TEE, and a paired t test of TEE by IRMS compared with OA-ICOS, for each of the calculation methods. We used model II regression, also known as reduced major axis or standard major axis regression, to compute the subject-wise relation between TEE by IRMS and TEE by OA-ICOS, using the *lmodel2* package in R (21, 22), because it is arbitrary which is the x or y variable (23).

In addition, we tested the effect of an increasing IRMS–OA-ICOS δ^{18}O difference on rCO_2 and TEE by increasing the OA-ICOS δ^{18}O values to generate differences with IRMS of up to ~25‰ at maximal ^{18}O enrichment (the first postdose sample).

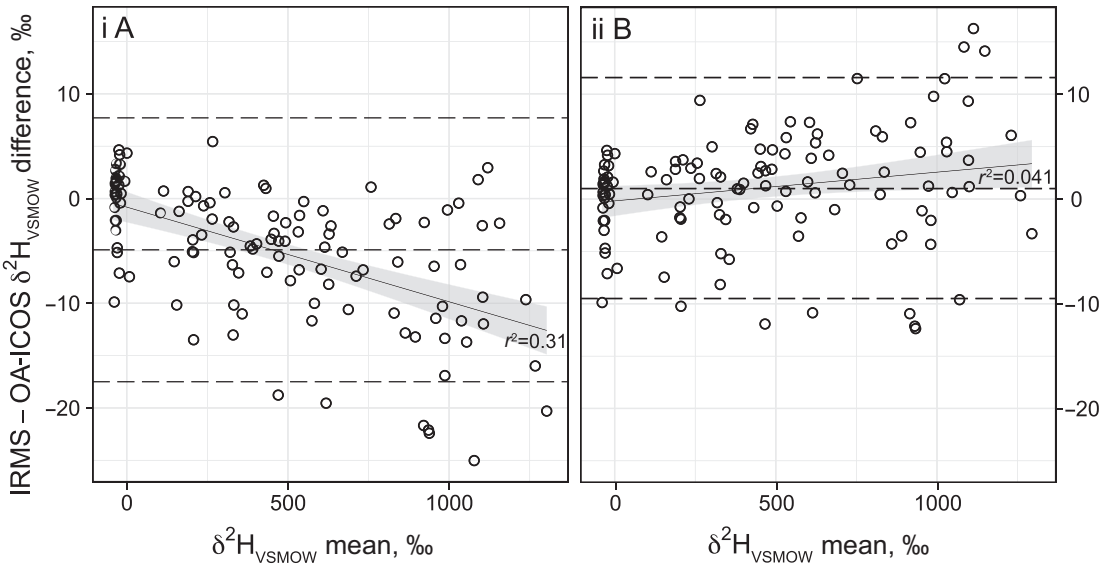

FIGURE 1 Difference in δ^2H between IRMS and OA-ICOS for each urine sample, with standards' nominal values (i) (method A) and with standards' revised values (ii) (method B). $n = 120$ in each panel. The cluster of values around and below δ^2H values of 0‰ are the baseline unenriched samples. The dashed horizontal lines represent the mean difference (bias) and bias ± 1.96SD, and the gray envelope is the 95% CI of the linear regression of the difference on the mean. IRMS, isotope ratio mass spectrometry; OA-ICOS, off-axis integrated cavity output spectroscopy; VSMOW, Vienna Standard Mean Ocean Water.

Results

Agreement of δ^2H and δ^{18}O values

The predose measurements, at natural isotopic abundance, were in excellent agreement between IRMS and OA-ICOS [δ^{18}O: mean difference: 0.03‰ ± 0.4‰ (1SD); mean absolute difference: 0.3‰ ± 0.2‰; mean: −3.5‰ ± 0.8‰ by IRMS and OA-ICOS (result A); and δ^2H: mean difference: 0.2‰ ± 3‰; mean absolute difference: 2.5‰ ± 2.2‰; mean: −27.6‰ ± 9.9‰ by IRMS and −27.8‰ ± 8.8‰ by OA-ICOS (result A)]. These values are within the limits of acceptable analytical reproducibility of δ^{18}O and δ^2H measurements for both techniques.

