

# A Simplified Method for Deuterium/Hydrogen Isotope Ratio Measurements on Water Samples of Biological Origin

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Equilibration of hydrogen gas with the water in a variety of biological sample materials was carried out in Vacutainers using platinum-on-alumina catalyst physically isolated from the liquid water. The equilibration takes 3 days at room temperature—much slower than with catalysts which float on the water surface—but this reduces the short-term temperature sensitivity of the procedure, and the inexpensive materials used allow convenient disposal of biologically contaminated samples after analysis. The slow equilibration also allows time for complete exchange with water contained in a complex sample matrix such as plant stems or soil without the need for prior isolation of the water sample. The method has particular application in studies of human total body water where repeated studies can be carried out over a short time period without the need to wait for the previous dose to wash out. Water turnover rates from total diurnal energy expenditure studies measured by this method were not significantly different from those obtained by the zinc reduction method.

## INTRODUCTION

A number of techniques used in physiological and biological research rely on the accurate measurement of hydrogen/deuterium (H/D) ratios in water. For example, deuterium oxide tracer dilution is used for measurement of total body water (TBW) and the water turnover rate ( $k_t$ ) required in total diurnal energy expenditure (TDEE) studies, the D/H ratio in the water of plasma, urine or saliva samples being measured before and at various times after the administration of low doses (0.1–0.3 g/kg body weight) of deuterium oxide. In plant physiology, studies of plant/soil water relationships rely on the measurement of natural differences in the isotopic composition of different water sources present in the soil from which the plant is drawing water and the appearance of these isotopic signatures in the bulk plant water. Adequate precision and accuracy for such measurements is only available by isotope ratio mass spectrometry (IRMS), almost always applied to hydrogen gas derived from the water in the sample. The sample preparation and analytical methods used are largely based on those developed for geochemical studies of natural abundance variations of the D/H ratio in samples such as precipitation and ground water. The natural abundance of deuterium, around 150 p.p.m., is very low compared with 0.2–4% for the rare stable isotopes of most other low atomic mass elements ( $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{18}\text{O}$ ,  $^{34}\text{S}$ ) commonly measured by IRMS.

This significantly restricts the dynamic range of measurement, and it makes stringent demands on the instrument performance to measure accurately the intensity of  $\text{H}_2$  and HD ion beams differing by a factor of 10 000.

A number of methods have been developed for converting water samples to  $\text{H}_2$  gas for IRMS analysis. Continuous flow uranium or zinc reduction furnaces have been employed, often as part of an integrated preparation and measurement system, but this approach is subject to memory effects often requiring up to ten replicate sample injections for reliable results. Batch processing of individual samples with zinc shot in evacuated glass or quartz tubes avoids the memory problem, but water entrained in the zinc may contaminate the sample, and different batches of zinc behave differently both in this respect and in their ability to reduce the water effectively.

In all the reduction methods it is important that the water is completely reduced so that the isotopic composition of the hydrogen gas is the same as the original water. Physiological fluids such as urine and plasma contain significant amounts of dissolved material which contaminates the metal surface, preventing complete reaction, and methods have been developed where the water and zinc are kept physically separate<sup>1</sup> or the water is cryogenically purified prior to reduction.<sup>2</sup> Plant and soil water must first be extracted, usually by azeotropic distillation with an immiscible organic solvent, prior to reduction.<sup>3</sup>

A quite different approach, similar in principle to the  $\text{CO}_2$  equilibration method for  $^{18}\text{O}$  in water, has employed platinum to catalyse the equilibration of added hydrogen gas with the water sample. The catalyst

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used consists of platinum supported on a porous hydrophobic polymer (Hoko beads) which either floats on the water surface<sup>4</sup> or is supported at the water/gas interface,<sup>5</sup> and this achieves equilibrium within an hour. When equilibrium is achieved at room temperature, the D/H ratio of the water is about 3.6 times that of the hydrogen gas, and this fractionation may exacerbate instrumental problems with measuring D/H ratios about a third of those obtained by direct reduction.<sup>5</sup> The fractionation factor is also temperature sensitive, requiring the equilibration system to be thermostated. The method has been applied to strong brine solutions<sup>6</sup> and samples from which H<sub>2</sub>S contamination has been removed with copper prior to analysis,<sup>5</sup> but its use with physiological fluids has not been reported. 'Hoko beads' are expensive and not readily available, and the existing methods are predicated upon the recovery and reuse of the catalyst. Clinical samples, which may be contaminated with pathogens, are best analysed in fully disposable systems, making recovery of the catalyst impracticable.

