

Evaluation of an Automated Equilibration Technique for Deuterium/Hydrogen Isotope Ratio Measurements with Respect to Assessing Total Energy Expenditure by the Doubly Labelled Water Method

F. Thielecke* and R. Noack

German Institute of Human Nutrition, Arthur-Scheunert-Allee 114/116, 14558 Potsdam-Rehbrücke, Germany

The doubly labelled water (DLW) method for measuring energy expenditure in free living subjects suffers from inaccuracy and imprecision mainly owing to analytical problems. An automated H/D/O equilibration device was tested for precision, internal validity and matrix effects with particular consideration of DLW studies. The precision was found to be 0.5‰ and 0.04‰ for ^2H and ^{18}O , respectively. Dilution series with water standards and urine showed a high linearity and no matrix effects. In addition, the automated preparation is less laborious and time consuming and reduces sources of analytical imprecisions in studies measuring energy expenditure by the DLW method. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

The doubly labelled water (DLW) method has been established as the first accurate technique for measuring energy expenditure in free living subjects.¹ It relies on the accurate measurement of $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$ isotope ratios in plasma, saliva or urine samples in order to calculate the flux of tracer and tracee across the physiological compartment of measurement. The predominant sample preparation for deuterium-enriched aqueous samples is a reduction method employing zinc reduction or continuous flow uranium furnaces. The latter approach is subject to memory effects, requiring frequent replicate sample injections. All reduction methods have in common that the water has to be completely converted into hydrogen to avoid isotope effects. These methods are laborious, time consuming and only accurate in the hands of an experienced technician. However, the quality of the isotope analysis largely determines the precision of the DLW method.

A different technique is to equilibrate water samples with added hydrogen. This is similar in principle to the equilibration of water with CO_2 for the determination of the $^{18}\text{O}/^{16}\text{O}$ isotope ratio. This approach employs platinum to catalyse the equilibration of added hydrogen with the water sample.^{2,3} The platinum catalyst increases the rate of deuterium exchange between H_2O vapour and H_2 gas. It is essential to control precisely

the temperature during equilibration since the reaction is strongly temperature dependent. The fractionation factor $\alpha_{\text{HD}/\text{H}_2\text{O}} = 0.2625$ at 25°C .^{4,5} Based on that technique, this paper describes the performance with respect to the analytical requirements of a commercially available, automated device for measuring $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$ isotope ratios in aqueous samples and demonstrates its benefits for DLW studies. The advantage over other methods for the determination of $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$ isotope ratios is that this automatic device can be used for both isotopic species, meaning that the $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$ isotope ratios can be obtained from the same sample. The equilibration device tested can be purchased from Finnigan MAT (Bremen, Germany).

EXPERIMENTAL

The equilibration system consists of a sample rack holding 24 glass bottles of 20 ml volume. The sample rack is mounted on a sled in such a way that the samples are immersed and shaken in a temperature-controlled water-bath. The temperature is held constant at 19°C ($\pm 0.05^\circ\text{C}$) by the means of a water chiller loop and precise counter heating. A temperature below room temperature was chosen in order to minimize condensation. The glass bottles are filled with sample fluid (5 ml) and the hydrophobic catalyst is added before transferring them to the sample rack. The platinum catalyst is attached to Pyrex rods with silicone glue, which facilitates handling of the catalyst. The rods are easy to place

* Correspondence to: F. Thielecke.

