



IAEA HUMAN HEALTH SERIES

No. 12

Introduction to Body
Composition Assessment
Using the Deuterium Dilution
Technique with Analysis
of Saliva Samples by
Fourier Transform Infrared
Spectrometry



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**INTRODUCTION TO
BODY COMPOSITION ASSESSMENT
USING THE DEUTERIUM DILUTION TECHNIQUE
WITH ANALYSIS OF SALIVA SAMPLES BY
FOURIER TRANSFORM INFRARED SPECTROMETRY**

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SPECTROMETRY

INTERNATIONAL ATOMIC ENERGY AGENCY
VIENNA, 2010

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FOREWORD

For many years, the IAEA has fostered the more widespread use of stable isotope techniques to assess body composition in different population groups to address priority areas in public health nutrition in Member States. The objective is to support national and regional nutrition projects through both the IAEA's technical cooperation programme and its coordinated research projects. In particular, during the last few years, the increased access to analyses of deuterium enrichment by Fourier transform infrared (FTIR) spectrometry has increased the application of this technique in Africa, Asia and Latin America.

This publication was developed by an international group of experts to provide practical, hands-on guidance in the use of this technique in settings where the analysis of deuterium enrichment in saliva samples will be made by FTIR. It is targeted at new users of this technique, for example nutritionists, analytical chemists and other professionals. More detailed information on the theoretical background and the practical application of state of the art methodologies to monitor changes in body composition can be found in an IAEA publication entitled Assessment of Body Composition and Total Energy Expenditure in Humans by Stable Isotope Techniques (IAEA Human Health Series No. 3).

The IAEA is grateful to the major contributors to this publication for sharing their technical expertise and extensive experience in stable isotope techniques in nutrition: L. Bluck (United Kingdom), C. Slater (United Kingdom) and M.E. Valencia (Mexico).

The IAEA officer responsible for this publication was L. Davidsson of the Division of Human Health.

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1. INTRODUCTION

1.1. BACKGROUND

For many years, the IAEA has fostered the more widespread use of stable isotope techniques to assess body composition in different population groups to address priority areas in public health nutrition in Member States. The objective is to support national and regional nutrition projects through both the IAEA's technical cooperation programme and its coordinated research projects. In particular, during the last few years, the increased access to analyses of deuterium enrichment by Fourier transform infrared (FTIR) spectrometry has increased the application of this technique in Africa, Asia and Latin America.

1.2. OBJECTIVE

This publication was developed by an international group of experts to provide practical, hands-on guidance in the use of this technique. It is intended for use in settings where the analysis of deuterium enrichment in saliva samples will be made by FTIR spectrometry.

1.3. SCOPE

This publication is targeted at new users of this technique, for example nutritionists, analytical chemists and other professionals. More detailed information on the theoretical background and the practical application of state of the art methodologies to monitor changes in body composition can be found in an IAEA publication entitled Assessment of Body Composition and Total Energy Expenditure in Humans by Stable Isotope Techniques (IAEA Human Health Series No. 3).

1.4. STRUCTURE

After this introduction, Section 2 defines the key concepts and terms. Section 3 outlines the equilibrium technique of estimating total body water by deuterium dilution. Section 4 gives a detailed description of the steps and procedures involved in the deuterium dilution method of estimating total body water. Section 5 describes the analysis of deuterium enrichment by FTIR

spectrometry. Section 6 describes how body composition is calculated. Section 7 examines quality control issues, while Section 8 summarizes the critical steps for good quality data. Section 9 presents some frequently asked questions. Appendix I presents general information on the safety of deuterium oxide, while Appendix II describes the principles of FTIR spectrometry. Appendix III provides a sample data sheet for the estimation of total body weight by deuterium oxide dilution, while Appendix IV provides an equipment list. Finally, Appendix V explains isotope fractionation.

2. BACKGROUND

2.1. BODY COMPOSITION

On its own, body weight is a relatively poor indicator of health and nutritional status. A more important indicator is body composition, namely, the components that comprise an individual's body weight. The human body is often subdivided into two component parts: fat mass (FM) and fat free mass (FFM). This is known as a 'two compartment model'.

2.2. TOTAL BODY WATER

Water is the largest component of the body. At birth, the body contains 70–75% water, but as the body matures, this proportion decreases to 50–60% in lean adults and to less than 40% in obese adults. Water is found exclusively within the FFM, which is approximately 73.2% water in adults. Total body water (TBW) includes both intracellular fluid and extracellular fluid. With an estimate of TBW, the amount of FFM can be estimated. Body FM is the difference between body weight and FFM.

In conditions where adequate food and drink are available, the body water compartment is in a constant state of flux, with water molecules entering and leaving the body. The circulatory system is responsible for providing a regular supply of nutrients to, and removal of waste from, all body cells. Each time we drink fluid, consume food containing moisture, or produce a water molecule during energy substrate oxidation, these molecules mix with the body water pool. At the same time, water is constantly leaving the body in different forms. This includes insensible water losses as water vapour from the lungs and skin, as well

as water loss in urine and faeces. In adults, the body water compartment remains relatively constant in size and rarely varies by more than a few per cent within or between days. During a typical day, the input and output of water is roughly equal, and the pool size remains relatively constant [1].

TBW can be measured in the field using the deuterium oxide dilution technique. An advantage of this technique is that it can be used to assess longitudinal changes in body composition before and after an intervention. Other field methods to estimate TBW include bioelectrical impedance analysis (BIA) and predictions from anthropometry (weight, height, sex, age). These are less accurate and require prediction equations to be derived for particular population groups by comparison with a reference method (probably TBW) in a representative sample [2, 3].

2.3. DEUTERIUM

Stable isotope techniques have been used in studies of human nutrition for over 50 years. Deuterium is a stable (non-radioactive) isotope of hydrogen, with the symbol ^2H . It is given orally as deuterium oxide ($^2\text{H}_2\text{O}$), and after mixing with body water, it is eliminated from the body in urine, saliva, sweat and human milk. Deuterium oxide is handled in the body in the same way as water, and is dispersed through the body water within a matter of hours. More information on deuterium oxide is included in Appendix I.

2.3.1. Analysis of deuterium enrichment in body water

Body water can be sampled in the form of saliva, urine, plasma or human milk, and the enrichment of deuterium can be measured by isotope ratio mass spectrometry (IRMS) [4] or FTIR spectrometry [5]. FTIR spectrometry is not as sensitive as IRMS and therefore requires a larger dose of deuterium oxide (approximately ten times more). FTIR spectrometry is not suitable for analysis of urine or human milk. However, FTIR instrumentation is easier to use and maintain than IRMS, and is less expensive to buy, and the cost of analysis is lower. It is therefore particularly suitable in resource limited settings. (For more information on FTIR spectrometry, see Appendix II.)

This manual describes the estimation of TBW by deuterium dilution using the equilibrium or plateau method with saliva sampling and analysis of deuterium enrichment by FTIR spectrometry.

2.4. THE EQUILIBRIUM VERSUS THE BACK EXTRAPOLATION METHOD OF ESTIMATING TOTAL BODY WATER

The equilibration technique is appropriate for use in adults and children, but in situations where the participants have high water turnover, such as infants and adults undergoing high physical activity, the back extrapolation technique gives more accurate results. Body composition is determined by back extrapolation as part of the doubly labelled water technique of estimating total energy expenditure [6], and in lactating mothers during the ‘dose to mother’ technique of estimating human milk intake in breastfed babies [7]. The back extrapolation technique measures water turnover over a two week period in adults and over seven days in infants (3–4 cycles of water turnover). An advantage of the equilibrium technique is that sample collection is completed in a single day.

3. THE EQUILIBRIUM TECHNIQUE OF ESTIMATING TOTAL BODY WATER BY DEUTERIUM DILUTION

3.1. SUMMARY

The following is a brief summary of the deuterium dilution technique:

- The body water pool naturally contains a small amount of deuterium (^2H). This represents the natural abundance of ^2H in body water and is usually close to 0.015 atom % (at.%) ^2H .
- After collection of a baseline sample, a known quantity of deuterium oxide (99.8 or 99.9 at% ^2H) is ingested (30 g for adults). Deuterium oxide is also known as D_2O .
- The $^2\text{H}_2\text{O}$ mixes with body water within a few hours (Fig. 1). The amount of deuterium in body water above that naturally present is known as the enrichment of body water. Enrichment reaches a ‘plateau’ after 2–5 h in body water.
- It is recommended that two post-dose saliva samples are collected 3 and 4 hours after the deuterium oxide is administered. In elderly participants and patients with a slow water turnover rate, saliva should be sampled 4 and 5 h after the dose is consumed.
- Participants should avoid drinking during the equilibration period, but if this is not possible, the volume of all fluids consumed should be recorded.

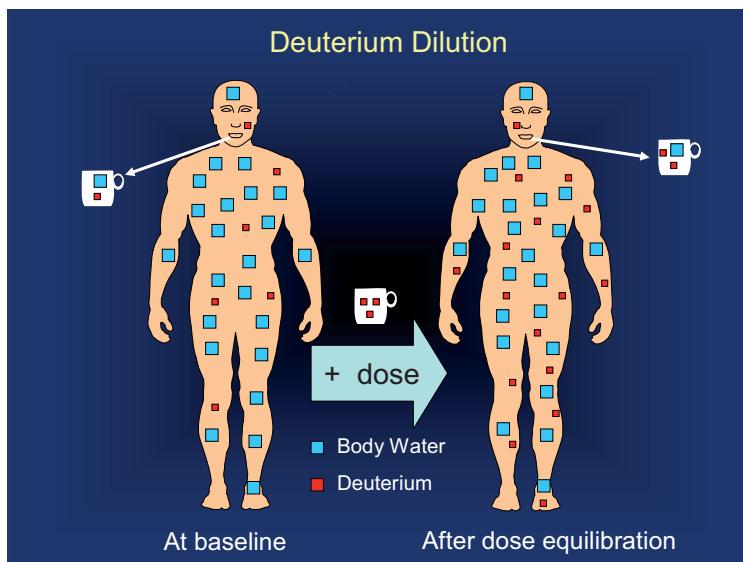


FIG. 1. Estimating TBW by deuterium dilution.

- The concentration of ^2H in saliva samples measured using FTIR spectrometry is actually the enrichment as the background is automatically subtracted during the measurement.

3.1.1. Calculation of TBW

When the enrichment of deuterium in saliva is measured by FTIR spectrometry, the results are given in mg $^2\text{H}_2\text{O}$ per kg H_2O (ppm):

$$\text{TBW (kg)} = \text{Dose } ^2\text{H}_2\text{O (mg)} / \text{enrichment } ^2\text{H in saliva (mg/kg)}.$$

3.2. ASSUMPTIONS OF THE TECHNIQUE

There are certain assumptions associated with the deuterium dilution technique for estimating TBW [1]:

- The deuterium oxide is distributed only in body water.
- The deuterium oxide is equally distributed in all body water compartments (e.g. saliva, urine, plasma, sweat, human milk).
- The rate of equilibration of deuterium oxide is rapid.
- Neither deuterium oxide nor body water is lost during the equilibration time.

Where the assumptions are not true, a correction factor must be included in the calculation of TBW or precautions taken to minimize the effect. Each of these assumptions will be discussed in the following sections.

3.2.1. Assumption 1: The deuterium oxide is distributed only in body water

This assumption is not true. Deuterium in body water enters other pools within the body, which is known as non-aqueous exchange:

- Deuterium exchanges with exchangeable hydrogen atoms in body protein. Exchangeable hydrogen atoms are those on amino ($-\text{NH}_2$), hydroxyl ($-\text{OH}$) and carboxyl ($-\text{COOH}$) groups of amino acids.
- Deuterium is also sequestered into fat and protein as these are synthesized.

Therefore, the volume of distribution (V), sometimes known as the dilution space, of deuterium is slightly greater than TBW. The ^2H space (V_D) is 1.041 times that of TBW.

This is accounted for by dividing the calculated dilution space (V_D) by 1.041 to achieve TBW (kg).

Therefore,

$$\text{TBW (kg)} = \text{Dose } ^2\text{H}_2\text{O (mg)}/\text{enrichment } ^2\text{H in saliva (mg/kg)}$$

should read:

$$V_D (\text{kg}) = \text{Dose } ^2\text{H}_2\text{O (mg)}/\text{enrichment } ^2\text{H in saliva (mg/kg)}$$

and

$$\text{TBW (kg)} = V_D (\text{kg})/1.041$$

It is important to note that the calculation of TBW from FTIR spectrometry data is different from the calculation of TBW from IRMS data (see Appendix II).

3.2.2. Assumption 2: The deuterium oxide is equally distributed in all body water compartments

This assumption is true for water in the body, but not for water leaving the body as water vapour, which is subject to isotopic fractionation. There is no fractionation in urine, faecal water or sweat. Sweat is excreted from the sweat glands as liquid water, and evaporation occurs after it leaves the body water; thus,

it is not fractionated as it leaves the body. However, water leaving the body as water vapour in breath and transdermal evaporation is subject to fractionation. Transdermal evaporation is insensible water loss from the skin through routes other than the sweat glands. The effect of increased insensible water losses, which contain less deuterium than body water, is to concentrate the deuterium oxide left behind, which leads to an underestimation of TBW and therefore an overestimation of body fat. It is important to avoid physical activity during the equilibration period to avoid increasing the rate of breathing and transdermal evaporation.

3.2.3. Assumption 3: The rate of equilibration of deuterium oxide is rapid

This is true in healthy participants, but water turnover is slower in the elderly, in pregnant women, and in patients with expanded extracellular water volume (such as malnourished children with oedema). Therefore, a longer equilibration time should be allowed for these subjects:

- Equilibration is the process whereby the D₂O is evenly mixed throughout the body water. After equilibration, all body water compartments will contain the same concentration of deuterium.
- Equilibration between the enriched dose and body water is not instantaneous. Equilibration of body water with saliva is rapid, but equilibration with urine, especially in elderly subjects with residual urine post voiding, can take a few hours. The question is, “How long is the delay before equilibration is complete?”
- In healthy participants, equilibration is usually achieved after 2–5 h (Fig. 2) and saliva samples can be collected at 3 and 4 h. In general, children have faster water turnover than adults, and elderly adults have slower water turnover than younger adults. Various disease states can also influence water turnover; therefore, it is important to carry out a small pilot study to determine the required sampling time before starting the main study. It may be necessary to collect saliva samples at 4 and 5 h, or even longer, after dosing.