This paper describes a modification of the equilibration method in which the catalyst used is a readily available platinum-on-alumina powder which is kept physically separate from the water sample, by being placed in a small glass tube. While this greatly reduces the equilibration rate, it brings the advantages of disposability and reduced sensitivity to short-term temperature fluctuations.

## METHODS

Additive-free 20 ml Vacutainer tubes (Becton Dickinson, Rutherford, New Jersey, USA, ref. 6433) were used for the equilibration. After removing the rubber septa, in the bottom of each was placed a 32 mm × 5 mm o.d. glass vial (MICRO-VI, Chromacol Ltd, London, UK) containing about 4 mg of catalyst (5% platinum-on-alumina, 325 mesh, Aldrich Chemical Company Ltd, Dorset, UK). Sample fluid (400 µl) was then placed in the bottom of the Vacutainer, taking care to avoid wetting the catalyst, and the septum replaced. Air was removed from the Vacutainer through a 23 gauge × 25 mm hypodermic needle attached to a three-way tap, which allowed the Vacutainer tube to be evacuated by a rotary oil pump and filled with hydrogen, without removing the needle. Each tube was evacuated for 120 s then filled to 4 p.s.i. (30 kPa) above atmospheric pressure with hydrogen from a cylinder, allowing 10 s for filling before being withdrawn from the needle. The tubes were kept vertical in a rack throughout and then left to equilibrate at  $20 \pm 1^\circ\text{C}$  for a minimum of 4 days.

IRMS measurements were carried out on a Finnigan MAT Delta D IRMS fitted with an HD collector. Standard H<sub>3</sub><sup>+</sup> corrections were carried out by software during the measurement. The reference hydrogen gas (GPS grade, BOC Ltd) contained about 150 p.p.m. HD and was also the gas used for the equilibration. Measurements were carried out at a mass 2 signal of 7 V, corresponding to an analyser pressure of  $1 \times 10^{-6}$  mbar, where small sample size variations had least effect

on the measured  $\delta\text{D}$ . Hydrogen was sampled through a 19 gauge needle connected via 15 cm of 27 gauge capillary to a U-trap cooled in liquid nitrogen to remove water vapour. The needle tip was sealed by partial insertion into the Vacutainer septum, the trap and IRMS inlet evacuated, then the needle was pushed through the septum and hydrogen allowed to enter the inlet for about 10 s. The sample pressure was then adjusted to give a mass 2 signal of 7 V. To check for air leaks into the sample, the IRMS analyser pressure was monitored for both sample and reference gas after balancing reference and sample signals. If the sample:reference pressure ratio was greater than 1.1, the sample was rejected.

## RESULTS AND DISCUSSION

### Equilibration time

The time course of the equilibration was observed by preparing a set of tap water samples and analysing these at various times over a 6-day period (Table 1). After 3½ days there were no significant changes in either the measured  $\delta\text{D}$  or in the precision of the measurement. Samples of a dilution series equilibrated for 4 weeks also gave satisfactory results (see below).

Some sample sets, however, gave very poor results, due to only a small amount of hydrogen remaining in the tube at the time of analysis. This was presumed to be due to oxygen remaining in or subsequently entering the tube after degassing, since, instead of pure hydrogen, the gas mixture remaining at the time of analysis contained nitrogen and argon in addition to residual hydrogen. This suggests that the catalyst also catalyses the combination of hydrogen and oxygen to form water, resulting in a reduction in the pressure in the tube sufficient to allow more air to leak in and continue the reaction until all the hydrogen is used up. This problem was minimized by ensuring an adequate degassing time through a sufficiently wide needle. Despite these precautions occasional batches (about one in 20) of samples have failed in this way, and this can only be attributed to different batches of Vacutainers which reseal poorly after removing the stopper. For critical applications, the use of standard vacuum glassware might be preferred to Vacutainers, but the overall success rate with the present method is at least as good as that with the zinc reduction method in our hands.