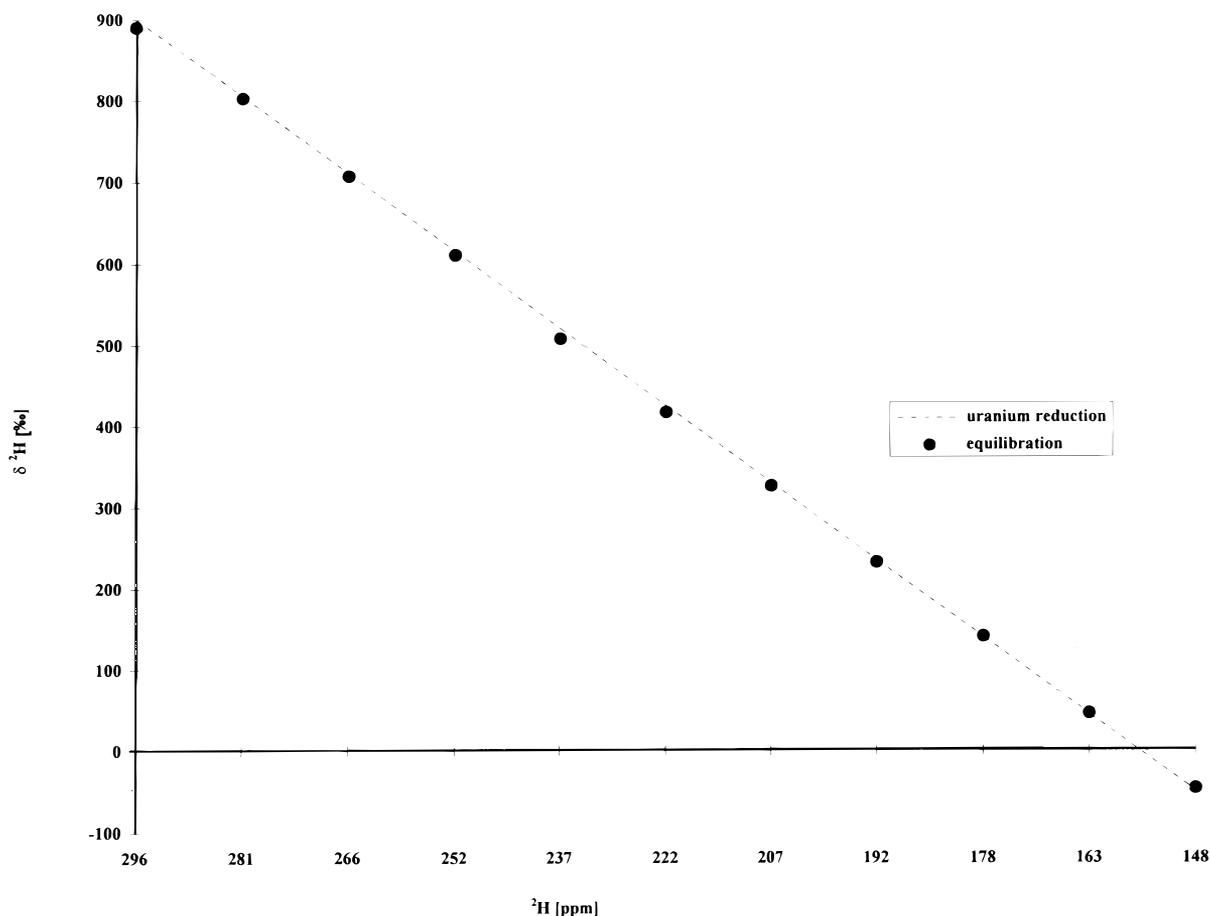


Figure 1. Deuterium oxide dilution series. Differences in measured concentrations between the uranium reduction technique (---) and the equilibration technique (●).

in the sample and easy to remove after the measurement. These catalysts are reusable; so far, we have used them 60–70 times without deterioration of performance. We only ensure that after the measurement the catalysts are rinsed thoroughly with distilled water and dried in an oven overnight at 60 °C. The catalysts are available from Finnigan MAT. The sample bottles are connected to their port valves (Nupro BK series) via a short capillary. After pump-out by a rotary oil pump (Edwards, 5 m³ h⁻¹), the sample bottles are filled with equilibration gas. The pressure is ~ 0.4 bar, resulting in an appropriate pressure in the inlet system of the isotope ratio mass spectrometer after equilibration and transfer of the sample gas. The equilibration time is 90 min for H₂ and 480 min for CO₂. After equilibration, the sample gas is sequentially transferred to the isotope ratio mass spectrometer (Delta S, Finnigan MAT). There is a cold trap (-70 °C, dry-ice in ethanol) surrounding the transfer line to prevent H₂O vapour from entering the spectrometer. Double collector measurements are performed so that after the hydrogen measurements are finished the equilibration system is evacuated and CO₂ is introduced automatically into the sample bottles and the standard port of the isotope ratio mass spectrometer and in this way the ²H/¹H and ¹⁸O/¹⁶O ratios are obtained from the same sample. This results in an increase in analytical capacity. Twenty-four samples can be analysed for ²H/¹H and ¹⁸O/¹⁶O within 24 h. In contrast, using the zinc reduction method in our labor-