In the isotopically enriched urine samples (postdose), there was fair agreement between the methods for δ^2H across the range of values measured here [up to ~1300‰, Figure 1(i); mean offset: –4.9‰ ± 6.4‰ (1SD) for all points and –9.9‰ ± 7.7‰ (1SD) for the highest enriched samples only]. However, δ^{18}O values showed a monotonous increasing divergence with higher δ^{18}O values [Figure 2(i)].

When the revised standard values were used (Table 1), the δ^{18}O differences between IRMS and OA-ICOS decreased

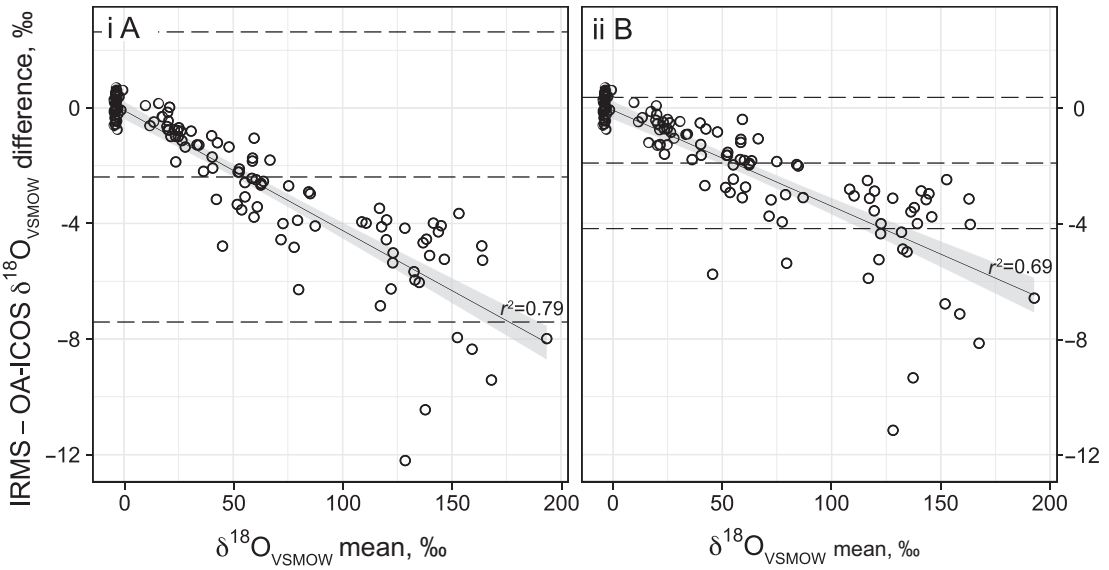

FIGURE 2 Difference in δ^{18}O (‰) between IRMS and OA-ICOS for each urine sample, with standards' nominal values (i) (method A) and with standards' revised values (ii) (method B). $n = 120$ in each panel. The cluster of values around and below δ^{18}O values of 0‰ are the baseline unenriched samples. The dashed horizontal lines represent the mean difference (bias) and bias ± 1.96SD, and the gray envelope is the 95% CI of the linear regression of the difference on the mean. IRMS, isotope ratio mass spectrometry; OA-ICOS, off-axis integrated cavity output spectroscopy; VSMOW, Vienna Standard Mean Ocean Water.

TABLE 2 rCO_2 calculated from data from IRMS and OA-ICOS by diet[1]

	rCO_2,[2] L/d				Compared with IRMS,[3] %			
	All diets	High CHO	Moderate CHO	Low CHO	All diets	High CHO	Moderate CHO	Low CHO
IRMS	439 ± 91	406 ± 67	446 ± 123	462 ± 70				
OA-ICOS A	438 ± 76	409 ± 57	445 ± 96	457 ± 69	−0.1	0.2	−0.1	−0.3
OA-ICOS B	429 ± 81	390 ± 63	443 ± 96	452 ± 73	−0.7	−1.2	−0.2	−0.6

[1]All diets, $n = 29$; high CHO, $n = 9$; moderate CHO, $n = 10$; low CHO, $n = 10$. CHO, carbohydrate; IRMS, isotope ratio mass spectrometry; OA-ICOS, off-axis integrated cavity output spectroscopy; rCO_2, carbon dioxide production rate.
[2]Values are mean ± 1SD.
[3]Difference/mean × 100.

slightly (results B, Figure 2) and the δ^2H differences became nonsignificant (results B, Figure 1) [mean difference: 1.0‰ ± 5.4‰ (1SD)].