3.2.4. Assumption 4: Neither deuterium oxide nor body water is lost during the equilibration time

This assumption is probably not true, but precautions should be taken to minimize losses. Body water is not a simple closed system; it is a dynamic system with a variety of inputs (drink, food and metabolic water) and outputs (urine, faeces, sweat, breath, etc.). In temperate climates, approximately 8% of body

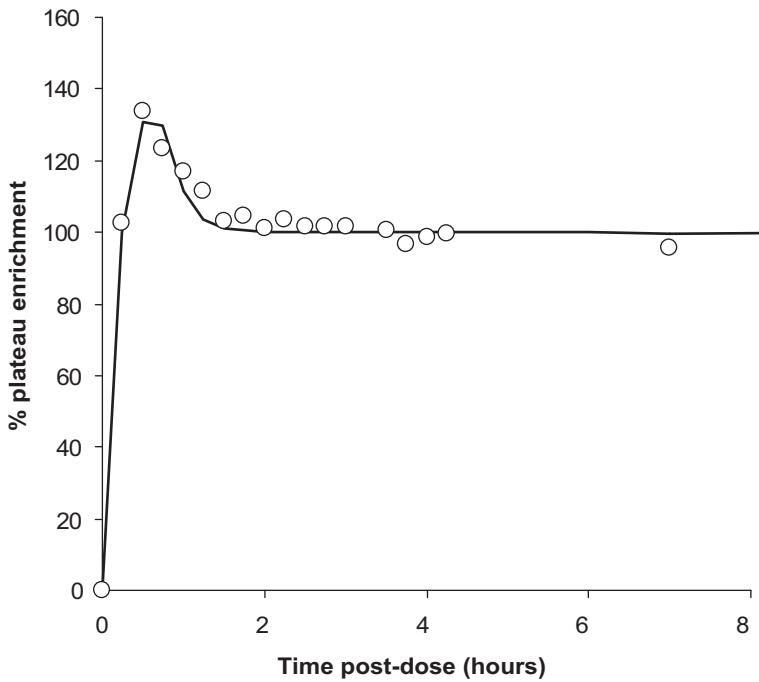


FIG. 2. Equilibration of deuterium oxide in body water. There is an early phase of overshoot where the deuterium enrichment appears above the eventual plateau since the deuterium oxide has not fully mixed with intracellular fluid. The plateau enrichment is maintained for several hours.

water is turned over in adults each day. Water turnover is 50–100% greater in tropical climates due to increased insensible water losses in the lungs and from the skin.

When TBW is measured using the equilibration technique described here, which lasts for 3–4 hours, participants can be asked to empty their bladder before the dose is taken, not to consume food or liquids, and to avoid physical activity during the equilibration period. As a result, the loss of deuterium in urine and sweat is minimized and can be ignored.

If it is not possible to fast during the equilibration period, a note should be kept of the volume of fluids consumed; this volume should be subtracted from the calculated TBW.

3.3. ASSESSMENT OF TBW IN INFANTS

It is preferable to use the back extrapolation procedure to assess TBW in infants [8]. It is advisable to seek the help of an expert if you are planning to assess TBW in infants, as special precautions must be taken to ensure that the dose is consumed correctly. Collection of saliva samples is very difficult in babies less than three months old.

3.4. HYDRATION OF FFM

The two compartment model of body composition divides the body into fat mass and FFM. Hydration of FFM refers to the proportion of water within the FFM. It is assumed that FFM contains 73.2% water in adults (aged 21 years and above):

$$\text{FFM (kg)} = \text{TBW (kg)}/0.732$$

Cellular hydration in all mammals is controlled within strict limits. The classic work of Pace and Rathbun is the source of the commonly used hydration coefficient, 0.732 [9]. In vivo studies in adults indicate that there is no effect of aging on the constant up to age 70 years [10, 11]. Factors that can result in individual variation in the hydration of FFM have been described by Wang et al. [12]. The hydration of FFM can vary by between 2% and 3% (standard deviation) in healthy adults [13, 14]. These values include both measurement errors and physiological variation. The true physiological variation in the hydration of FFM in healthy adults cannot be estimated without knowledge of the measurement errors. Schoeller estimates the average measurement error to be 1%. The within-laboratory standard deviation in the measurement of in vivo hydration is estimated to be 1.1% and the physiological variation to be 0.5%, which is small [1].

3.4.1. Variation of FFM hydration during infancy and childhood

The adult hydration factor of 0.732 is not appropriate for use in children and infants. The hydration of lean tissue is known to vary as the body develops during infancy. Newborn infants have relatively little muscle mass in proportion to their body weight. As the proportion of muscle mass increases, the hydration of FFM decreases during childhood [15, 16]. Lohman provides hydration factors for children and adolescents (Table 1). In infants, the Fomon hydration factors (Table 2) are often applied to convert TBW to FFM [17]. Data on body composition in infants are also available from Butte et al. [18]. The hydration of

TABLE 1. HYDRATION OF FFM (%)
IN CHILDREN AND ADOLESCENTS

Age (years)	Boys	Girls
1	79.0	78.8
1–2	78.6	78.5
3–4	77.8	78.3
5–6	77.0	78.0
7–8	76.8	77.6
9–10	76.2	77.0
11–12	75.4	76.6
13–14	74.7	75.5
15–16	74.2	75.0
17–20	73.8	74.5

Reference [16].

TABLE 2. HYDRATION OF FFM (%)
IN INFANTS

Age (months)	Boys	Girls
Birth	80.6	80.6
1	80.5	80.5
2	80.3	80.2
3	80.0	79.9
4	79.9	79.7
5	79.7	79.5
6	79.6	79.4
9	79.3	79.0
12	79.0	78.8
18	78.5	78.4
24	78.1	78.2

Reference [17].

FFM in infants was reviewed by Fomon and Nelson [19]. Corresponding data for prematurely born infants are lacking. Thus, until more information becomes available, any assessment of body fat in premature infants should use three or four compartment models of body composition [20].

3.4.2. Variation of FFM hydration during pregnancy and lactation

During pregnancy, the water content of FFM (the hydration coefficient) increases [21]. There is presently no consensus on the most appropriate hydration coefficients for different stages of pregnancy. Therefore, the deuterium dilution technique is not recommended for a two compartment model assessment of body composition in women in the second and third trimesters of pregnancy.

The conventional hydration coefficient, 0.732, is generally used in lactating women and women in the first trimester of pregnancy.

4. PROCEDURES

The following sections give detailed descriptions of the steps and procedures involved in the deuterium dilution method of estimating TBW, which include:

- Planning the study;
- Preparation and storage of deuterium oxide doses;
- Procedure for measuring TBW including anthropometric measurements of participants;
- Sampling saliva;
- Outputs.

The analysis of the enrichment of deuterium in saliva samples by FTIR spectrometry is described in Section 5.

4.1. PLANNING THE STUDY

Careful planning is essential for a successful outcome in any study. The most important task in the study is to determine its purpose. It is important to concentrate on one main issue. What is the hypothesis being tested? In addition:

- How many participants are required to address the issue? Perform a sample size calculation. Consult a biostatistician for advice.
- How will the data be handled? What statistical tests will be performed? Seek expert advice at the planning stage, not after the data have been collected.
- What is the procedure for obtaining ethical approval?

4.1.1. Ethics

All studies involving human participants must be reviewed and approved by the local ethics committee. Most leading journals will not accept a paper for publication without a statement of its approval by this committee, which is usually composed of medical doctors, scientists and lay people, including religious and community leaders, and a legal person such as an advocate or lawyer. It could be based at the Ministry of Health, Ministry of Science or the local university. The committee should be contacted at an early stage to determine the process for seeking ethical approval and obtain copies of the required documentation.

Participants must be informed of the purpose of the study in language appropriate to the local situation. Participants must give their voluntary, informed consent to take part, and be informed that they are free to withdraw at any time during the study.

The following is an example of the kind of information required by the ethics committee, but the details will vary, depending on local circumstances:

- The purpose of the proposed study, clearly stated;
- A summary of the study design and methodology, including details of the proposed sample size, giving indications of the calculations used to determine the required sample size;
- An outline of the ethical considerations involved in the proposal;
- Details of how consent is to be obtained, including an information sheet written in simple, non-technical language;
- Who will have access to the data and what measures will be adopted to maintain confidentiality of the participants;
- Who are the investigators (including assistants) who will conduct the study, and what are their qualifications and experience;
- Location(s) where the project will be carried out;
- Proposed start date and proposed completion date.

Appendix I contains information about the safety of deuterium oxide, which may be useful in the preparation of applications for ethical approval.

4.1.2. Preparation of the participant data sheet

Data from each participant should be appropriately recorded. An inexpensive way is to keep paper records in the field, which can be transferred to an electronic spreadsheet later. The minimum information required is shown in Table 3, but additional information specific to the study will also be required, such as information on the participant's health. The information sheet should be designed at the planning stage, so that it can be evaluated, and if necessary, amended during the pilot study. Information sheets can be designed using a word processor or a spreadsheet and reproduced as many times as necessary. An example of a participant data sheet is included in Appendix III.

4.1.3. Pilot study

If this technique has not been used before, it is advisable to carry out a pilot study. Such a study is important to:

- Practice and test the procedures, including sampling, analysis of samples and data handling;
- Train all people involved;
- Develop routine and team work;
- Develop strategies to overcome practical difficulties. 's

TABLE 3. MINIMUM INFORMATION REQUIRED FOR THE PARTICIPANT'S DATA SHEET

Project name/code
Participant's study ID
Name/initials of researcher
Date
Participant's weight (kg)
Dose number
Dose weight (g)
Time of baseline sample
Time dose taken
Time of 1st post-dose sample
Time of 2nd post-dose sample
Volume of water consumed (L)

A pilot study is usually conducted with a relatively small number of participants. It can be used to check the equilibration time in the particular circumstances encountered in the study. Water turnover is affected by the physiological condition of the participants. Age, health status and climate all affect water turnover; therefore, saliva specimens should be collected 3, 4 and 5 h after the dose has been consumed in adults, and 2, 3 and 4 h after the dose has been consumed in children. In the final study, it will only be necessary to collect two post-dose saliva samples, but the time of these samples will have been determined empirically under the local circumstances.

4.1.4. Sample size calculation

Any study must ensure that an appropriate number of participants are included to be able to obtain a reliable answer to the question asked. Sample size or power calculations are an important component of any study design and are required by ethical review committees and funding bodies. A power calculation can be used to determine the sample size necessary to obtain a reliable answer. Consult a biostatistician for advice on sample size determination. Power calculations can be performed using statistical software.

To calculate the required sample size, it is necessary to know the standard deviation (SD) of body composition parameters in a population similar to that being studied, and to define the difference between study groups that will be regarded as significant (δ). Before visiting the biostatistician, perform a literature search to obtain this information. The number of participants required in a public health situation, where measurements are conducted at field centres, will be higher than for measurements conducted under carefully controlled conditions in a research laboratory.

The ‘power’ of a study is usually expressed as the percentage of time that a study will detect a significant result when there is a true difference. A power of 80% is usually selected, which means that if there is a true difference and a study was performed 100 times, 80 would detect a statistically significant result and 20 would not (the value of 20 would detect a false negative result). The significance level (α) is fixed at some low value (usually 0.05). This is the probability of a false positive result.

4.1.4.1. Example

If the effect of a nutritional intervention on body composition of adults living with HIV is to be studied, it is necessary to determine the SD of TBW in the population and the magnitude of change that would be clinically important (δ). In a recent study in Africa, the SD (σ) of TBW in 150 HIV sero-positive

adults was 5 kg (personal communication). The mean body weight of participants was approximately 60 kg. An increase of FFM equivalent to 5% body weight might be considered clinically important. A total of 3 kg of FFM is equal to 2.2 kg of TBW (3×0.732). Therefore, if we assume $\sigma = 5$ kg and $\delta = 2.2$ kg of TBW, with a power of 80% and a significance level of 0.05, and two study groups (control and intervention), the required sample size (n) can be calculated using the following equation:

$$n = 2 \times 7.85 \times \left(\frac{\sigma}{\delta} \right)^2$$

where 7.85 is the multiplication factor $f(\alpha, \text{power})$ for power 80% and $\alpha = 0.05$ obtained from statistical tables, therefore:

$$n = 2 \times 7.85 \times \left(\frac{5}{2.2} \right)^2 = 81$$

At least 81 participants in each group are required to achieve statistically significant results. If more power or a higher level of significance is required, then more participants will be required for the study. It is also advisable to add a factor to allow for dropouts based on local experience. If we assume a 25% attrition rate, then about 110 participants would need to be recruited in each group.

4.2. PREPARATION AND STORAGE OF DEUTERIUM OXIDE DOSES

Correct preparation of the dose is a critical step in this method and will affect the quality of the data obtained.

4.2.1. Equipment

All equipment used for preparing doses must be completely dry to avoid contamination by water. An equipment list is included in Appendix IV. Dose bottles must be screw capped and leak proof (e.g. 60 mL wide mouth, leak proof, autoclavable, polypropylene bottles) to avoid losses during storage and contamination by moisture from the atmosphere. It is not necessary to autoclave the bottles, but these bottles will not crack or leak if stored in a freezer.

The balance used to weigh the dose must have a weighing range that is adequate for the amount **and** the container to be weighed. A balance weighing to 0.01 g is recommended. The dose must be weighed to four significant places, e.g. 30.05 g for adults or 6.034 g for children.