atory we are able to analyse only 8–10 samples for ²H/¹H in 24 h.

Isotope ratio mass spectrometric measurements were carried out on a Finnigan MAT Delta S instrument equipped with an H/D collector. It is desirable to have a low and constant H₃⁺ factor; the constancy is the most important aspect for precise measurements. The H₃⁺ factor was routinely measured ten times every morning over a period of 1 month and the average value was found to be 4.16(±0.07) mA⁻¹. The contribution of H₃⁺ to the mass 3 ion current was then quantitatively corrected. For the purpose of this work the ion source was tuned in such a way that resulted in an H₃⁺ factor of 4.32(±0.02) mA⁻¹. Measurements were carried out at a mass 2 signal of 1.54 A⁻¹, corresponding to an analyser pressure of 1 × 10⁻⁷ mbar. Interfering masses 14 and 18 were monitored with every measurement in order to check for air leaks and H₂O vapour.

RESULTS AND DISCUSSION

External precision

In order to test for external precision, ten water samples were analysed for ²H/¹H and ¹⁸O/¹⁶O isotope ratios. The precision expressed as relative standard deviation was found to be 0.5‰ and 0.04‰ for ²H and ¹⁸O,

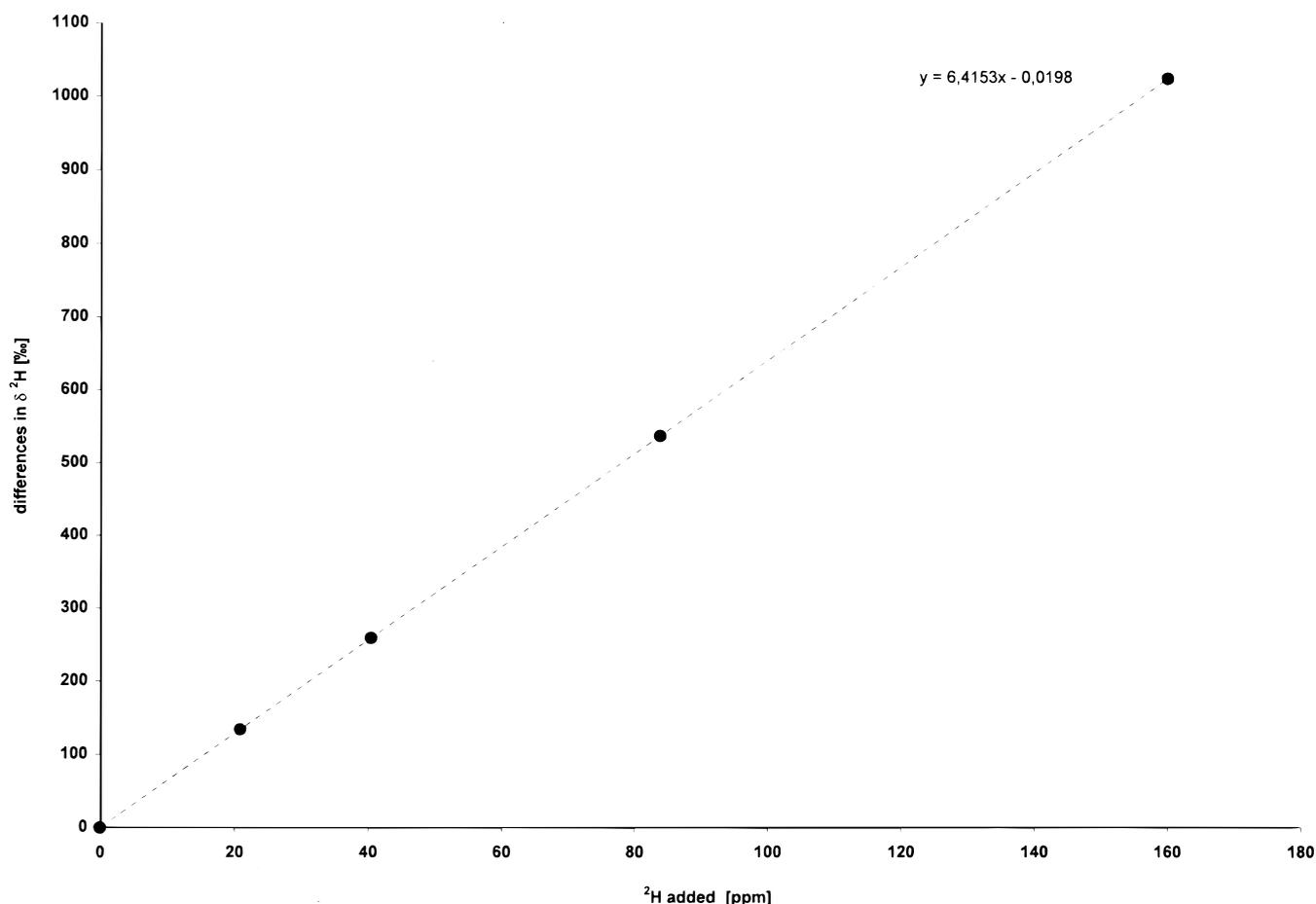


Figure 2. Matrix effects. Plot of differences in measured concentrations between urine and urine with added D₂O vs. proportion of D₂O added.

respectively. The results are given in delta notation as proposed by Craig:⁶

$$\delta_x(\text{‰}) = \left(\frac{R_{\text{sa}}}{R_{\text{st}}} - 1 \right) \times 1000$$

whereas R_{sa} is the isotope ratio of the sample and R_{st} is that of the standard and subscript x represents ²H or ¹⁸O.

Linearity of deuterium oxide dilutions

The between-laboratory variation for the DLW method was found to be much larger than expected.