Carbon dioxide production and TEE

The derived rCO_2 and TEE were in very good agreement between IRMS and OA-ICOS, on average, for all data-processing methods (**Tables 2** and **3**, Supplemental Tables 2 and 3). The energetic difference trends between the 3 diet groups noted in the original study (12) were maintained with both IRMS and OA-ICOS with the much smaller subset of data used here (**Figure 3**).

The mean ± SD bias in TEE [defined as the mean of each pairwise difference (24)] between IRMS and OA-ICOS was 0.03 ± 1.0 MJ/d (calculation method A, **Table 4**) and 0.23 ± 1.1 MJ/d (calculation method B, Table 4). The bias values were not different from 0, within 1 SD; there was no difference on average between the TEE computed using IRMS or OA-ICOS (paired t test, $P = 0.87$ for A, $P = 0.26$ for B). In addition, there was no trend in the difference in TEE between IRMS and OA-ICOS as a function of mean TEE (linear regressions of bias on mean TEE: $P = 0.063$ and $P = 0.30$ for A and B, respectively). **Figure 4** shows TEE by OA-ICOS compared with IRMS.

With this data set, the modeled effect on TEE with a shift in $\delta^{18}O$ was −0.022 ± 0.010 MJ · d^{-1} · $‰^{-1}$ (mean ± 1SD; range: −0.042 to 0.0012 MJ · d^{-1} · $‰^{-1}$). That is, a 1‰ shift in $\delta^{18}O$ (by OA-ICOS) at the highest enrichment resulted in a −0.022 MJ/d change in TEE. The equivalent change in rCO_2 was −0.91 ± 0.42 L · d^{-1} · $‰^{-1}$ (mean ± 1SD).

Discussion

The percentage difference between IRMS and OA-ICOS was small, and was slightly different between results A and B (Tables 2 and 3). Calculation results A and B were within 1.2% for rCO_2 and 4.1% for TEE, with most comparisons returning <2.2% differences in TEE. This level of uncertainty is reasonable in comparison with the magnitude of expected uncertainty in estimation of energy expenditure by the DLW technique. Recently compiled differences between rCO_2 by DLW and calorimetry are −0.4% ± 7.7% (mean ± 1SD) using the revised equations also used here (20). The differences between calculations A and B did not depend on diet group, so that the energetic differences between the diet groups were reproduced by OA-ICOS (Figure 3). These acceptably small differences suggest that using OA-ICOS for DLW studies in humans (using urine) produces data of good quality. This recapitulation of the between-group TEE trends was achieved using a subset of the time points and individuals (4 compared with 10 time points; 29 compared with 162 individuals) from the original study (12).

Melanson et al. (8) compared rCO_2 via DLW measured with OA-ICOS and IRMS to whole-room indirect calorimetry, using 17 subjects. They found a ∼10% difference in rCO_2 using the intercept method between IRMS and OA-ICOS [Figure 3 in Melanson et al. (8)], with OA-ICOS agreeing more closely with indirect calorimetry (∼3%) than did IRMS. However, in 5 other DLW validation studies (2, 25–28), rCO_2 calculated from isotopic data using IRMS showed good agreement with indirect calorimetry. Using an older-version CRDS instrument, other researchers found TEE differences using the laser-based instrument and IRMS of 0.5% ± 6% (mean ± 1SD), but highlighted the need to be attentive to memory effects from successive injections (10). Our results here compare very favorably in terms of yielding low percentage differences between the 2 methods (Table 2).

The divergence of $\delta^{18}O$ between IRMS and OA-ICOS at higher $\delta^{18}O$ values (Figure 2) was not significant enough to cause much of a discrepancy in rCO_2 or TEE in this study. At the highest ^{18}O enrichment (a mean $\delta^{18}O$ value of 135‰), at day 1, the mean ± SD absolute difference of $\delta^{18}O$ between IRMS and IA-ICOS was 5.7‰ ± 2‰ (calculation method A) and 4.6‰ ± 2‰ (calculation method B).