4.2.2. Dose preparation for adults

The standardized dose of deuterium oxide for estimating total body water is 30 g for all adult body weights. Doses must be accurately weighed to at least 0.01 g. In a laboratory notebook, keep a record of the batch number of the stock solution of deuterium oxide used to make the doses, the date the doses were prepared, the dose number, the weight of the bottle, the weight of the bottle plus dose, and the weight of the dose. This information can be transferred to a spreadsheet later. Doses should be prepared in a clean area, e.g. a food preparation area. It is not good practice to prepare doses for human consumption in a chemistry laboratory, because the balance may have previously been used to weigh toxic compounds.

The procedure for preparing doses is illustrated in Fig. 3.

If an electronic scale is used:

- The bottle plus lid should be tared.



FIG. 3. Dose preparation — doses should be prepared in a food preparation area, not a laboratory.

- 30 mL of D₂O should be added to the bottle using a measuring cylinder and the lid replaced.
- The exact weight of D₂O in each dose should be recorded.
- The weight of D₂O will not be exactly 30 g, as the density of D₂O is greater than the density of water (density of D₂O at 25°C is 1.105 g/mL. The density of H₂O is 1.000 g/mL). This does not matter as long as the exact weight is recorded and used in subsequent calculations.

Note that if this is the first time this technique is being used, a few millilitres of the D₂O stock solution will need to be retained to make a calibration standard for analysis with the saliva samples. Preparation of the calibration standard is described in Section 5.2.

4.2.3. Dose preparation for children

The dose for children is approximately 0.5 g of D₂O per kg body weight. In large studies, it is easier to give all participants the same dose. A standard dose can be prepared based on the average weight of the children, or several standardized doses for studies involving children of different ages and body weight. Suggested doses are given in Table 4.

For children <30 kg body weight, a dilution of the D₂O can be prepared to avoid dispensing small volumes, which are more prone to loss by evaporation. A 1 in 5 dilution of the D₂O should be prepared by adding 800 g (800 mL) of local drinking water to 200 g (180 mL) of D₂O (Fig. 4). The weight of both the D₂O and the added drinking water should be recorded to 0.01 g. For children >30 kg body weight, undiluted D₂O should be used.

TABLE 4. STANDARDIZED DOSES OF D₂O FOR THE ESTIMATION OF TBW IN CHILDREN

Body weight (kg)	Weight of D ₂ O required (g)	Approximate volume of 1 in 5 dilution (mL)
<10	3 ^a	15
10–20	6 ^a	30
20–30	10	50
30–50	20	Not applicable
>50	30	Not applicable

^a This amount can also be given undiluted using a disposable plastic syringe, if preferred. The syringe should be weighed before and after dosing to determine the exact amount consumed.

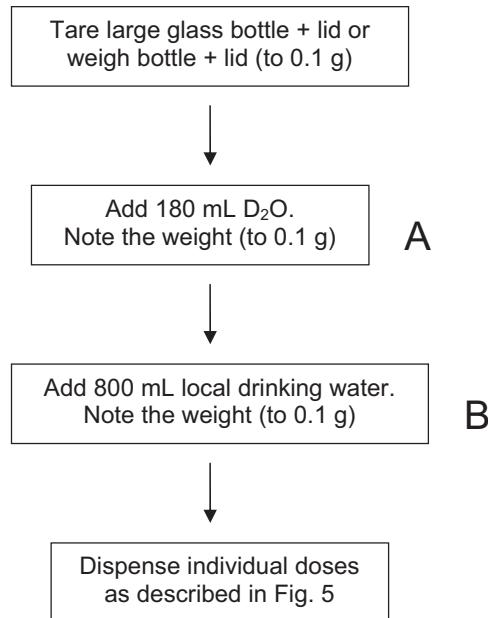


FIG. 4. Preparation of a 1 in 5 dilution of D₂O.

Doses containing the correct amount of D₂O can be prepared by dispensing an aliquot of the diluted D₂O into weighed (or tared) screw cap bottles, as described below and in Fig. 5.

4.2.3.1. Preparation of a 1 in 5 dilution

This dilution can be prepared using local drinking water. A clean 1 L (or larger) capacity borosilicate screw cap bottle is required. A new borosilicate reagent bottle that has a screw cap with a PTFE facing disc would be suitable. Use of a bottle that has previously been used to store chemical reagents should be avoided. A balance that can weigh 2 kg to 0.1 g is required. Note that the density of D₂O is 1.105 g/mL at 25°C; therefore, 180 mL weighs 200 g. The density of water (H₂O) is 1.000 g/mL at 25°C; therefore, 800 mL weighs 800 g.

The lid on the bottle must be kept on during weighing to avoid loss by evaporation.

If an electronic scale is used:

- The bottle plus lid must be tared. If not, the bottle plus lid must be weighed;
- 180 mL of 99.8 or 99.9 at% D₂O should be added to the bottle and the lid replaced.

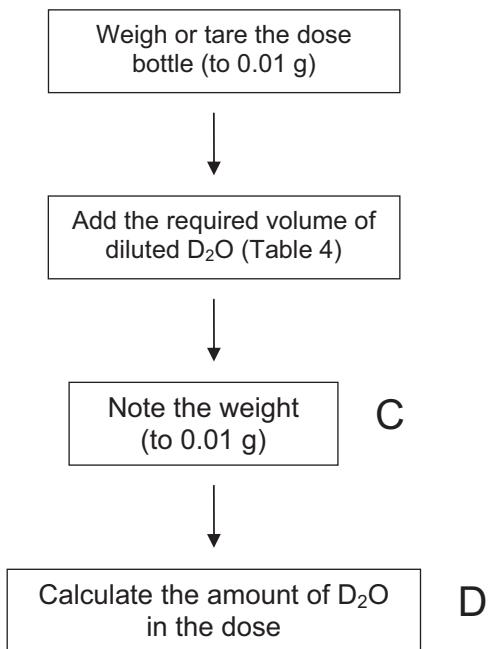


FIG. 5. Preparation of individual doses for children.

- The weight of D₂O in the bottle should be recorded (A, approximately 200 g).
- 800 mL of local drinking water should be added to the bottle and the lid replaced. The weight of the D₂O plus drinking water should be recorded (B, approximately 1000 g).

4.2.3.2. Preparation of individual doses for children

Individual doses must be accurately weighed to at least 0.01g. If an electronic scale is used,

- The bottle plus the lid should be tared; otherwise they should be weighed.
- The required volume of 1 in 5 D₂O should be added to the bottle using a measuring cylinder or pipette. The lid should be replaced.
- The exact weight of D₂O (or D₂O plus water) in each dose bottle should be recorded (to 0.01g, C).
- The amount of D₂O in each dose (D) can by calculated by proportion.

Thus, the weight of D₂O in the dose (g) = weight of water in the dose bottle (g) × weight of D₂O in the 1 in 5 dilution (approx 200 g)/weight of D₂O plus drinking water in the 1 in 5 dilution (approx 1000 g):

$$\text{Weight of D}_2\text{O in the dose, } D = C \times A/B \text{ (g)}$$

where D is the weight that should be used to calculate the TBW after conversion to milligrams.

Figures 4 and 5 summarize the procedure for the preparation of doses for children.

4.2.4. Dose storage

The doses can be made in batches and stored in a refrigerator until required.

To ensure good hygiene and avoid cross-contamination, doses should not be stored in the same place as saliva samples. Samples will have an enrichment of D₂O of up to 1000 mg/kg (ppm D₂O), whereas the doses are enriched to approximately 999 000 mg/kg (ppm D₂O). Therefore, the dose contains approximately 1000 times as much deuterium as the biological specimens. In addition, doses should not be stored with saliva samples in order to avoid microbial cross-contamination.

When transporting doses to and from the field, separate boxes for doses and saliva samples should be used.

4.3. PROCEDURE FOR MEASURING TBW

The procedure for measuring TBW by deuterium dilution is summarized in Fig. 6.

The participant should have normal fluid and food intake on the day before the estimation of TBW and avoid vigorous exercise after the final meal of the previous day to avoid dehydration and depletion of glycogen stores.

For accurate measurements of TBW, participants should be asked to empty their bladder before starting. This will ensure that each time body weight is measured under the same conditions in longitudinal studies and that water in urine is not included in TBW.

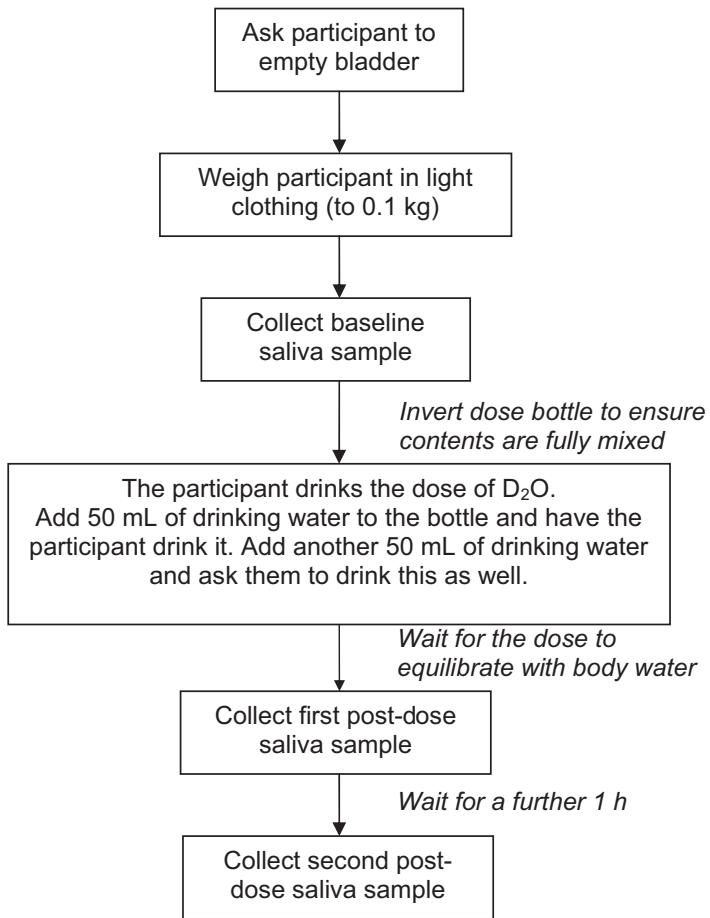


FIG. 6. Flow chart describing the procedure for measuring TBW by deuterium oxide dilution.

4.3.1. Anthropometric measurements

An accurate measure of body weight is required because body fat is estimated by the difference of FFM from body weight. Participants should be asked to empty their bladder (and if possible bowels) before being weighed in light clothing. Standardizing conditions in this way is particularly important in longitudinal studies. The accuracy of scales used for measuring body weight should be checked daily using a calibration weight of known mass. Much care is taken to ensure the accuracy and precision of isotopic data. Body composition

results will be compromised if equal care is not taken to ensure the accuracy of anthropometric measurements.

4.3.1.1. Measuring weight and height in adults and children

Measuring weight

- The participant's weight must be measured to the nearest 0.1 kg using electronic scales or any balance with adequate precision.
- The balance must be placed on a level surface. The balance should be checked using a spirit level, if possible.
- Participants should wear minimal clothing and no shoes (Fig. 7). If they do not wish to wear minimal clothing during the weighing procedure, their clothes should be weighed separately afterwards, and the weight of their clothes subtracted to obtain an accurate measure of body weight.
- Record the weight on the participant's information sheet to 0.1 kg.
- In longitudinal studies measuring changes in body composition over relatively short periods of time, an accurate measurement of body weight is essential. Account must be taken of the weight of any clothing worn during the measurement.
- The accuracy of the scale should be checked daily using a calibration weight of known mass.



FIG. 7. Weight is measured in light clothing without shoes.



FIG. 8. Measuring height: hair down, eyes forward.

Measuring height

- Height must be measured to the nearest 0.1 cm using a stadiometer.
- The stadiometer must be placed on a level surface. Check using a spirit level, if possible. The accuracy of the stadiometer should be checked periodically using measure rods of known length.
- Height is measured without shoes.
- The participant should stand upright with their heels to the wall or touching the vertical post on the stadiometer. Their knees should be straight.
- The participant should be asked to look straight ahead and their eyes should be at the same level as the ears (Fig. 8).
- Lower the beam until it just touches the top of the head. Elaborate hair arrangements must be undone. The height should be recorded in centimetres (to the nearest 0.1 cm) on the participant's information sheet. The measurement should be repeated. After recording both measurements, the mean should be calculated.

In children, height is measured above 85 cm and length is measured below 85 cm (see next section). WHO has developed detailed instructions on how to measure a child's weight and length/height. The materials can be downloaded from the following site: www.who.int/childgrowth/training/en/index.html.



FIG. 9. Measuring an infant's weight.

4.3.1.2. Measuring weight and length in infants

Measuring weight in infants

- Infants must be weighed without clothes using scales accurate to 0.01 kg (Fig. 9).
- A cloth should be placed in the weighing pan to prevent chilling the child.
- The scales should be adjusted to zero with the cloth in the pan.
- The naked child should be placed gently on the cloth in the weighing pan.
- Wait for the child to settle and the weight to stabilize.
- The weight (to the nearest 10 g, 0.01 kg) should be measured and recorded immediately.

Standardization (levelling) of the scales should be performed weekly or whenever the scales are moved.

Checking the scales

Known weights of 3, 5, 10 and, if appropriate, 20 kg should be weighed. If calibration weights are not available, sealed bottles containing water can be used. These must have been accurately weighed on a calibrated balance, and the weight should be checked periodically.

To check tared weighing: weigh a 20 kg weight, tare the scale, and then add a 3 kg weight. The scale should indicate the 3 kg weight.

If the weights are not accurate, the scale should be calibrated if possible; if not possible, the scale needs to be replaced.



FIG. 10. Measuring an infant's length.

Measuring length in infants

The infant's length is measured using a measuring board (sometimes called an 'infantometer'). Two people are needed to measure the child's length (Fig. 10).