⁷ In the absence of international standards, laboratories must prepare their own standards to test for internal validity. As a working standard we used Potsdam tap water which had previously been measured against standard V-SMOW, SLAP and GISP waters, distributed by the IAEA (Vienna, Austria). In addition, regular inter-laboratory comparisons should be made, in order to exclude possible error sources for between-laboratory variations. For this purpose a series of deuterium oxide dilutions were kindly provided and pre-measured by W. A. Coward (Cambridge, UK), employing a continuous flow uranium furnace. The dilution series covered a

range from as high as +900‰ to −50‰ relative to V-SMOW in order to simulate a biological elimination curve in studies measuring energy expenditure by the DLW method. The results of triplicate measurements are shown in Fig. 1. They are normalized to V-SMOW as discussed elsewhere.⁸ The standard deviation of triplicate measurements for the points in Fig. 1 ranged from 0.07 to 1.57‰ and showed no trend with increasing enrichment. In addition, the samples had previously been measured by the uranium reduction technique, which served as a reference method. The same samples measured by the equilibration method showed an average difference in the ²H/¹H isotope ratios of 1.18‰ when compared with the reference method. This is just within the error range of both techniques. The results demonstrate both good internal validity for ²H from water standards and good comparability with the uranium reduction method.

Matrix effects

Another source of analytical variation in DLW studies is matrix effects, i.e. effects due to some non-aqueous material in the sample that interferes with the isotope analysis. Scrimgeour *et al.*⁹ found considerable variability in measured body water pools when employing the

Table 1 Residuals for the best-fit line for the flux rate of ^2H (k_{H}) for 14 days from catalytic equilibration

Time (decimal days)	$\text{Ln}_{\text{H}}^{\text{a}}$	Fit ^b	Residuals ^c
0.224	-7.7798	-7.7928	0.0130
0.963	-7.4829	-7.8425	-0.0004
5.036	-8.1290	-8.1163	-0.0127
8.911	-8.3866	-8.3768	-0.0098
13.088	-8.6683	-8.6576	-0.0107
14.005	-8.6987	-8.7193	0.0206

^a Ln_{H} refers to log normalized enrichment of k_{H} .