We modeled the effect of an even greater shift in $\delta^{18}O$ by OA-ICOS on calculated TEE values using the data here. We increased the difference between OA-ICOS and IRMS in

TABLE 3 TEE calculated from data from IRMS and OA-ICOS by diet[1]

	TEE,[2] MJ/d				Compared with IRMS,[3] %			
	All diets	High CHO	Moderate CHO	Low CHO	All diets	High CHO	Moderate CHO	Low CHO
IRMS	10.6 ± 2.4	9.3 ± 1.5	10.7 ± 2.9	11.8 ± 1.8				
OA-ICOS A	10.6 ± 2.0	9.4 ± 1.3	10.7 ± 2.3	11.7 ± 1.7	−0.3	0.8	−0.3	−1.0
OA-ICOS B	10.4 ± 2.1	8.9 ± 1.5	10.6 ± 2.3	11.5 ± 1.9	−2.2	−4.1	−0.8	−2.2

[1]All diets, $n = 29$; high CHO, $n = 9$; moderate CHO, $n = 10$; low CHO, $n = 10$. CHO, carbohydrate; IRMS, isotope ratio mass spectrometry; OA-ICOS, off-axis integrated cavity output spectroscopy; TEE, total energy expenditure.
[2]Values are mean ± 1SD.
[3]Difference/mean × 100.

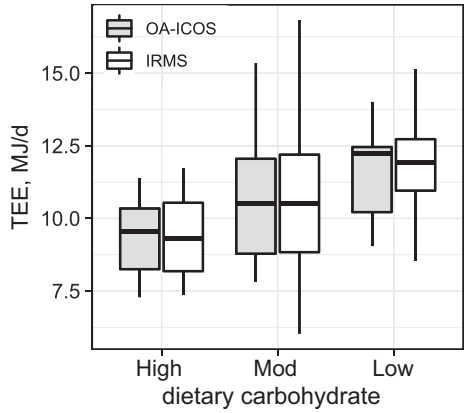

FIGURE 3 TEE by diet group [high ($n = 9$), moderate ($n = 10$), and low ($n = 10$) carbohydrate] using isotope data generated by OA-ICOS (shaded boxes) and IRMS (light boxes), using results A with standards' nominal values (provided by the supplier). The thick horizontal line is the median, the hinges (the horizontal box edges) are the first and third quartiles, and the whiskers extend from the hinge to either the smallest or largest value, or not more than 1.5 times the interquartile range. No differences between methods are noted but TEE does vary by diet. IRMS, isotope ratio mass spectrometry; OA-ICOS, off-axis integrated cavity output spectroscopy; TEE, total energy expenditure.

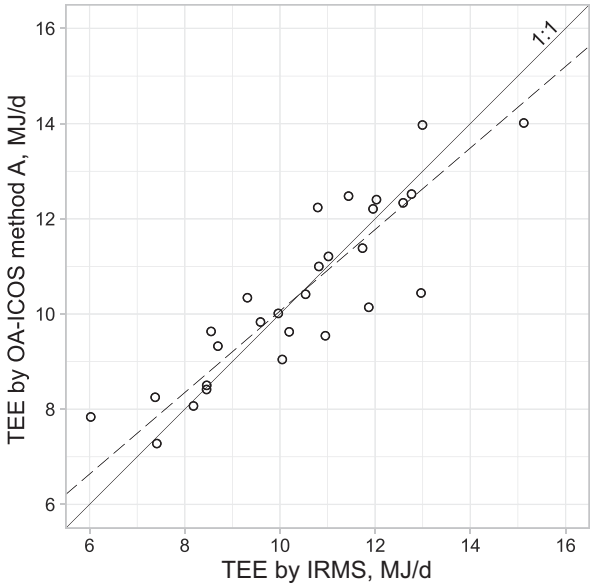

FIGURE 4 TEE using isotope data generated by OA-ICOS with calculation method A compared with IRMS, for each subject ($n = 29$). The dashed line is the model II regression (standard major axis) line, given by TEE (OA-ICOS) = 0.86 TEE (IRMS) + 1.5, where 95% CIs are 0.73–1.0 for the slope and −0.13 to 2.9 for the intercept (21, 22). The solid line is the line of identity. IRMS, isotope ratio mass spectrometry; OA-ICOS, off-axis integrated cavity output spectroscopy; TEE, total energy expenditure.