One person should:

- Assist in positioning the child face-up on the measuring board, supporting the head and placing it against the headboard.
- Position the crown of the head against the headboard, compressing the hair. Check that the child lies straight along the centre line of the board and does not change position. Shoulders should touch the board, and the spine should not be arched.

Usually, this person stands or kneels behind the headboard.

The second person should:

- Support the trunk as the child is positioned on the board.
- Lay the child flat along the board.
- Place one hand on the shins above the ankles or on the knees and press down firmly. With the other hand, he or she should place the foot piece firmly against the heels. Ensure that the toes do not prevent the footplate coming into contact with the heels.
- Measure the length (to the nearest 0.1 cm) and record it immediately.

The measuring board should be kept clean and stored at normal indoor temperature, protected from humidity. The board should be checked for accuracy every week.

4.3.2. Dose administration

In adults and children, the dose should be consumed at least 2 h after the last meal, preferably after an overnight fast. If this is not possible, a small meal may be given one hour after the dose is taken. The meal should be simple and less than 1250 kJ (300 kcal). This allows the dose to empty from the stomach before the meal, but water in the meal to equilibrate with body water before the post-dose saliva samples are collected. In infants, the dose is usually given with a meal. In breastfed babies, a disposable syringe can be used to administer the dose immediately before feeding. If a disposable syringe is used, determine the dose accurately by weighing the syringe while full and again after the dose has been given. In bottlefed babies, the deuterium oxide can be given in the milk. If the dose is not consumed completely, the infant cannot be included in the study. It is recommended to seek advice from experienced individuals in measuring TBW in babies.

Furthermore,

- Baseline saliva samples must be obtained before the dose is consumed.
- The dose of D₂O (labelled water) is 30 g for adults (see Table 4 for child doses).
- If the dose has been frozen, it should be completely thawed before use.
- Whether stored in a fridge or thawed following freezer storage, the bottle should be inverted several times to mix any condensation on the cap into the bulk of the liquid. This should be done immediately before the dose is consumed. This is because the condensation is fractionated relative to the bulk liquid. (See Appendix V for more information on fractionation.)
- The bottle should not be opened until it is time for the dose to be consumed.

When talking to participants, it is often better to use the term ‘heavy water’ or ‘special water’, rather than ‘deuterium labelled water’ or ‘stable isotope labelled water’, since there can be confusion over the word ‘isotope’, which is often associated with radioactivity.

There is no radiation hazard associated with the use of D₂O.

The specific steps are as follows:

- The bottle number and the time the dose was taken should be noted on the participant’s data sheet.



FIG. 11. Dose administration — the participant drinks the dose through a straw to avoid spillage.

- Participants should drink the dose through a straw to avoid spillage (Fig. 11).
- About 50 mL of drinking water should be added to the dose bottle and the participant asked to drink it through the same straw. The procedure should be repeated with another 50 mL of drinking water. This will ensure that no labelled water is left in the bottle.

4.3.3. Food and drink intake, and physical activity during the equilibration period

If possible, participants should not eat or drink during the equilibration period; if this is not possible, e.g. in children and lactating women, a small meal can be given 1 h after the dose. The volume of all drinks taken should be noted during the equilibration period, including the 100 mL used to rinse the dose bottle. This volume should be subtracted from the calculated TBW. If no additional fluid is consumed during the equilibration period, the 100 mL of water used to rinse the dose water is usually ignored. Participants should not drink and eat between the 3 and 4 h saliva specimens to minimize any short term effect of water intake on deuterium enrichment in saliva.

Participants should avoid physical activity during the equilibration period to minimize water loss in breath and evaporation from the skin (insensible water loss). There is less deuterium in water vapour than in body water due to isotopic fractionation. Therefore, an increase in insensible water loss will lead to an error in the calculation of the TBW.

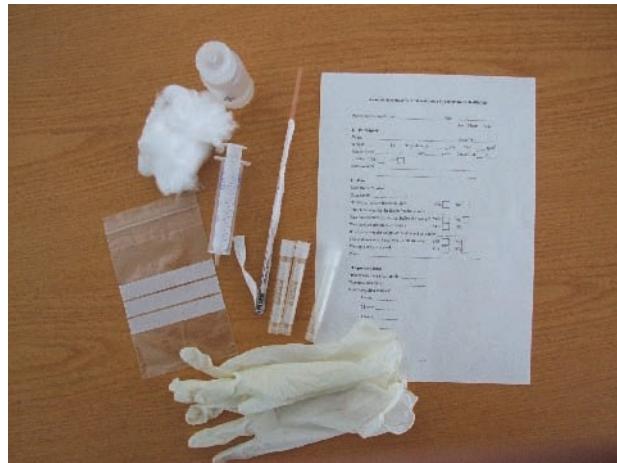


FIG. 12. Equipment required for saliva sampling.

4.3.4. Saliva sampling

4.3.4.1. Preparing for saliva sampling

Good preparation before taking the samples and a clear understanding of the procedure are very important for accurate results. The procedure should be clearly explained to the participant before sampling.

The availability of the following items should be confirmed before starting (Fig 12).

Cotton wool and swabs:

- Cotton wool balls to collect saliva samples from adults and children;
- Cotton wool swabs with extra cotton wool to collect saliva samples from infants.

Sample storage vials:

- These vials must be screw capped with a seal to prevent losses, fractionation and cross-contamination during storage, e.g. 4 mL cryovials.
It is a good idea to use different coloured caps for baseline and post-dose samples, e.g. blue caps for baseline samples and red caps for post-dose samples.
- They must be completely dry before use.

- They must not be reused to prevent cross-contamination between enriched (post-dose) samples and unenriched (baseline) samples.
- They should be labelled with the participant's identification number, and the date and time the sample was taken. Names should not be written on sample vials to preserve confidentiality.

Disposable 20 mL syringes:

- The syringes must be completely dry before use.
- They must not be reused to prevent cross-contamination between enriched (post-dose) samples and unenriched (baseline) samples.

Gloves:

- New disposable gloves must be worn by the person taking the saliva sample.
- The gloves must be discarded before moving on to the next participant.
- The dose bottle must not be touched after putting on gloves to take the baseline saliva sample until after the sampling is complete.

Zip-lock bags:

- Two small zip-lock bags are needed for each participant: one for the baseline sample and one for the post-dose samples.
- Another zip-lock bag is needed to keep all the samples from the participant together.
- All bags must be labelled permanently with the participant's identification number.

Labels:

- Ensure labels are of good quality and cannot come off the containers.
- Use a permanent marker to write on the labels to prevent the writing from being smudged or removed, in particular when the samples are thawed.

Participant data sheets:

- Printouts of data sheets for each participant need to be available before the first sampling (baseline).
- To preserve confidentiality, do not write names on the data sheets; the names and corresponding participant IDs must be recorded separately.

An example of a participant data sheet is shown in Appendix III.

4.3.4.2. Sampling times

Sample saliva from adults and children at baseline and then 3 h and 4 h after the dose is taken. In elderly participants and those with expanded extracellular water volume, it is recommended that post-dose samples are taken at 4 h and 5 h after the dose is consumed. Two samples at plateau enrichment confirm that the dose has fully equilibrated with body water. In infants, saliva should be sampled two times between 2.5 h and 4 h after the dose was taken, but not within 15 min of feeding.

4.3.4.3. Saliva sampling using cotton wool balls

The procedure for saliva sampling in adults and children is described below and illustrated in Fig. 13.

The following should be noted:

- The dose bottle should not be touched during sample collection, from the time gloves are put on until the baseline saliva sampling procedure is completed;
- Clean gloves should be used for each participant.
- When collecting samples, ensure that the participant does not eat or drink anything for at least half an hour before saliva collection.

The following procedure is recommended:

- (1) The participant should be given a cotton wool ball to soak up saliva. He/she should be asked to move it around in the mouth for 2 min, or until sodden, keeping the mouth closed. Asking them to think about his/her favourite food increases salivation.
- (2) The plunger from a new 20 mL disposable syringe should be removed.
- (3) The participant should be asked to transfer the cotton wool to the front of the mouth and transfer it directly from the mouth into the body of the syringe (Fig. 13).
- (4) Replace the plunger in the body of syringe.
- (5) A sample storage vial should be labelled with the participant's ID, and the date and time of collection.
- (6) The lid from the vial should be removed and the syringe plunger used to extract saliva from the cotton wool into the sample storage vial (Fig. 13). The lid should be replaced to avoid evaporation and subsequent isotope fractionation.



FIG. 13. Saliva sampling using cotton wool balls.

- (7) If there is not at least 2 mL of saliva, the above steps should be repeated using a new cotton wool ball. If possible, 4 mL should be collected to allow for repeat analysis.
- (8) Syringes, cotton wool and gloves should be discarded between participants. Sample vials or syringes should not be reused.
- (9) Each vial should be labelled with the participant's identification number, together with the date and time the sample was taken. In addition, all dates and times of saliva collection should be recorded on the participant's data sheet. This information should be copied to a spreadsheet as soon as possible.

Participants should avoid physical activity until the final saliva samples have been taken.

4.3.4.4. Saliva sampling in infants using cotton wool swabs

The procedure for saliva sampling in infants is described below and illustrated in Fig. 14:

- Clean gloves should be used for each infant.
- When collecting samples, ensure at least 15 min since the infant was last fed so that there is no residual milk or other food in the mouth.
- In infants, saliva is sampled using a cotton wool swab (Fig 14). An extra piece of cotton wool should be wrapped around the swab. Saliva should be collected by moving the swab around the infant's mouth until the cotton wool is sodden. The time required for this will vary between infants, and

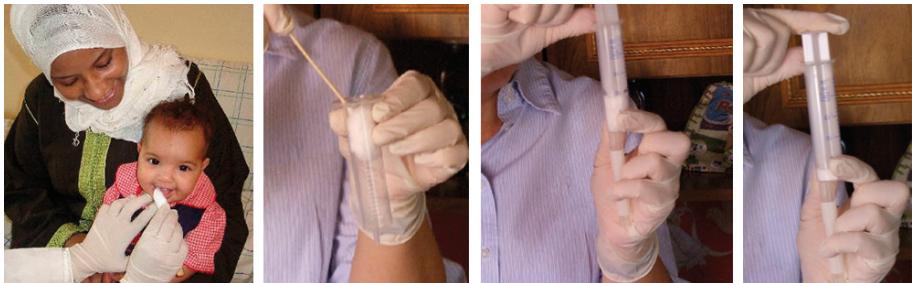


FIG. 14. Saliva sampling using a swab.

patience is required. It may take several attempts to collect the required volume (minimum 2 mL, preferably 4 mL).

The following procedure is recommended:

- (1) The plunger from a new 20 mL disposable syringe should be removed. The cotton wool from the swab should be removed and placed in the barrel of the 20 mL syringe (Fig. 14).
- (2) The plunger should be replaced in the body of the syringe.
- (3) A sample storage vial should be labelled with the participant ID, date and time of collection.
- (4) The lid should be removed from the vial and the syringe plunger used to extract saliva from the cotton wool into the sample storage vial. The lid should be replaced to avoid evaporation and subsequent isotope fractionation.
- (5) If there is not at least 2 mL of saliva, the above steps should be repeated with a new cotton wool ball or swab. If possible, 4 mL should be collected to allow for repeat analysis.
- (6) The swab, syringe, cotton wool and gloves should be discarded between participants. Sample vials or syringes should not be reused.
- (7) The infant's identification number should be recorded, and the date and time the sample was taken written on each vial. All dates and times of saliva collection should be copied to the participant's data sheet. This information should be recorded on a spreadsheet as soon as possible.

4.3.4.5. Storage of saliva samples

Since a large study will generate hundreds of samples, careful management and labelling of saliva samples is essential. Proceed as follows:

- The containers must be firmly closed to prevent the loss of water by evaporation and cross-contamination between samples.
- Zip-lock bags can be used to keep all samples for a single participant together and prevent cross-contamination. A small bag should be used for the baseline sample and another small bag for the post-dose samples. The two bags should then be placed in a third, larger one, so that the samples from a single participant are kept together.
- The participant identification number should be written on both the sample vials and the zip-lock bags.
- A log of the samples should be kept in a spreadsheet.

Saliva samples should be stored frozen (at -20°C) until analysis to minimize bacterial growth. If this is not possible, samples should be stored in a refrigerator or a cool box until they can be transferred to a freezer. To avoid contamination of samples:

- Samples and doses should never be stored together.
- The cap of the sample bottles should be tightly closed to avoid losses by evaporation and contamination by moisture from the atmosphere.

5. ANALYSIS OF DEUTERIUM ENRICHMENT BY FOURIER TRANSFORM INFRARED SPECTROMETRY

The enrichment of deuterium in saliva samples can be measured by FTIR spectrometry [5]. A typical example of FTIR instrumentation is shown in Fig. 15. A brief description of the principles of FTIR spectrometry can be found in Appendix II. The exact details of the procedure will depend on the make and model of the FTIR instrument, but the main principles and precautions are summarized in the following sections.

5.1. THE FTIR LABORATORY

- The FTIR instrument should be sited in a well ventilated room to avoid buildup of CO_2 in the atmosphere. Ideally, the room should be airconditioned with controlled temperature and humidity. The bench on which the FTIR instrument is placed should not be subject to vibration from nearby equipment or external sources.



FIG. 15. Typical FTIR instrumentation.

- The FTIR instrument should not be moved once it has been installed. If it is necessary to move it, an engineer should be called to check the alignment of the mirrors.
- The humidity in the FTIR instrument should be less than 60%. The desiccant in the FTIR chamber should be changed when the indicator changes colour. This could be once a week in humid climates.

5.1.1. Cleaning the FTIR instrument

A water dampened cloth should be used to wipe the exterior of the FTIR instrument to keep it dust free. It is not advisable to wipe the inside of the sample compartment. If spillage from the cell occurs inside the compartment, it should be cleaned up immediately with an absorbent, lint free cloth.