**Table 1. Effect of equilibration time on observed  $\delta\text{D}$  for samples of tap water prepared simultaneously**

Equilibration time (days)	$\delta\text{D}$ (‰)	SD	n
(0)	(0)		
1.5	-597	10.6	4
2.5	-674	4.2	4
3.5	-682.5	0.5	4
6.5	-682.5	0.7	7

### Sample size

After equilibration, the isotopic composition of the hydrogen gas depends on the  $H_2:H_2O$  mole ratio as well as the isotopic composition of the equilibrating gas and the water sample. Thus the volume of water used and the  $H_2:H_2O$  ratio must be kept constant for reproducible analyses or else the measured  $\delta D$  value must be corrected for variations in sample size. Using 400  $\mu l$  with pure  $H_2$  in 20 ml tubes there is little error due to small variations in the amount of hydrogen present (arising from variations in pressure or the volume of the tubes), and replicate analyses are within 1–2‰ as shown above. For most of the applications described below a constant sample volume of 400  $\mu l$  was used and the measured  $\delta D$  used directly in subsequent calculations.

Changing the water volume while keeping the other variables constant results in significant changes in measured  $\delta D$ , for example from –660‰ for 500  $\mu l$  tap water to –635‰ for 100  $\mu l$ . The measured and true (that is, extrapolated to zero added  $H_2$ )  $\delta D$  for such an equilibration are related as follows:<sup>1</sup>

$$\delta_c = \delta_m + (\delta_m - \delta_{st}) \times \alpha/k$$

where  $\delta_c$  is the corrected  $\delta D$ ,  $\delta_m$  the measured  $\delta D$ ,  $\delta_{st}$  the  $\delta D$  of the equilibrating gas relative to the mass spectrometer working standard,  $\alpha$  is a constant at constant temperature and  $k$  the mole ratio of  $H_2O$  to  $H_2$ .

When the equilibrating gas is also used as the mass spectrometer working standard,  $\delta_{st}$  is zero, and  $k$  is proportional to the weight of water (wt) if the amount of hydrogen is constant. Thus:

$$\delta_c = \delta_m(1 + \alpha'/wt)$$

where  $\alpha'$  is a composite constant. Rearranging:

$$1/\delta_m = 1/\delta_c + (1/\delta_c) \times \alpha'/wt$$

and a plot of  $1/\delta_m$  against  $1/wt$  will have an intercept of  $1/\delta_c$  and slope  $\alpha'/\delta_c$ .

By analysing a set of water samples of different weights, and plotting  $1/\delta_m$  against  $1/wt$  (see Fig. 1) the constant  $\alpha'$ , characteristic of the equilibration volume and temperature, can be obtained. Hence  $\delta_c$  for any sample of known weight may be obtained. If the system is calibrated by using variable weights of Standard Mean Ocean Water (SMOW) and Standard Light Antarctic Precipitation (SLAP) (or working standards of known relationship thereunto) then the corrected  $\delta D$  values can be normalized to SMOW in the normal way.<sup>1</sup>

Since the normalization involves expansion of the measured  $\delta D$  differences by about 3.6 times (the equilibrium constant for the reaction) the precision of normalized values will be correspondingly reduced to about 5%. This reduced precision is about at the limit of usefulness for studies of natural variation such as hydrology and plant/soil water relationships, and for such applications the method might have to be refined, by improved thermostating and accurate control of the  $H_2:H_2O$  ratio.