stepwise increments (≤25‰ at the highest enrichment) and recomputed the TEE at each step. As a result we were able to estimate the effect of changing $\delta^{18}O$ on TEE. The mean shift of –0.022 ± 0.010 MJ · d^{-1} · ‰$^{-1}$ means that a 10‰ $\delta^{18}O$ difference results in a 0.22 MJ/d change in TEE, several times smaller than the mean difference between the diet groups (1.1–1.4 MJ/d). The direction of the shift was the same for all but 1 of the subjects. Both these factors (small mean shift, all but 1 in the same direction) mean that a fairly large $\delta^{18}O$ offset would not affect the between-group differences and subsequent interpretations in dietary intervention studies such as the one used as a test case here. However, in smaller animals (including human infants) where higher ^{18}O doses are generally applied (4), caution is warranted. If the trend of increasing offset from IRMS continues to higher enrichments [Figure 2(i)], unacceptable error in estimated TEE may occur.

TABLE 4 Bias and 95% CIs between IRMS- and OA-ICOS-derived results[1]

	δ^2H bias, ‰	$\delta^{18}O$ bias, ‰
A	−4.9 ± 6.4 (−17.5, 7.7)	−2.4 ± 2.6 (−7.4, 2.6)
B	1.0 ± 5.4 (−9.5, 11.6)	−1.9 ± 1.2 (−4.2, 0.4)
	rCO_2, L/d	TEE bias, MJ/d
A	1.1 ± 40 (−77, 80)	0.03 ± 1.0 (−1.9, 2.0)
B	9.8 ± 45 (−79, 99)	0.23 ± 1.1 (−1.9, 2.4)
	N_d bias, kg	N_o bias, kg
A	0.39 ± 0.32 (−0.24, 0.97)	1.5 ± 0.57 (0.43, 2.66)
B	−0.06 ± 0.30 (−0.65, 0.54)	1.2 ± 0.53 (0.15, 2.21)
	k_d bias, × 10^{-3}/d	k_o bias, × 10^{-3}/d
A	−0.2 ± 1.2 (−2.5, 2.2)	0.5 ± 1.5 (−2.5, 3.4)
B	0.0 ± 1.2 (−2.3, 2.4)	0.0 ± 1.6 (−3.1, 3.1)

[1] $n = 29$. Values are mean ± 1SD (−1.96 SD, +1.96 SD). IRMS, isotope ratio mass spectrometry; k_d, ^2H elimination constant; k_o, ^{18}O elimination constant; N_d, hydrogen dilution space; N_o, oxygen dilution space; OA-ICOS, off-axis integrated cavity output spectroscopy; rCO_2, carbon dioxide production rate; TEE, total energy expenditure.

The use of the revised standard values (B, with standards measured at USGS, Table 1) resulted in a slightly smaller $\delta^{18}O$ and near-zero δ^2H offset from IRMS [Figures 1(ii), 2(ii)]; however, there was a somewhat less good agreement of rCO_2 and TEE with the results using IRMS (Tables 2 and 3). The intent of remeasuring the isotope ratios of some of these reference materials was to obtain an additional independent verification of their isotopic composition, and to conduct an exercise to see what effect shifts in the values have on resultant sample isotopic values. We unequivocally do not suggest these new values be used; they were measured only once in a single laboratory, which is not an adequate characterization for adoption as a standard material. However, given the offset between the $\delta^{18}O$ measured by OA-ICOS and IRMS methods coupled with the limited availability of verified highly enriched oxygen isotope water standards, care must be taken that an appropriate range of enriched water standards are used in an analysis.

The test of OA-ICOS on isotopically enriched urine samples for DLW studies presented by Melanson et al. (8) does not include the measured OA-ICOS or IRMS $\delta^{18}O$ and δ^2H values. However, Berman et al. (9) found <0.8‰ $\delta^{18}O$ and <2.5‰ δ^2H differences in urine measured by IRMS and OA-ICOS, at enrichments of ≤80‰ and ≤725‰, for $\delta^{18}O$ and δ^2H, respectively—lower maximal values than in this present work. Those authors also noted excellent agreement between methods in pure water at $\delta^{18}O$ values of up to ~275‰. Our findings highlight the benefit of checking consistency between a particular OA-ICOS instrument and a reference method: the need to examine the analytical stability of highly enriched ^{18}O in the sample type of interest and not to rely solely on the behavior of pure water values in the system. If IRMS is available for comparison, an "instrument-specific" OA-ICOS correction

can be determined (see **Supplemental Discussion**, Supplemental Figures 1 and 2), assuming this offset remains constant for the instrument and the particular sample type. We note that a blind round-robin test of CRDS and OA-ICOS instruments on unenriched water samples resulted in more accurate results for δ^2H determination than for δ^{18}O in general (29).