5.2. PREPARATION OF THE CALIBRATION STANDARD

A large volume of a calibrating or standard solution of approximately 1000 mg/kg (ppm), or 1 g/L, should be prepared (gravimetrically) by weighing deuterium oxide and diluting in normal drinking water from the region. Note that the density of D₂O is 1.105 g/mL at 25°C. The following points should be noted:

- It is convenient to make 1 L of the calibration standard in a volumetric flask and then transfer it to a borosilicate bottle with a PTFE lined screw cap for storage until required. A second bottle containing 1 L of the water used to make the dilution should also be retained. It is a good idea to store the calibration standards in several smaller, tightly sealed bottles (e.g. 250 mL borosilicate bottles with PTFE lined screw caps). Only one enriched and one natural abundance bottle should be in use at any time, as working standards. The remainder should remain sealed until required. The calibration standards will last for several years if stored in a cool, dark place. The bottles must be well sealed to prevent ingress of water from the atmosphere. They should not be stored in the same place as the deuterium oxide.
- The D₂O should be weighed on an analytical balance accurate to 0.0001 g, or preferably 0.000 01 g. Suitable analytical balances and glassware will probably be available in the chemistry department of the local college or university. The standard should be prepared in two stages, as described below, but it is important that the weight of deuterium oxide is known to 0.0001 g. Balances must be levelled and calibrated before use.
- Distilled water should not be used to make the calibration standard since it is subject to fractionation. Local drinking water should be used to make the 1000 mg/kg (ppm) standard. A similar volume of the local drinking water should be kept to serve as a zero standard. If the quality of the local drinking water is poor, the shelf life of the standard will be improved if the water is passed through a sterile 0.22 µm filter.

All glassware must be clean and dry before use.

The following procedure is recommended:

- Using an analytical balance (accurate to 0.0001 g), a clean, dry 50 mL volumetric flask with its stopper in place should be weighed or another similar container can be used, e.g. a clean, dry glass bottle with a cap.
- A small volume (~20 mL) of drinking water should be added to the flask, the cap replaced and the weight should be recorded again.
- 1 g of D₂O should be added to the bottle. If an adjustable pipette is being used to transfer 1 g of D₂O, then the volume selected should be 0.9 mL, since the density of the D₂O is higher than water (1.105 g/mL and 1.000 g/mL, respectively at 25°C). The stopper or cap should be replaced to avoid losses by evaporation, and the weight noted. The weight of the D₂O in the bottle should be calculated.
- A clean dry 1 L volumetric flask should be weighed with its stopper. At this stage, a balance weighing to 0.1 g can be used.

- The water from the 50 mL container should be quantitatively transferred into the 1 L volumetric flask using a funnel. Local drinking water should be added to the smaller container and poured into the larger container. This procedure should be repeated at least three times to ensure that all the D₂O is transferred. Avoid spillage.
- Local drinking water should be added to the 1 L volumetric flask up to the mark. After replacing the stopper, the flask should be weighed again.
- After noting the weight, the calibration standard should be transferred to a clean, dry glass bottle with a PTFE lined screw cap.
- A similar volume of the local drinking water should be kept for use as a zero standard or blank to measure the background spectrum.
- The enrichment of the calibration standard should be calculated as follows:
 - If (A) is the weight of D₂O, (B) is the weight of drinking water plus D₂O in the 1 L flask, then the weight of added drinking water is (B – A).

For example,

- If the weight of D₂O = 1.0015 g (A)
- Weight of drinking water plus D₂O in the 1 L flask = 1000.1 g (B)
- Then the weight of added drinking water = 1000.1 g – 1.0015 g
= 999.0985 g (B – A)
- Enrichment of D₂O in the calibration standard = $A/(B - A) \times 10^6$ mg/kg
= 1.0015 g/999.0985 g $\times 10^6$ mg/kg
= 1002 mg/kg (ppm)

Note: 1 mg/kg = 1 mg/L as the density of H₂O is 1.0 kg/L at 25°C; therefore, the calibration standard is approximately 1000 mg/L.

The enrichment of the FTIR calibration standard can be verified by having it independently analysed in a reference laboratory. The measured enrichment of the calibration standard solution should be close to the enrichment obtained gravimetrically, i.e. calculated as described above.

5.2.1. Shelf life of the calibration standards

The shelf life of the calibration standards will depend on the quality of the local drinking water. The bottles should be stored in a cool dark place out of direct sunlight, but not in the same refrigerator as the highly enriched D₂O. Wrapping bottles in aluminium foil helps to protect the contents from light. Bottles must have good seals and be tightly closed to prevent ingress of atmospheric moisture. Some laboratories recommend storing the bottles upside

down. Should there be a leakage, these are less likely to suffer fractionation. Some laboratories recommend storing the calibration standards in several 100 mL or 250 mL bottles, rather than 1 L bottles. This has the advantage of only exposing a small portion of the calibration standard to the atmosphere at any time, but the disadvantage that, as the calibrant is used, it will be more prone to fractionation effects. See Appendix V for more information on fractionation.

5.3. PREPARATION OF A STANDARD CURVE

Once the FTIR instrument has been installed, the accuracy of deuterium analysis over the range of enrichments likely to be encountered should be checked using gravimetrically prepared standards. Smaller volumes (e.g. 100 mL) of these standards can be prepared by diluting deuterium oxide with local drinking water in a volumetric flask, as described above. The enrichment should range from 0 (natural abundance drinking water) to 2000 mg/kg, i.e. enrichment above that which is likely to be encountered in saliva samples.

Standards should be made (in 100 mL local drinking water) according to Table 5. The D₂O can be pipetted into the volumetric flask (column 2), but it must be accurately weighed (column 3). Also, the weight of the drinking water added to make up the volume should be noted (column 4). The actual enrichment (mg/kg) can be calculated from the weights, as described previously. The procedure is illustrated in Fig. 16. An example of a standard curve is shown in Fig. 17.

The balance used for preparing standards must be on a stable table away from open windows and draughts.

TABLE 5. PREPARATION OF FTIR STANDARDS

Target enrichment (mg/kg D ₂ O)	µL D ₂ O	Weight of D ₂ O (g) to 4 decimal places	Weight of drinking water added (g)
0	0		
100	10		
200	20		
400	40		
600	60		
800	80		
1000	90		
1500	140		
2000	180		

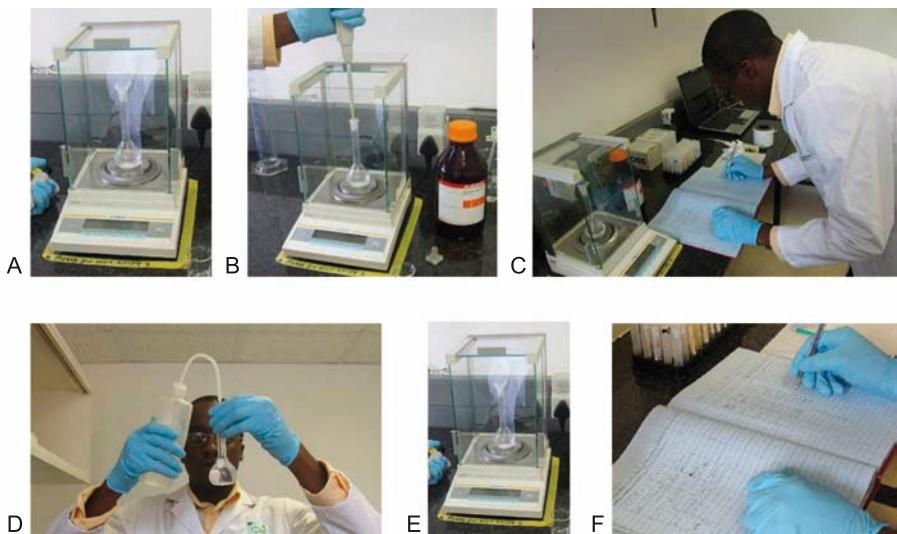


FIG. 16. Preparation of calibration standards.

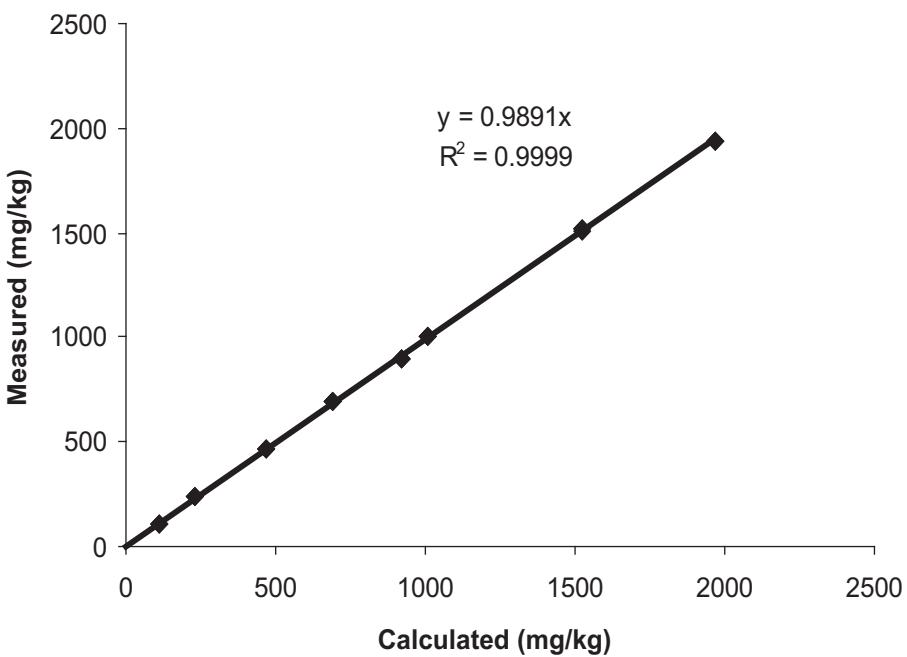


FIG. 17. Deuterium calibration curve measured by FTIR spectrometry.

Figure 16 illustrates the preparation of calibration standards as follows:

- A. Weigh the volumetric flask with its lid.
- B. Place the pipette of D₂O into a weighed quantity of water.
- C. Weigh again and note the weight.
- D. Fill to the mark.
- E. Weigh again.
- F. Note the weight. Calculate the enrichment of D₂O in the standard.

If the gradient of the calibration curve is not close to 1, there is a problem with the weighing, with the calculations or with the analysis. The data input should be checked and, if necessary, the procedure should be started again and new standards made.

5.4. OPERATION OF THE FTIR INTRUMENTATION

The FTIR instrument should be switched on 30–40 min before use to allow the electronics to stabilize. Both the interface and the mirror should be checked to make sure that they are working properly. Finally, ensure that the following are set:

- Measurement mode: *Absorbance*
- Apodization: *Square triangle*
- No. of scans: 32
- Resolution: 2.0
- Range (cm⁻¹): Minimum 2300 Maximum 2900

A ‘background’ scan should be performed using unenriched (natural abundance) water, e.g. the water used to make the calibration standard (zero standard). The instrument should be calibrated using the 1000 mg/kg (ppm) standard.

The peak due to deuterium should have a maximum at about 2504 cm⁻¹ (Fig. 18).

The instrument can automatically perform a background correction. When the absorption spectrum of the local drinking water is ratioed to that of the reference, then the instrument is calibrated directly in terms of mg/kg (ppm) excess of deuterium.

Body water samples can be treated in the same way, but in this case the background correction should be performed using the baseline (time 0) saliva sample. The facility to perform this background correction is included in the instrument’s software.

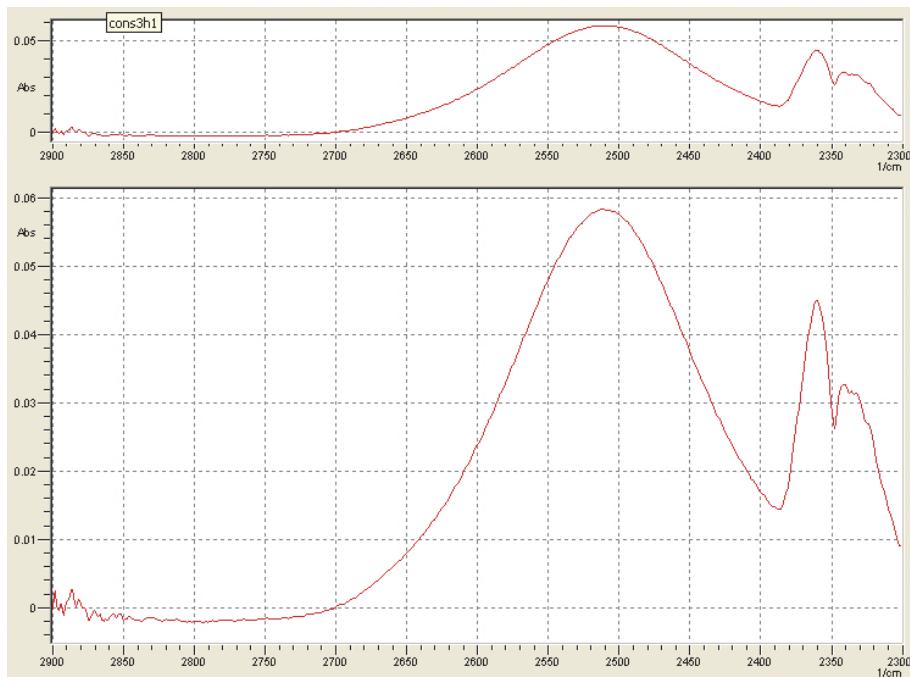


FIG. 18. Typical FTIR spectrum from an enriched water sample after background correction.

The dynamic range of the FTIR spectrometer for deuterium analysis is much greater than the concentrations likely to be encountered in studies of human milk intake and body composition, but enrichments below approximately 100 mg/kg (ppm) ^2H should be interpreted with care.