The linearity of the  $1/\delta$  versus  $1/wt$  plots in Fig. 1 suggests that there is negligible fractionation error due to water loss during evacuation of the Vacutainers, at least over the range of water samples studied (0.04–0.4

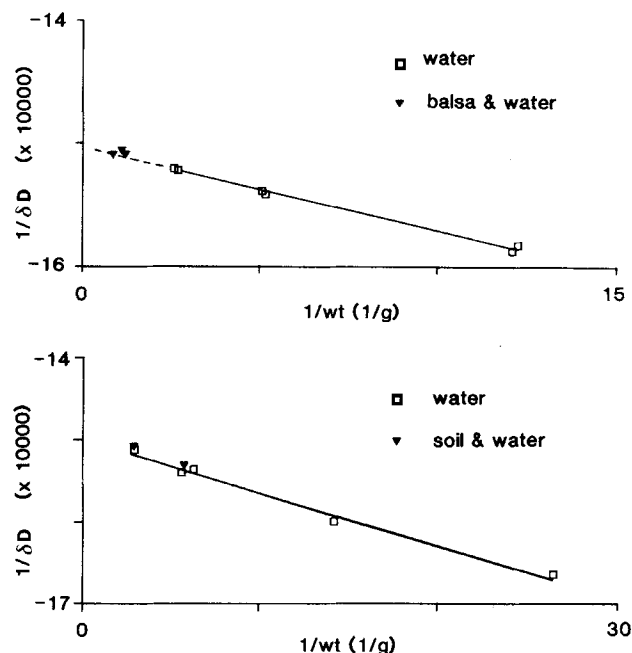


Figure 1. Plots of  $1/\delta D$  vs.  $1/wt$  for tap water and (a) balsa wood soaked in water, and (b) dried soil with water added. The heterogeneous samples show no significant deviation from the water lines.

g). Coplen *et al.*<sup>5</sup> used a similar-sized capillary for degassing sample tubes and they also found no significant fractionation error for samples down to 100  $\mu l$ . They recommended freezing samples less than 100  $\mu l$  during evacuation, and a similar precaution might be appropriate for this method when attempting natural abundance measurements on small water samples.

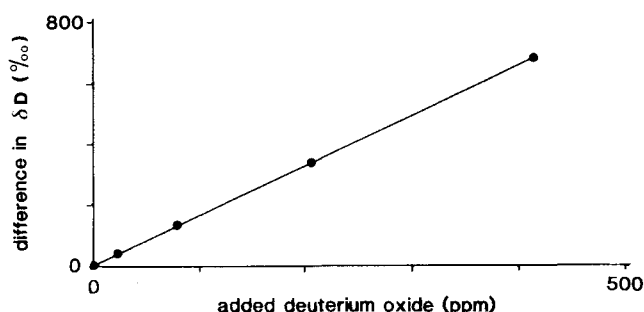
### Nature of sample

Samples of local tap water as well as plasma and urine obtained at the same time from one subject were analysed after 5 days' equilibration (Table 2). There is no difference in the precision of the values obtained for the different samples, and the urine and plasma samples give the same value within the precision of the measurement, which is as expected since there is no fractionation between urine and plasma.<sup>7</sup>

Model experiments were carried out to test the suitability of the equilibration method for the direct measurement of plant and soil water, on the basis that diffusion of water within the sample matrix would be no slower than the gas water equilibration, and that exchange with all the water in the matrix would occur within a few days. Topsoil, oven-dried at 100°C overnight, was contained in 1.5 ml plastic tubes which could fit in a Vacutainer, and 0.25 and 0.5 ml water was added

Table 2. Observed  $\delta D$  for tap water, urine and plasma

Sample	$\delta D$ (‰)	SD	n
Tap water	–674.0	1.0	3
Urine	–665.3	0.4	3
Plasma	–664.8	1.0	3



**Figure 2.** Deuterium oxide dilution series. Plot of difference in measured  $\delta D$  between tap water and tap water with added deuterium oxide versus proportion of deuterium oxide added.

to the soil. These were then dropped into the Vacutainer, followed by the tube of catalyst, and the Vacutainer was closed and filled with hydrogen as normal. Similarly, balsa wood strips  $3 \times 3 \times 35$  mm were soaked in water for 7 days, then removed; the surplus water was quickly removed and 90% of the wood surface was wrapped in PTFE tape to simulate an impervious bark. These were then prepared for equilibration in a similar manner to the soil samples. Samples (0.1–0.5 g) of the tap water and the water in which the balsa had been soaked were prepared as normal and all the samples were allowed to equilibrate for 5 days. After analysis the Vacutainer septa were removed, the tubes weighed, dried at  $100^\circ\text{C}$  overnight and reweighed to determine the amount of water in each sample.