Practical considerations

We have found that the throughput of OA-ICOS for urine samples is similar to our experience of using IRMS by Thermal Conversion Elemental Analysis for δ^2H and δ^{18}O determination on the same sample aliquot. The OA-ICOS takes a short time (~2 min) for each sample injection, but in order to account for memory effects inherent in the instrumental operation, many injections per sample are needed [although some mathematical strategies may reduce this (30)]. The need for more injections is increased with saline, biologically derived, and highly enriched samples like these, because the variability between injections is greater than for pure water. Further optimization and reduction in the number of injections could be made; for example, we observed that the isotope values of the ninth injection were typically the same as those of the last 3. We have also found, as mentioned before (31), that frequent cleaning of the injection block is needed, typically at least daily, and this necessarily interrupts the instrument's operation.

However, compared to IRMS, OA-ICOS's advantages include the previously mentioned lower initial cost, no need for specialty compressed gases, and less specialist user training. The instrument can also be run largely unattended between injection block cleanings. We found that it is useful to monitor the data as they are generated, so that the operator can intervene to stop the instrument and change the injection block. The main indications of a needed block change are increasing isotopic variability between injections of the same sample, a shift in the isotope value of a pure water control sample interleaved throughout the run, and a decrease in sample amount reaching the analysis chamber to unacceptable levels due to blockage of the injection port with mineral and organic precipitate. Without this careful monitoring and frequent injection block changes, the risk of erroneous data is high.

Reference material availability and implications

Stable isotope ratio measurements are dependent on having reference materials with accepted values for data normalization. Primary reference materials or standards have internationally agreed isotope ratios that set the common reference scale to which all data are normalized. For ^{2}H- and ^{18}O-enriched waters, few primary reference materials are available. Only 1 primary standard is currently available that has suitable isotope ratios: a relatively new IAEA standard (IAEA 607, δ^2H = 802.4‰, δ^{18}O = 99.02‰) which is close to, but below, the highest values measured in this study. Isotopic data should be standardized using materials whose isotopic ratios bracket those of the samples, so ^{2}H- and ^{18}O-enriched waters are needed.

Secondary reference materials or working standards have isotopic values that are determined in relation to primary reference materials, and are therefore 1 step away from the internationally agreed reference scale, VSMOW-SLAP for δ^2H and δ^{18}O. These materials are intended to be used on a day-to-day basis to normalize results. They are less costly and available from several sources. The waters available cover the ranges of δ^2H and δ^{18}O values encountered in DLW studies in humans: an important attribute, because isotopic data should be standardized using materials whose isotopic ratios bracket those of the samples. However, the paucity of primary reference materials in a suitable isotopic range means that many of these secondary reference materials were calibrated by extrapolation to higher δ^2H and δ^{18}O values from VSMOW2 and SLAP (which have unenriched δ^2H and δ^{18}O values, e.g., VSMOW2 δ^2H = δ^{18}O = 0‰). We hope that the increased use of laser-based instruments will prompt the creation of commercially available working standards that are calibrated using the enriched primary reference waters now available (e.g., IAEA 607). This is not unique to laser-based instruments: we note that IRMS is also subject to the need for good reference materials that bracket the range of the samples. For DLW experiments that require higher isotopic enrichments (e.g., small animals), 2 certified waters (IAEA 608 and 609) may prove useful in developing secondary standards.

Conclusions

We have shown that rCO_2 and TEE can be reliably determined using an OA-ICOS instrument in the case where differences are sought between diet or treatment groups. In agreement with 1 previous study (8), we found deviations at high δ^{18}O values of urine between IRMS- and OA-ICOS-generated values that are not observed in pure waters of similar isotopic enrichment [e.g., (9)]. Whether the deviation between IRMS and OA-ICOS in urine at high δ^{18}O is solely due to urea is not known. It is possible that the partitioning of ^{18}O/^{16}O among water vapor, free water, and the urea–water cluster may be altered in high-^{18}O conditions (32). Efforts to remove urea and maintain isotopic fidelity have not been exhaustively attempted, but simple addition of activated charcoal is unlikely to remove a substantial amount of urea. Our attempt at removal with activated charcoal resulted in a <25% loss of urea, which is in agreement with the complex challenge of urea removal highlighted in other studies (33).