5.4.1. Typical FTIR spectra

Atmospheric CO_2 causes a sharp doublet on the shoulder of the D_2O signal. These peaks can be either positive (Fig. 18) or negative (Fig. 19). Negative peaks are obtained if there is a lower CO_2 concentration present in the sample compartment when the enriched sample is scanned than when the background was scanned.

The large peak at 2504 cm^{-1} is the D_2O peak. The double peak at 2350 cm^{-1} is due to CO_2 in the sample chamber.

Precautions must be taken to minimize the size of the CO_2 peak on the tail of the D–O peak.

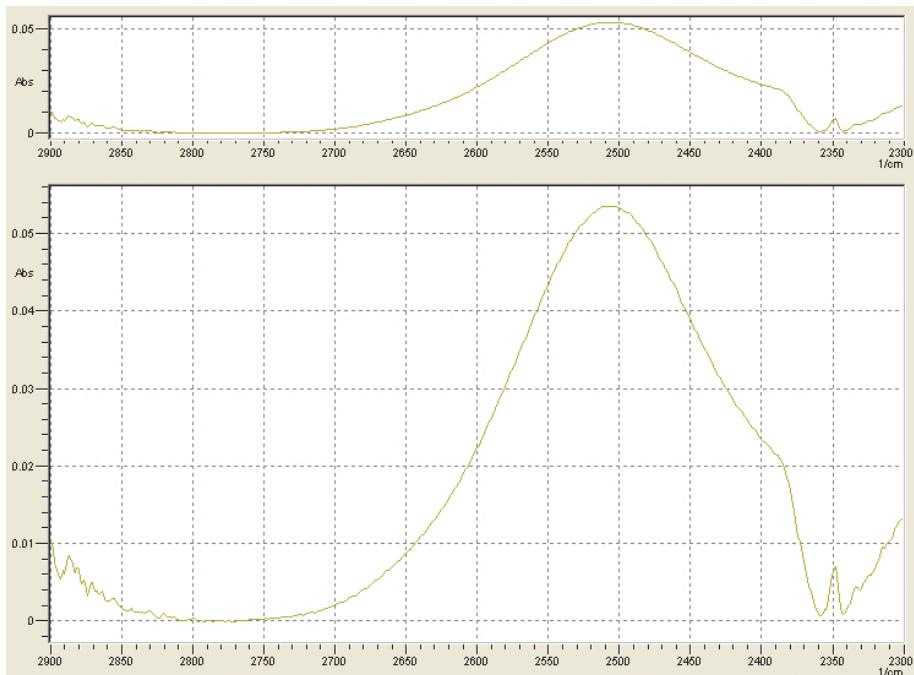


FIG. 19. Typical FTIR spectrum after background correction showing negative CO₂ peak.

- The FTIR instrument should be placed in a well ventilated or airconditioned room;
- The cells should be filled carefully, as described in Section 5.6, to avoid air bubbles.
- Samples containing bubbles should not be analysed. They should be flushed out by adding more sample.

5.5. THE SEALED CELL ASSEMBLY

Calcium fluoride cells with a cell thickness (path length) of 10^{-4} m ($100\text{ }\mu\text{m}$) are recommended for analysis of deuterium in saliva samples. These cells cannot be used for the analysis of urine samples, because they are damaged by the ammonium and phosphate content of urine. Figure 20 shows a schematic diagram of the FTIR cell. A demountable cell is also available. The cell is illustrated in Fig. 21. The procedure for filling the cell is described in Section 5.6. Sodium chloride cells, which are often supplied with the FTIR instrument, are not suitable for analysis of samples containing water.

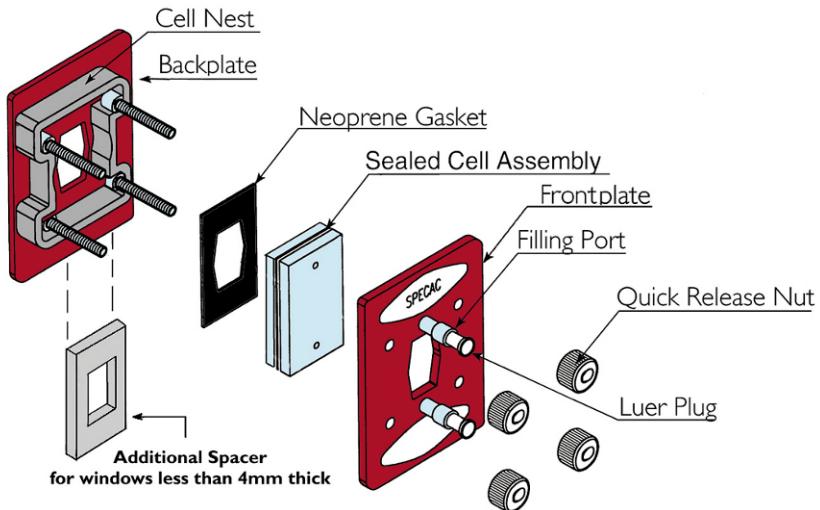


FIG. 20. Schematic diagram of the sealed cell assembly. (Copyright Specac Ltd., United Kingdom. Reproduced with permission.)



FIG. 21. FTIR cell with a 1 mL syringe in the filling port. The cell is raised at one end by propping on any suitable object, such as a pencil.

5.5.1. Care of the cells

When not in use, the cells should be stored in their original packaging. They should be wiped only with a lint free cloth. Slight scratches and other imperfections can be removed from the cell windows using a commercially available polishing kit (available from the supplier of the cells). To test for flatness after polishing, an optical flat can be used. This is usually provided with the kit.

5.6. FILLING THE FTIR CELL

The cells are filled using a 1 mL disposable syringe (Fig. 21).

5.6.1. Introduction

- Saliva samples must be completely thawed before analysis.
- The vials should not be opened until it is time to fill the FTIR cell, in order to prevent loss of water by evaporation, and hence fractionation of the specimen.
- The vials containing specimens of saliva should be centrifuged for at least 10 min at 1000g (with the caps on) to move any condensation in the lid down into the bulk of the specimen, and to remove bubbles.
- When filling the FTIR cells, it is important not to have bubbles in the sample. Bubbles cause the light to be scattered, resulting in a severely distorted baseline.
- The window of the cell should be cleaned with lens tissue before starting.
- The capacity of the cell is approximately 150 µL. By pushing through 1 mL saliva or reference water, traces of the previous sample are removed.

5.6.2. Recommended procedure for filling the FTIR cell

- (1) A 1 mL syringe should be filled with the sample (standard or saliva).
- (2) Folded absorbent paper should be firmly pressed over the exit port to absorb excess sample and prevent ingress of air.
- (3) The cell should be filled by gently pushing the syringe plunger or using firm taps on the plunger with the index finger.
- (4) Excess/splashes from the outside of the cell window should be removed by using absorbent paper.



FIG. 22. Procedure for filling the cell. The cell should be filled using a 1 mL syringe, checked for bubbles by holding the cell up to a light, and placed in the sample chamber of the FTIR.

- (5) Check for bubbles by holding the cell up to a light (Fig. 22).
- (6) If there are visible bubbles in the cell, add more sample as described above until all of the bubbles have been pushed out.
- (7) The absorbance should be measured from 2300–2900 cm⁻¹.
- (8) The sample should be removed using the same syringe that was used for filling. After that, the syringe should be discarded.
- (9) A new syringe should be used for each sample to avoid cross-contamination.
- (10) For the next sample, step 1 should be repeated.
- (11) When all the samples have been analysed, the cell should be rinsed with drinking quality water before storing.

6. CALCULATION OF BODY COMPOSITION

The dilution space of ²H (V_D) is 4.1% higher than TBW due to exchange of H with non-aqueous H in the body:

$$\text{TBW (kg)} = V_D / 1.041$$

where

$$V_D \text{ (kg)} = \text{Dose D}_2\text{O (mg)} / \text{enrichment } ^2\text{H in saliva (mg/kg)}$$

The hydration of FFM is assumed to be 73.2 % in adults:

$$\text{FFM (kg)} = \text{TBW (kg)} / 0.732.$$

Lohman [16] or Fomon [17] hydration factors in children should be used (see Tables 1 and 2, Section 2.4.1).

Fat mass (FM) is calculated by the difference between body weight and FFM:

$$FM \text{ (kg)} = \text{body weight (kg)} - FFM \text{ (kg)}.$$

Results are often expressed as % body weight:

$$FM \text{ (\%)} = FM \text{ (kg)} / \text{body weight (kg)} \times 100.$$

6.1. EXAMPLE CALCULATIONS

Table 6 shows example data from two adults with the same BMI, but different body composition. An example of data from a child is also shown. The dose of deuterium oxide consumed is lower in children than adults. The hydration of FFM appropriate for the age of the child is used.

TABLE 6. EXAMPLE CALCULATIONS

	Adult 1	Adult 2	Child
Study ID	A001	A002	Child1
Study date	15 Aug. 2007	15 Aug. 2007	15 Aug. 2007
Date of birth	9 Sep. 1944	29 Apr. 1979	1 Apr. 2000
Age (years)	62	28	7
Body weight (kg)	90	90	25
Height (cm)	180	180	130
BMI (kg/m^2)	27.8	27.8	14.8
Dose weight (g) = Weight D_2O (g) consumed	30.03	29.99	10.05
Dose weight (mg) = Dose (g) $\times 1000$	30030	29990	10050
${}^2\text{H}$ enrichment in saliva (mg/kg)	674	498	610
${}^2\text{H}$ pool space (V_{D} , kg) = Dose (mg) $/ {}^2\text{H}$ conc. (mg/kg)	44.6	60.2	16.5
Non-aqueous exchange factor	1.041	1.041	1.041

TABLE 6. EXAMPLE CALCULATIONS (cont.)

	Adult 1	Adult 2	Child
Total body water (kg) = V_D (kg)/1.041	42.8	57.8	15.8
Hydration factor (adults 0.732; child age 7 female 0.776)	0.732	0.732	0.776
FFM (kg) = TBW (kg)/hydration factor	58.5	79.0	20.4
Fat mass (kg) = Body weight (kg) – FFM (kg)	31.5	11.0	4.6
Fat mass (% body weight) = FM (kg)/ body weight (kg) × 100	35.0	12.2	18.4
FFM (% body weight) = FFM (kg)/body weight (kg) × 100	65.0	87.8	81.6
TBW (% body weight) = TBW (kg)/ body weight (kg) × 100	47.6	64.3	63.3

7. QUALITY CONTROL ISSUES

7.1. INSTRUMENT CALIBRATION

All analyses are compared with natural abundance and enriched water standards of known deuterium content. The FTIR instrument is calibrated daily at the beginning of each batch of samples. The standards (0 and 1000 mg/kg) are analysed as samples at the beginning and end of each working day. The full standard curve should be analysed when the instrument is new and when the instrument has been out of use for some time before starting to analyse samples.

7.2. ANALYTICAL PRECISION

Analysis of replicate samples can be used to estimate analytical precision. When using FTIR spectrometry precision (CV) of <1% should be routine. CV = coefficient of variation = SD/mean × 100.

7.3. MEASUREMENT OR ASSAY VARIATION

Comparison of TBW calculated from each post-dose sample can be used to estimate measurement or assay variation, which includes equilibration, sampling, handling and analytical precision. The 3 h and 4 h post-dose saliva samples should give values that are within 2% of their mean.

If the difference is more than 5%, it is possible that the dose was not properly equilibrated at the time of the first post-dose sample. The measured enrichment will be more than 100 mg/kg higher in the first sample than the second (Fig. 2), due to the early phase of overshoot where the deuterium enrichment in saliva appears above the eventual plateau, as the deuterium oxide has not fully equilibrated with intracellular fluid. The second post-dose sample should be used to calculate TBW in these circumstances. If this occurs regularly in the pilot study, it is advisable to sample at a later time point, e.g. 4 and 5 hours post-dose.

7.4. SCREENING THE DATA

The data should screened as follows:

- The equivalence of deuterium enrichment should be checked in the first and second post-dose samples (see above).
- The enrichment of deuterium in saliva should be between 600 and 1200 mg/kg, depending on body size. The body of large people contains a larger volume of water than the body of small people. If all participants receive the same dose, it will be diluted more in large people than in small people, and therefore the enrichment of saliva will be higher in smaller people.
- If the enrichment is <600 mg/kg and consistent between the first and second post-dose samples, there may have been a problem with dosing, unless the participant is an unusually large person. The calculated body fat will be very low, and the data must be rejected.

7.5. IDENTIFYING OUTLIERS

Measured TBW can be compared with a predicted value using the method of Bland and Altman [22] and the data flagged for checking or reanalysis if outside a normal range. If no other prediction is available, the relationship with height³ can be used: $TBW = 7.4 \times \text{height}^3$ (m³), validated in children and

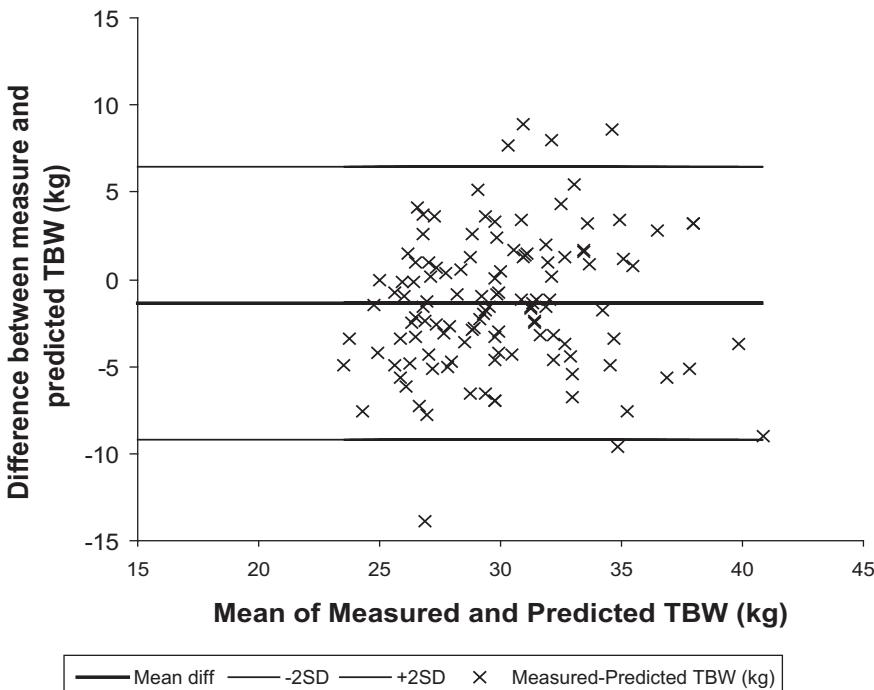


FIG. 23. Bland–Altman (residual) plot of TBW measured by deuterium dilution and TBW estimated from height.

adults [23]. If the measurements fall outside the 95% confidence intervals of this relationship, the data and calculation should be checked and the samples re-analysed if necessary. However, one would expect 2.5% of measurements to be more than +2SD from the mean difference (in obese participants with high BMI) and 2.5% to less than the mean difference minus 2SD (in participants with low body-mass index (BMI)).