^b Fit reflects the linear regression of the log normalized enrichment.

^c Residuals are the differences between observed data and calculated values.

zinc reduction method. This problem is largely caused by a variable error in measuring the enriched samples, which showed a higher scatter than the basal samples. It was concluded that this is due to occluded water in the zinc, which has been reported to interfere with the analysis.¹⁰ Ritz *et al.*¹¹ demonstrated that urine solids may alter the apparent isotope abundance of urinary water when urine is directly reduced on zinc. In order to test for matrix effects, a dilution series was prepared using sample fluid, i.e. urine in this case. The urine was analysed without pre-treatment such as distillation or filtration. Similarly to the dilution series with water standards, the urine samples covered a range from +1000‰ to -50‰ relative to V-SMOW. It was prepared according to an arithmetic dilution series. The results of triplicate measurements are shown in Fig. 2. The slope of the best-fit line had a relative standard deviation of 0.08%. The relative standard deviation for the points in Fig. 2 ranged from 0.25 to 1.40‰ independently of the magnitude of enrichment. This relative standard deviation is approximately four times better than that of the zinc reduction technique when applied to urinary water, indicating that the hydrogen equilibration technique gives accurate results and improved precision compared with the zinc reduction technique.¹²

Table 2. Residuals for the best-fit line for the flux rate of ^{18}O (k_{O}) for 14 days from catalytic equilibration

Time (decimal days)	$\text{Ln}_{\text{O}}^{\text{a}}$	Fit ^b	Residuals ^c
0.224	-7.7293	-7.7650	0.0157
0.963	-7.8418	-7.8318	-0.0096
5.036	-8.2076	-8.1999	-0.0077
8.911	-8.5544	-8.5502	-0.0042
13.088	-8.9287	-8.9277	-0.0010
14.005	-9.0038	-9.0106	0.0068

^a Ln_{O} refers to log normalized enrichment of k_{O} .

^b Fit reflects the linear regression of the log normalized enrichment.

^c Residuals are the differences between observed data and calculated values.

Total energy expenditure measurement

In order to demonstrate the benefits of H/D/O equilibration for DLW studies, the total energy expenditure was measured in a healthy volunteer. The subject was a non-smoking, weight stable healthy man, age 43 years, weight 75.7 kg. Written informed consent was obtained prior to the start of the investigation. A baseline urine sample was collected prior to oral dosing. The dose, sufficient to raise ^2H enrichment up to $\sim 750\text{‰}$ and for ^{18}O up to $\sim 130\text{‰}$ relative to V-SMOW, was weighed before and after administration. The subject ingested 0.12 g of $^2\text{H}_2\text{O}$ and 2.5 g of H_2^{18}O per kg of total body water from the same mixture of two stock solutions (99.8 at% ^2H and 10.2 at% ^{18}O). The volunteer fasted for 6 h after dosing. This period was assumed to be sufficient for complete equilibration, and the first post-dose urine sample was collected. The volunteer was instructed to maintain his normal lifestyle. Urine samples were collected daily for a period of 14 days. All urine samples and samples of the dose given to the subject were stored at -20°C until analysis. All analyses were carried out in duplicate.

The carbon dioxide production (r'_{CO_2}) was calculated based on the slope-intercept method according to Coward,¹³ which also provides a measurement of precision.^{14,15} If fractionation is ignored, r'_{CO_2} is calculated as

$$r'_{\text{CO}_2} = \frac{1}{2} (k_{\text{O}}N_{\text{O}} - k_{\text{D}}N_{\text{D}})$$

where units are moles per day, k is the flow rate, N is the pool and subscripts O and D refer to ^{18}O and ^2H , respectively. General calorimetric principles were then employed to calculate energy expenditure.¹⁶

The measured $^2\text{H}/^1\text{H}$ isotope ratios were logged and a linear regression of the logged normalized enrichments *vs.* time was calculated. In order to obtain a measure for the quality of the determination of the flux rates of ^2H (k_{H}), the residuals between the observed data and the calculated values from the linear regression of the logged normalized enrichments *vs.* time were computed and are presented in Table 1. The residuals are in good agreement with the calculated data from the regression analysis. This is confirmed by examining the residuals for the ^{18}O data as shown in Table 2.