If another accepted technique, such as IRMS, is available for comparison, an "instrument-specific" OA-ICOS correction on the sample type in question can be determined. It is conceivable that highly enriched δ^{18}O values vary by instrument and therefore caution should be exercised in assuming that the results at high ^{18}O enrichments will be comparable to results published using other OA-ICOS instruments.

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References

1. Lifson N, McClintock R. Measurement of total carbon dioxide production by means of D_2O^{18}. J Appl Physiol 1955;7(6):704–10.
2. Schoeller DA, Ravussin E, Schutz Y, Acheson KJ, Baertschi P, Jéquier E. Energy expenditure by doubly labeled water: validation in humans and proposed calculation. Am J Physiol Regul Integr Comp Physiol 1986;250(5):R823–30.
3. Wong WW, Lee LS, Klein PD. Deuterium and oxygen-18 measurements on microliter samples of urine, plasma, saliva, and human milk. Am J Clin Nutr 1987;45(5):905–13.
4. Speakman JR. Doubly labelled water: theory and practice. New York: Springer; 1997.

5. Westerterp KR. Doubly labelled water assessment of energy expenditure: principle, practice, and promise. Eur J Appl Physiol 2017;117(7):1277–85.
6. Wong WW, Clarke LL. A hydrogen gas-water equilibration method produces accurate and precise stable hydrogen isotope ratio measurements in nutrition studies. J Nutr 2012;142(11):2057–62.
7. Wong WW, Clarke LL. Accuracy of $\delta^{18}O$ isotope ratio measurements on the same sample by continuous-flow isotope-ratio mass spectrometry. Rapid Commun Mass Spectrom 2015;29(23):2252–6.
8. Melanson EL, Swibas T, Kohrt WM, Catenacci VA, Creasy SA, Plasqui G, Wouters L, Speakman JR, Berman ESF. Validation of the doubly labeled water method using off-axis integrated cavity output spectroscopy and isotope ratio mass spectrometry. Am J Physiol Endocrinol Metab 2018;314(2):E124–30.
9. Berman ESF, Fortson SL, Snaith SP, Gupta M, Baer DS, Chery I, Blanc S, Melanson EL, Thomson PJ, Speakman JR. Direct analysis of $\delta^{2}H$ and $\delta^{18}O$ in natural and enriched human urine using laser-based, off-axis integrated cavity output spectroscopy. Anal Chem 2012;84(22):9768–73.
10. Thorsen T, Shriver T, Racine N, Richman BA, Schoeller DA. Doubly labeled water analysis using cavity ring-down spectroscopy. Rapid Commun Mass Spectrom 2011;25(1):3–8.
11. Berman ESF, Swibas T, Kohrt WM, Catenacci VA, Creasy SA, Melanson EL, Speakman JR. Maximizing precision and accuracy of the doubly labeled water method via optimal sampling protocol, calculation choices, and incorporation of ^{17}O measurements. Eur J Clin Nutr 2020;74(3):454–64.
12. Ebbeling CB, Feldman HA, Klein GL, Wong JMW, Bielak L, Steltz SK, Luoto PK, Wolfe RR, Wong WW, Ludwig DS. Effects of a low carbohydrate diet on energy expenditure during weight loss maintenance: randomized trial. BMJ 2018;363:k4583.
13. Wong WW, Roberts SB, Racette SB, Das SK, Redman LM, Rochon J, Bhapkar MV, Clarke LL, Kraus WE. The doubly labeled water method produces highly reproducible longitudinal results in nutrition studies. J Nutr 2014;144(5):777–83.
14. Reynard LM, Burt N, Koon HE, Tuross N. Limits and possibilities in the geolocation of humans using multiple isotope ratios (H, O, N, C) of hair from east coast cities of the USA. Isot Environ Health Stud 2016;52(4–5):498–512.
15. Elemental Microanalysis. Certificate of analysis (stable isotope) [Internet]. Okehampton, United Kingdom: Elemental Microanalysis; 2007. Available from: https://www.elementalmicroanalysis.com/Downloads/B2191-WA103-IA-R055.pdf (Accessed May 2021).