An example is shown in Fig. 23. TBW was assessed in 120 adults with HIV using the method described in this manual. The mean difference between TBW calculated from deuterium enrichment and TBW estimated from height was 1.4 kg. The 95% confidence interval for the difference was -9.1 to +6.4 kg. The participant with a difference between measured and predicted TBW of -14 kg had a BMI of 12.2. At extremely low BMI values, body composition estimated from TBW should be interpreted with caution, as the assumptions regarding the hydration of FFM may not be valid.

8. SUMMARY OF CRITICAL STEPS FOR GOOD QUALITY DATA

DOSE PREPARATION

- Doses should be weighed to the correct precision (at least 0.01 g). This should preferably be undertaken by trained scientists in the analytical laboratory.

IN THE FIELD

- Well trained field workers can help with anthropometry, dose administration and collection of saliva samples, but it is important that they appreciate the importance of care in making measurements of height and weight, and accurate record keeping. Training of field workers is therefore crucial.
- Participants should have normal food and fluid intake the day before the measurement and should not take part in strenuous activities after the final meal of the previous day to avoid dehydration and depletion of glycogen stores.
- Participants should not eat or drink for 30 min (15 min for infants) before saliva sampling.
- Participants should be weighed wearing minimal clothing (to 0.1 kg); the weight of any clothing worn during the measurement must be subtracted from the measured weight to give body weight.
- Before opening, the dose bottle should be inverted a few times to mix in condensation on the lid.
- The bottle should not be opened until it is time for the dose to be consumed.
- Make sure that 100% of the dose is consumed by adding water to the bottle and asking participants to also drink this.
- Sampling time: Enough time should be allowed for tracer equilibration (3–4 h). In elderly and sick participants, 4–5 h should be allowed.
- Sample vials should be labelled with the participant's ID, time and date.
- All data should be recorded on a patient information sheet.
- Data should be transferred to a spreadsheet, e.g. Microsoft Excel, as soon as possible.
- Paper records should be kept as a backup.

IN THE LABORATORY

- The FTIR instrument should not be moved after it has been installed. If it is necessary do so, an engineer must check the alignment of the mirrors.
- The instrument should be placed in a well ventilated room.
- The humidity in the FTIR instrument should be less than 60%. The desiccant in the FTIR chamber should be changed when the indicator changes colour. This could be once a week in humid climates.
- Saliva samples should be properly thawed before analysis.
- Specimen vials containing saliva should be inverted to mix in condensation on the lid.
- The saliva samples should be centrifuged at 1000g for 10 min before analysis to ensure that any droplets in the lid are mixed into the bulk of the specimen, and to remove air bubbles that interfere with the FTIR analysis.

9. FREQUENTLY ASKED QUESTIONS

- Q. Why did I get a negative value for % body fat?
- A. Negative values occur when the deuterium dose has not had sufficient time to fully equilibrate with body water, or the dose was not completely consumed. This is seen as low deuterium enrichment, resulting in an overestimation of the size of the body water pool and hence high FFM and low % body fat.
- Q. How soon can I repeat a measurement?
- A. For adults, it takes about five weeks for the D₂O dose to wash out of the body water and the concentration of deuterium to return to baseline levels in tropical regions, and ten weeks in temperate regions. However, since body water is calculated from the difference in deuterium concentration between the pre-dose and post-dose specimens, it is not necessary to wait for this length of time before repeating a measurement using the equilibration method, which takes only a few hours. A second baseline specimen should be collected on the day of the repeat measurement. When the time between measurements is short, water intake between the time of the dose and final saliva specimen on the day of the repeat measurement should be minimized. Water intake at this time will have a larger dilution effect during the repeat measurement than during the first measurement.

- Q. Why is it necessary to collect two samples at the plateau enrichment?
- A. It is necessary to collect two samples after the dose has equilibrated to ensure that the plateau enrichment has been reached. Two samples with the same enrichment (within 2%) confirm that the dose has fully equilibrated with body water. Occasionally, in participants with slow water turnover (e.g. the elderly or people with certain diseases), the dose will not have fully equilibrated with body water at 3 h. The 4 h sample will confirm this and the enrichment in that sample can be used to calculate TBW. If there is only one post-dose sample, it is not possible to be certain whether the plateau enrichment has been reached. Do a pilot study to confirm the equilibration time under your local conditions before commencing the main study. If the equilibration time is longer than 3 h, it will be necessary to collect samples at 4 and 5 h post-dose. Consult with more experienced people to evaluate the results of the pilot study.
- Q. Is it necessary to fast during the protocol?
- A. Fasting during the protocol gives a more accurate estimate of TBW. However, in some circumstances this is difficult, e.g. participants who are ill and lactating mothers. In these circumstances, the participant can be given a small meal 1 h after the dose was consumed. Keep a record of the volume of fluids taken and subtract this from the calculated TBW. Participants should not eat or drink anything for 30 min before saliva is sampled.

Appendix I

GENERAL INFORMATION ON THE SAFETY OF DEUTERIUM OXIDE

I.1. ISOTOPES OF HYDROGEN

An atom consists of a central nucleus composed of neutrons and protons, which is surrounded by electrons that orbit around the nucleus. Protons carry a positive charge of 1 and have a mass of about 1 atomic mass unit (amu). Neutrons are electrically neutral and have a mass of about 1 amu. Electrons carry a negative charge of 1 and have a mass of 0.000 55 amu.

Atoms with different numbers of protons are called elements. For example, hydrogen has one proton, carbon has six protons and oxygen has eight. Isotopes of an element have the same number of protons and different number of neutrons. Stable isotopes are not radioactive and are present naturally in the environment, including in the human body, in proportions known as the ‘natural abundance’ of the isotope. Most elements are a mixture of various stable isotopes. All atoms of an element have the same number of protons in their nucleus, while the number of neutrons may differ if more than one stable combination is possible. Stable isotopes of several elements (carbon, hydrogen, oxygen and nitrogen) have been used extensively in biomedical research.

Hydrogen consists of a nucleus with one proton (which is positively charged) and one electron (which is negatively charged). One proton represents mass 1 and thus the mass of hydrogen is 1; this stable isotope is also called protium. In deuterium, a heavier stable isotope of hydrogen, the nucleus contains one proton and one neutron (which has no charge and represents mass 1). Thus, the mass of deuterium is 2. The mass of an element is often shown at the top left of the letter indicating the element. Thus, hydrogen is ^1H and deuterium is ^2H . Deuterium is also commonly indicated as D. Deuterium was discovered in 1932.

Hydrogen has one proton in the nucleus	^1H (stable isotope)
If one neutron is present in the nucleus, this is deuterium	^2H (stable isotope)
If two neutrons are present in the nucleus, this is tritium	^3H (radioactive isotope)

The natural abundance of deuterium is 0.015%. This means that an adult woman weighing 55 kg with 30 kg of body water contains about 4.5 g of deuterium in her body water.

Deuterium oxide is water ($^2\text{H}_2\text{O}$) in which 99.8 or 99.9% of the hydrogen atoms are in the form of deuterium. This is referred to as 99.8 (or 99.9) atom% $^2\text{H}_2\text{O}$ or as D_2O . Deuterium oxide can be used to measure the size of the body water pool (total body water) by isotope dilution, and the flow of water from one pool to another (e.g. from the mother's body water to the baby's body water in human milk).

I.2. DEUTERIUM OXIDE SAFETY

Stable isotopes have been used in human metabolic studies for over half a century. Although stable isotopes of hydrogen do not emit potentially harmful radiation, the mass of deuterium is 2 (^2H) and the mass of hydrogen is 1 (^1H). The mass difference between deuterium and hydrogen (a factor of 2) is greater than for any other stable isotopes of the same element. This mass difference may cause significant 'isotope effects' at very high concentrations (>15%) of deuterium oxide in the tissues.

Isotope effects are caused by the fact that the presence of deuterium in a molecule shortens covalent bonds making them stronger and more resistant to breakage. Therefore, molecules containing deuterium display slightly different reaction rates than those containing only hydrogen. The difference in rate constants between a reaction involving a molecule containing only hydrogen and that involving a molecule containing deuterium is known as the 'kinetic isotope effect', and can occur during enzyme catalysed reactions in the body.

Animal studies have shown that tissues containing more than 15% of deuterium labelled water exhibit a multitude of effects, including impaired protein and nucleic acid synthesis, altered conformation and stability of biopolymers, altered rates of enzymatic reactions, impaired cell division and morphological changes [24]. The overall effect of deuterium labelling appears to be a depression of tissue metabolism due to lower reaction rates of deuterium labelled compounds *in vivo*. While some toxic effects of deuterium labelling are reversible, very high concentrations may prove lethal.

In mammals, concentrations of deuterium of below 15% have not been associated with harmful effects. Levels of deuterium labelling of 15% must be maintained by continual dosage before adverse effects become evident [24]. However, lesser effects, such as transitory episodes of vertigo, have been reported in human adults consuming an amount of deuterium oxide sufficient to enrich body water to 0.35–0.65 % [24]. It has been suggested that a threshold for

noticeable transitory side effects exists when body water is enriched above 0.2%. The threshold of deuterium toxicity has been defined as 15% and is far in excess of concentrations conceivable for use in human studies [24]. The amount of deuterium consumed in studies of human milk output and body composition enrich body water to a maximum in the region of 0.1% in the mother and less than half of this in her baby. At this level, no adverse side effects have been reported.

Appendix II

FOURIER TRANSFORM INFRARED SPECTROMETRY

The enrichment of deuterium in saliva samples can be measured by FTIR spectrometry. Practical aspects of using it, including preparation of standards and filling cells, are described in Section 5. This appendix gives an introduction to the principles of FTIR spectrometry.

II.1 PRINCIPLES OF FTIR

Absorbance in the middle of the infrared region of the electromagnetic spectrum is due to molecular vibrations. Water exhibits three vibrational modes, which can be regarded as modes of vibration of the O–H bond (Fig. II.1).

The energy of vibration depends on the mass of the atoms between which the bond is made. The effect of substitution of deuterium for hydrogen is a shift to a lower energy (Fig. II.2).

Peak positions are commonly expressed in terms of wave number (cm^{-1}), frequency (THz), or wavelength (μm) (Fig. II.3). The peak due to D_2O is at 2504 cm^{-1} (75.07 THz or 3.994 μm).

An FTIR instrument is composed of a source of infrared radiation, a beam splitter, two mirrors (one fixed and one moving) and a detector (Fig. II.4). The beam splitter and mirrors make up the interferometer. One of the mirrors is fixed, while the other is mounted on an assembly, which is designed to move back and forth at constant velocity (the moving mirror). Radiation from the source is

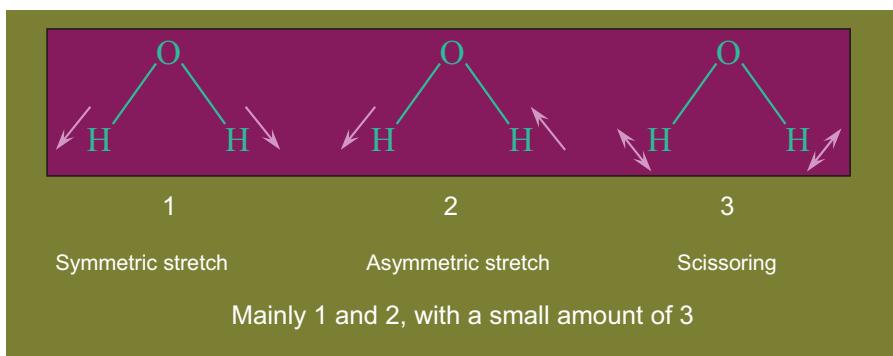


FIG. II.1. Modes of vibration of the O–H bonds in water.

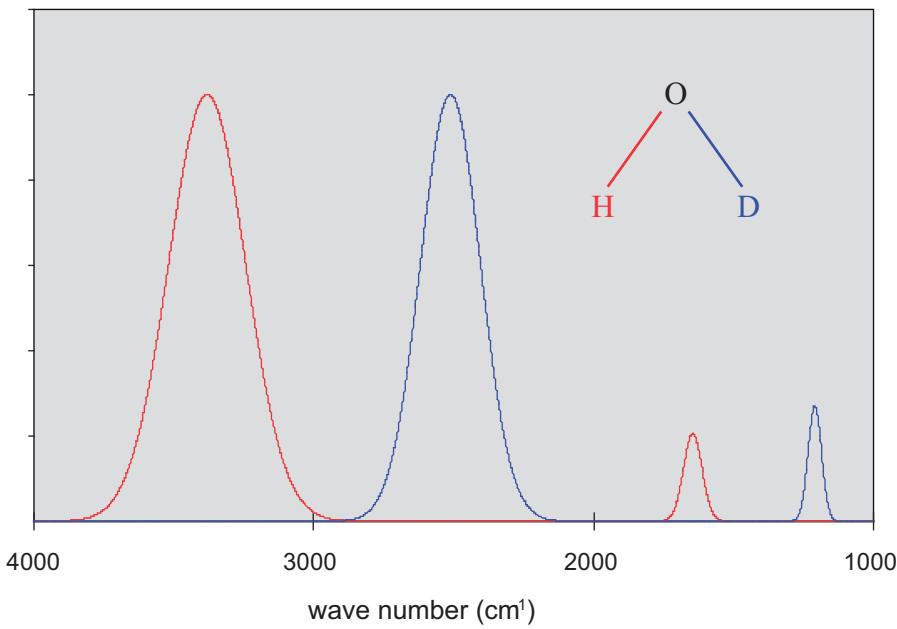


FIG. II.2. Schematic diagram of the infrared spectrum due to O–H and O–D bonds.