The overall good performance is also reflected by the relative standard deviation for the measurement of CO_2 production, which is 2.54% for the present example. With knowledge of the natural abundance variation, an estimation of the imprecision in r'_{CO_2} arising from the natural abundance can be calculated.¹⁷ Considering that the variability in the measurement of r'_{CO_2} arises from both physiological and analytical variations, it is therefore possible to obtain an additional measure for the error arising from analytical imprecisions.

CONCLUSIONS

We have demonstrated good precision and accuracy in the determination of $^2\text{H}/^1\text{H}$ isotope ratios in waters and sample matrices, e.g. urine covering a wide range of

enrichments. When tested for internal validity, the H/D/O equilibration device showed good linearity and no matrix effects. The small relative standard deviation in an experiment where total energy expenditure was measured further suggests good analytical performance.

We conclude that the automated H/D/O equilibration gives accurate results and improved precision, which fulfil the analytical requirements for measuring total energy expenditure by the DLW method, i.e. a relative standard deviation not higher than 3%. Moreover, it could be hypothesized that the improved precision could possibly lead to a lower enrichment of the

deuterated water dose given to a subject. However, it needs to be considered that precision is only one aspect among others, e.g. background variation and the period of sample collection. The determination of ^{18}O after ^2H by the equilibration method results in a considerable decrease in laborious and time-consuming preparation.

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REFERENCES

1. D. A. Scholler and E. van Santen, *J. Appl. Physiol.* **53**, 955 (1982).
2. J. Horita, *Chem. Geol.* **72**, 89 (1988).
3. J. Horita, A. Ueada, K. Mizukami and I. Takatori, *Int. J. Radiat. Appl. Instrum.* **40**, 301 (1989).
4. J. H. Rolston, J. den Hartog and J. P. Butler, *J. Phys. Chem.* **80**, 1064 (1976).
5. T. B. Coplen, J. D. Wildman and J. Chen, *Anal. Chem.* **63**, 910 (1991).
6. H. Craig, *Geochim. Cosmochim. Acta* **12**, 133 (1957).
7. S. B. Roberts, W. Dietz, T. Sharp and G. E. Hill, *Obes. Res.* **3** (Suppl. 1), 3 (1995).
8. W. W. Wong, L. S. Lee and P. D. Klein, *Am. J. Clin. Nutr.* **45**, 905 (1987).
9. C. M. Scrimgeour, M. M. Rollo, S. M. K. T. Mudambo, L. L. Handley and S. J. Prosser, *Biol. Mass. Spectrom.* **22**, 383 (1993).
10. C. Kendall and T. B. Coplen, *Anal. Chem.* **57**, 1437 (1985).
11. P. Ritz, P. G. Johnson and W. A. Coward, *Br. J. Nutr.* **72**, 3 (1994).
12. T. B. Coplen and I. T. Harper, *Biol. Mass. Spectrom.* **23**, 437 (1994).
13. W. A. Coward, in *The Doubly-Labelled Water Method for Measuring Energy Expenditure*, edited by A. M. Prentice, p. 48, IAEA, Vienna (1990).
14. T. J. Cole and W. A. Coward, *Am. J. Physiol.* **263**, E965 (1992).
15. W. A. Coward and T. J. Cole, *New Techniques in Nutritional Research*, p. 139, Academic Press, San Diego, CA (1991).
16. M. Elia, *Am. J. Physiol.* **260**, E75 (1991).
17. P. Ritz, T. J. Cole, C. Couet and W. A. Coward, *Am. J. Physiol.* **270**, E164 (1996).