16. Wong WW, Klein PD, Parr RM, Clements SA. Interlaboratory analysis of reference water samples enriched with deuterium and oxygen-18. Appl Radiat Isot 1993;44(3):561–6.
17. Sharp ZD. Principles of stable isotope geochemistry [Internet]. 2nd ed. 2017. Available from: https://digitalrepository.unm.edu/unm_oer/1/ (Accessed April 2021).
18. International Atomic Energy Agency (IAEA). Reference sheet for international measurement standards VSMOW2 Vienna Standard Mean Ocean Water 2, water ($\delta^{2}H_{VSMOW\text{-}SLAP}$, $\delta^{18}O_{VSMOW\text{-}SLAP}$), SLAP2 Standard Light Antarctic Precipitation 2, water $\delta^{2}H_{VSMOW\text{-}SLAP}$, $\delta^{18}O_{VSMOW\text{-}SLAP}$) [Internet]. Vienna, Austria: IAEA; 2017. Document RS_VSMOW2_SLAP2_rev1/2017-07-11. Available from: https://nucleus.iaea.org/sites/ReferenceMaterials/Shared%20Documents/ReferenceMaterials/StableIsotopes/VSMOW2/VSMOW2_SLAP2.pdf (Accessed April 2021).
19. International Atomic Energy Agency (IAEA). Assessment of body composition and total energy expenditure in humans using stable isotope techniques [Internet]. IAEA Human Health Series No. 3. Vienna, Austria: IAEA; 2009. Available from: https://www.iaea.org/publications/7982/assessment-of-body-composition-and-total-energy-expenditure-in-humans-using-stable-isotope-techniques.
20. Speakman JR, Yamada Y, Sagayama H, Berman ESF, Ainslie PN, Andersen LF, Anderson LJ, Arab L, Baddou I, Bedu-Addo K, et al. A standard calculation methodology for human doubly labeled water studies. Cell Rep Med 2021;2(2):100203.
21. R Core Team. R: a language and environment for statistical computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2020. Available from: https://www.R-project.org/.
22. Legendre P. lmodel2: model II regression. R package version 1.7-3 [Internet]. 2018. Available from: https://CRAN.R-project.org/package=lmodel2.
23. Smith RJ. Use and misuse of the reduced major axis for line-fitting. Am J Phys Anthropol 2009;140(3):476–86.
24. Giavarina D. Understanding Bland Altman analysis. Biochem Med (Zagreb) 2015;25(2):141–51.
25. Schoeller DA, Webb P. Five-day comparison of the doubly labeled water method with respiratory gas exchange. Am J Clin Nutr 1984;40(1):153–8.
26. Westerterp KR, Brouns F, Saris WHM, Ten Hoor F. Comparison of doubly-labelled water with respirometry at low activity and high activity levels. J Appl Physiol 1988;65(1):53–6.
27. Ravussin E, Harper IT, Rising R, Bogardus C. Energy expenditure by doubly labeled water: validation in lean and obese subjects. Am J Physiol 1991;261(3 Pt 1):E402–9.
28. Seale JL, Conway JM, Canary JJ. Seven-day validation of doubly labeled water method using indirect room calorimetry. J Appl Physiol 1993;74(1):402–9.
29. Wassenaar LI, Terzer-Wassmuth S, Aggarwal PK, Coplen TB. Seeking excellence: an evaluation of 235 international laboratories conducting water isotope analyses by isotope-ratio and laser-absorption spectrometry. Rapid Commun Mass Spectrom 2018;32(5):393–406.
30. van Geldern R, Barth JAC. Optimization of instrument setup and post-run corrections for oxygen and hydrogen stable isotope measurements of water by isotope ratio infrared spectroscopy (IRIS). Limnol Oceanogr Meth 2012;10(12):1024–36.
31. Berman ESF, Melanson EL, Swibas T, Snaith SP, Speakman JR. Inter- and intraindividual correlations of background abundances of ^{2}H, ^{18}O and ^{17}O in human urine and implications for DLW measurements. Eur J Clin Nutr 2015;69:1091–8.
32. Kakiuchi M, Matsuo S. Fractionation of hydrogen and oxygen isotopes between hydrated and free water molecules in aqueous urea solution. J Phys Chem 1985;89(21):4627–32.
33. Urbańczyk E, Sowa M, Simka W. Urea removal from aqueous solutions—a review. J Appl Electrochem 2016;46(10):1011–29.