On plots of  $1/\delta D$  versus  $1/\text{wt}$  (Fig. 1), the soil and balsa wood samples matched the best-fit line for their corresponding water, within experimental error. This method, including the normalization to SMOW discussed above, has potential as a minimal sample preparation approach to natural abundance studies of plant/soil/water relationships. Ripe woody twigs gave results within the expected range, but further validation of this approach is required, comparing results with azeotropic or vacuum-extracted water from real twig and soil samples, and extending this approach to  $^{18}\text{O}$  measured by equilibration with  $\text{CO}_2$  *in situ*.

### Linearity of deuterium oxide dilutions

A series of deuterium oxide dilutions was prepared covering a range up to 0.4 g  $\text{D}_2\text{O}$  per kilogram of tap water. The results after 5 days' equilibration of triplicate samples are shown in Fig. 2 and were not significantly different for sets analysed after 3 or 4 weeks' equilibration. The SD for the points ranged from 0.6 to 2.0 and showed no trend with increasing enrichment. The slope of the best-fit line had a coefficient of variation (CV) of 0.05%.

## APPLICATIONS

### Total body water measurement

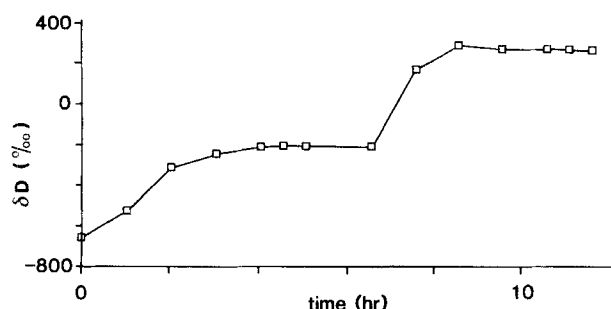
$^{18}\text{O}$  dilution remains the gold standard method for human total body water measurement, both for the ease

and precision of its measurement and because the measured dilution pool only minimally overestimates the true body water pool. However, the increasing cost and limited supply of  $^{18}\text{O}$ -labelled water makes its use in studies in large numbers of adults impractical.  $\text{D}_2\text{O}$  is cheap and in abundant supply but the analysis by zinc reduction is time-consuming and prone to variable error due to water trapped in the zinc.  $\text{D}_2\text{O}$  dilution also gives a larger discrepancy between the true and measured water pool due to the greater number of exchangeable hydrogens in protein and carbohydrate. However, this discrepancy is well established at 3% in normal adults and has been evaluated for extremes of altered body composition.<sup>8</sup>

Using zinc reduction we have found considerable variability in measured body water pools—a problem largely caused by a variable error in measuring the enriched plateau samples, which often showed a much higher scatter than the basal samples. This is due to occluded water in the zinc, which has been reported to interfere with the analysis.<sup>9</sup> Thus, with various batches of zinc, we have observed errors (SD  $n = 3$ ) of 1–4‰ for basal samples but of 4–10‰ for enriched samples resulting from a dose of 0.25 g/kg total body water (TBW). This results in an analytical error of up to 2% based on a single measurement, which might be reduced to about 1% by multiple measurements. Since the interference is due to water at natural HD abundance, any water in the zinc vitiates an increase in precision which might otherwise be expected from increasing the  $\text{D}_2\text{O}$  dose, and the amount by which the dose could be increased is also limited by the dynamic range of the IRMS.

In contrast, the equilibration method does not show an increasing error with enrichment and is highly linear over a useful range of dilution. A dose of 0.3 g/kg TBW results in a measured change in  $\delta D$  of about 600‰ with an SD of 3‰, giving an analytical precision of 0.5%. Furthermore, the compression of the  $\delta D$  scale caused by the equilibration means that a much wider range of actual water enrichments can be measured. This allows either larger  $\text{D}_2\text{O}$  doses to be given or, more usefully, repeated studies can be made over a short period in which there has not been time for the previous enrichment to drop to near basal levels (taking 10 days or more). Since the dilution versus  $\delta D$  difference is highly linear, the body water pool can be calculated directly by comparing the  $\delta D$  difference of the body water (as urine, saliva or plasma) with that of a known dilution.

As an example, TBW was measured in a volunteer subject when normally hydrated and again immediately after running 20–25 km under heat stress conditions. A dose of 0.3 g  $\text{D}_2\text{O}$  per kilogram estimated TBW was given at the start of each measurement, immediately after collection of a basal urine sample. Plateau urine samples were collected between 3 and 5 h after the isotope dose was given, and no rehydration was allowed until the last urine sample was taken. On two separate occasions the body weight fell by 4.0 kg after the exercise, and the measured fall in TBW was 4.0 and 4.1 kg, in good agreement with the change in body weight assuming this was all due to water loss during the exercise period. Figure 3 shows the time course of the measured  $\delta D$  for this experiment. The instrumental



**Figure 3.** Time course of measured  $\delta D$  from urine samples obtained before and after exercise under heat stress. A dose of 0.3 g  $D_2O$  per kilogram TBW was given at time zero and at 6.5 h immediately following 1.5 h of exercise.

measuring limit of about 2500‰ would have been approached had the true  $\delta D$  of the water been measured on  $H_2$  produced by zinc reduction. Using catalytic equilibration and a dose of 0.3 g/kg TBW (to ensure adequate precision) two further measurements could be carried out without the need to allow the earlier enrichment of the body water to decay.

#### Energy expenditure studies by doubly labelled water

The catalytic equilibration method was applied to three sets of urine samples from doubly labelled water energy expenditure studies. These sets were also analysed by our standard zinc reduction method,<sup>10</sup> essentially the same as that of Wong *et al.*<sup>1</sup> The subjects for these studies had all received a  $D_2O$  dose of 0.25 g/kg TBW. The calculated water turnover rates ( $k_h$ ) obtained by the two methods (shown in Table 3) are identical within the precision of the methods.

For the first set the residuals between the observed data and the values calculated from the best-fit line (Table 4) also show good agreement between the two methods, confirming that the rather large error in this set reflects genuine scatter in the samples rather than random analytical error, and this is confirmed by examining the residuals for the  $^{18}O$  data. An additional

**Table 3.** Comparison of  $k_h$  values obtained by catalytic equilibration and zinc reduction methods

Sample set	Catalytic equilibration			Zinc reduction		
	$k_h$	SD	CV%	$k_h$	SD	CV%
1	0.1088	0.0050	4.6	0.1096	0.0047	4.3
2	0.0947	0.0008	0.9	0.0974	0.0019	2.0
3	0.1094	0.0021	2.0	0.1122	0.0023	2.1
3	0.1088	0.0028	2.6			

advantage of this simple Vacutainer method is that the same samples can be analysed for  $^{18}O$  after HD analysis, simply by re-evacuating and filling with an air- $CO_2$  mixture as normal.<sup>10</sup> This results in a considerable reduction in the labour of sample preparation. Samples analysed in this way and for  $^{18}O$  directly showed no significant difference in  $\delta^{18}O$ .

**Table 4.** Residuals for best-fit lines for  $k_h$  from catalytic equilibration and zinc reduction methods and  $k_o$  from  $^{18}O$  analysis

Time (days)	Catalyst	Reduction	$^{18}O$
0.21	0.0203	0.0300	0.0180
1.01	0.0312	0.0293	0.0349
2.25	-0.1053	-0.0887	-0.1190
4.37	0.0128	-0.0199	0.0058
6.44	0.0173	0.0185	0.0337
8.08	0.0421	0.0297	0.0591
9.90	0.0389	0.0557	0.0454
12.08	-0.0572	-0.0547	-0.0779

#### Acknowledgements

This work was supported by Europa Scientific Ltd, the University of Dundee, the Arthritis and Rheumatism Council and the Prior Provincial of the Carmelites, Dublin.

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