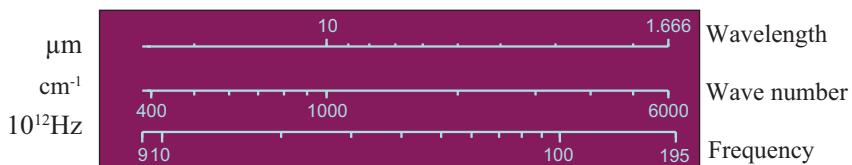


FIG. II.3. Comparison of wavelength, absorbance and frequency. (Note: As energy increases, frequency and wave number increase, but wavelength decreases.)

directed towards the beam splitter. This is a semi-transparent/semi-reflective material, which reflects half the incident radiation towards the fixed mirror and transmits half towards the moving mirror. After reflection from the mirrors, the two beams are recombined at the beam splitter and their sum passed through the sample and focused on the detector. When the beams are recombined, they interfere. As the mirror moves, the interference pattern changes from constructive to destructive and back cyclically (Fig. II.5).

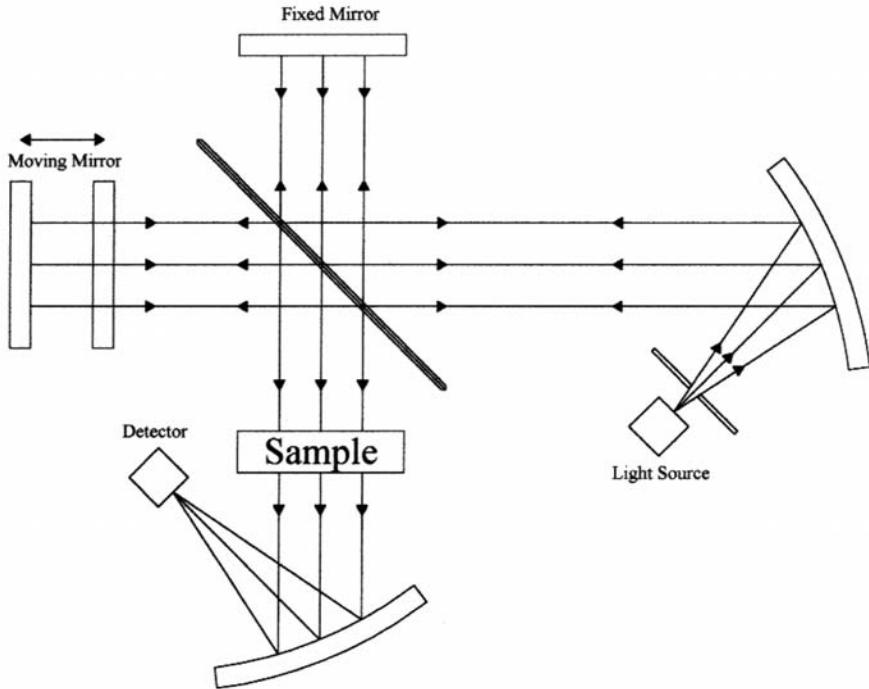


FIG. II.4. Schematic diagram of an FTIR instrument.

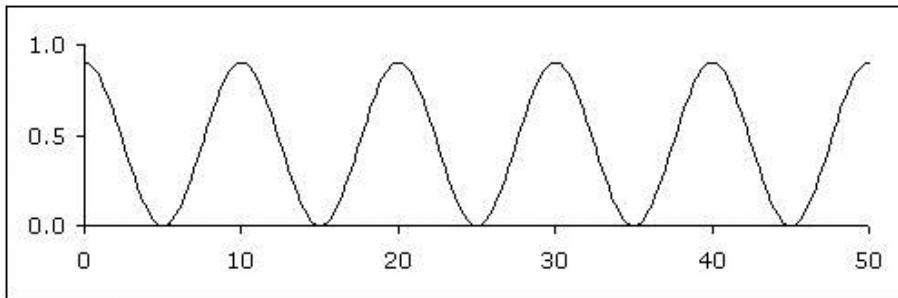


FIG. II.5. Cosine wave formed from the recombined beam from a single wavelength.

This occurs for all the different wavelengths of light simultaneously. These are summed to give the total output or interferogram (Fig. II.6).

If the output from the interferometer is passed through a sample, which absorbs some of the frequencies more than others, the amplitudes of the individual cosine waves will be different, and the interferogram will be modified accordingly.

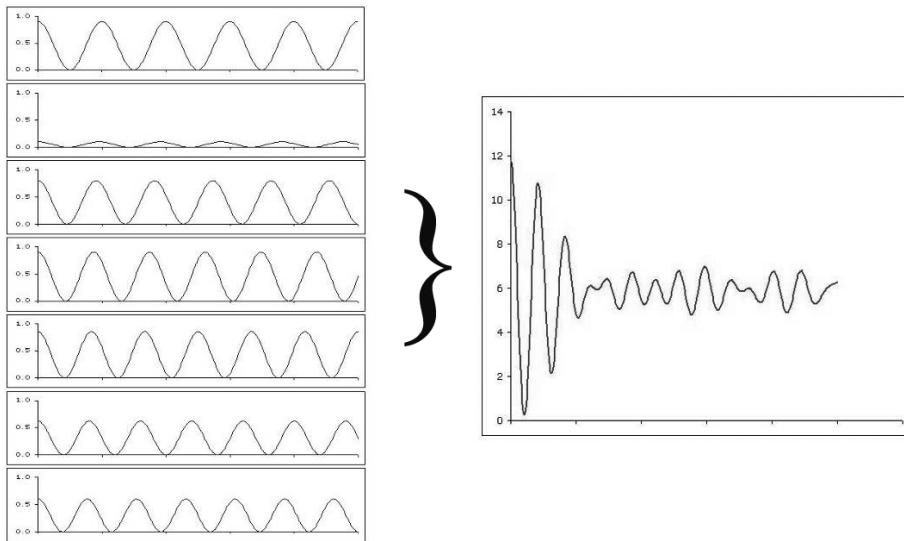


FIG. II.6. Representation of interferogram formed from seven different wavelengths.

The detector in an FTIR spectrometer is usually a pyroelectric material, such as triglycine sulphate (TGS), which has the property of producing an electrical signal if its temperature changes. The output from the detector is converted into a time varying voltage, which is an accurate representation of the total intensity of light passing through the sample. This is transformed back to the usual kind of spectrum mathematically, by the process of Fourier transformation.

As the fraction of deuterium is very small (Fig. II.7), approximately 1000 mg/kg (ppm) and the dynamic range of the detectors is not great enough to allow accurate measurement of the intensities of the peaks due to O–H and O–D in the same sample, only the intensity of the O–D peak is used, and the concentration of deuterium is estimated using the Beer–Lambert law. This law states that:

“for a parallel beam of monochromatic radiation passing through a homogeneous solution, the amount of radiation absorbed (A) is proportional to the product of the concentration (c) and pathlength (l)”:

$$A \propto c l$$

$$A = \epsilon c l$$

$$c = A/\epsilon l$$

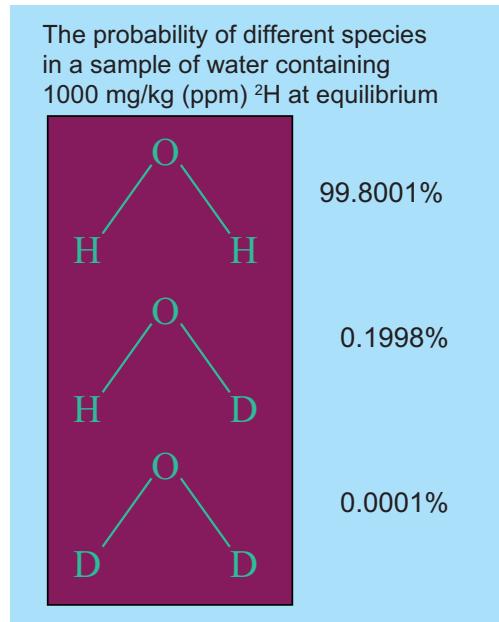


FIG. II.7. Deuterium content of a sample of water containing 1000 mg/kg (ppm) ^2H .

where ε is known as the extinction coefficient.

For D–O, the extinction coefficient at 2504 cm^{-1} , $\varepsilon_{2504} = 7150 \text{ M}^{-1} \text{ m}^{-1}$, and for quantitation, a cell thickness (path length) of 10^{-4} m ($100 \mu\text{m}$) is used.

At the low levels of deuterium encountered, the O–D signal appears as a small peak superimposed on the tail of the much larger peak due to the O–H bond (Fig. II.8). In addition, atmospheric CO_2 causes a sharp doublet on the shoulder of the O–D signal. This makes the estimation of the baseline under the O–D peak difficult when using air as a background reference. This difficulty is averted by using a sample of local drinking water as a background reference, removing much of the O–H background and revealing the O–D peak at 2504 cm^{-1} (Fig. II.9). The absorbance from the enriched samples is automatically corrected for the background by the instrument software. When analysing saliva samples, the baseline sample is used for the background correction.

A mathematical method for comparing the spectrum of the calibrant and sample, which automatically fits baselines to the peaks, has been described in the literature [6]. This method is now available as a programme¹ which runs under Microsoft Windows on the same data system as supplied with the instrument, and operates directly on text files generated by the FTIR vendor. Since most

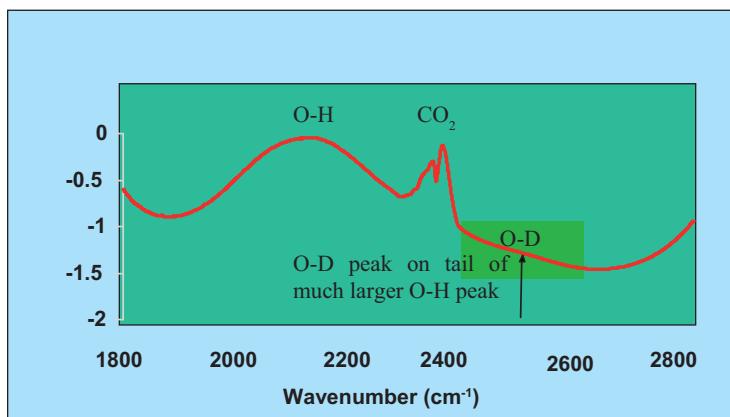


FIG. II.8. FTIR absorbance spectrum from water containing 1000 mg/kg (ppm) ²H using air for the background correction.

manufacturers export files in slightly different formats from each other, the HNR software must be preconfigured to match the instrument used. Versions are available for use with instruments made by Thermo, Unicam and Shimadzu.

II.2 UNITS

In FTIR spectrometry, enrichment is usually expressed as the concentration of deuterium in parts per million (ppm) by weight (mg/kg), above the amount naturally present:

The enrichment entered into the ‘isotope.exe’ software should be in mg/kg.

Note: The units of enrichment in IRMS are at.% excess ²H, also sometimes reported as ppm excess ²H. These parts per millions are a molar ratio, ppm (mol/mol), and not a weight ratio (mg/kg). The two kinds of ppm are not the same and are not interchangeable. This affects the calculation of the pool space. For example, when calculating TBW using FTIR data, the dose (g deuterium oxide) is

¹ The software comprises two files ‘isotope.exe’ and “vbrun300.dll”: the former is the executable (program) file that has been specially written by HNR; the latter is the Visual Basic runtime library. Both of these files need to be copied to the computer. They can be obtained from the Medical Research Council Collaborative Centre for Human Nutrition Research web site (www.mrc-hnr.cam.ac.uk). MRC-HNR, Elsie Widdowson Laboratory, Fulbourn Road, Cambridge CB1 9NL, United Kingdom. Telephone (+44 1223) 426357.

converted into mg and total body water is in kg. However, when calculating TBW using IRMS data, it is necessary to convert the weight of deuterium oxide consumed to moles. TBW will then be in moles and must be converted to kg. Calculating TBW from FTIR data is much simpler than from IRMS data. It is important to ensure that spreadsheets for calculating TBW contain the correct calculations, depending on the method used to analyse deuterium enrichment.

Appendix III

SAMPLE DATA SHEET FOR TBW ESTIMATION BY DEUTERIUM OXIDE DILUTION

Person performing the test: _____ Date: ____ / ____ / ____
Day Month Year

I. Participant

Name: _____ Code/ID: _____

Weight: ____ . ____ kg Height/length: ____ . ____ cm BMI ____ kg/m²

Date of birth: ____ / ____ / ____ Age: ____ years Gender: M F

Healthy: YES NO

Notes (health): _____

II. Dose

Dose bottle number: _____

Dose weight: ____ . ____ g

Did the participant fast overnight? YES NO

If not, how long was the fast before the dose? _____

Was the container opened just before the dosage? YES NO

Was the dose consumed correctly? YES NO

If not, what was the weight of the dose not consumed. ____ . ____

The container was rinsed with 2 × 50 mL water. YES NO

The same straw was used. YES NO

Notes: _____

III. Specimen times

Time of baseline saliva sample: ____ : ____

Time dose was taken: ____ : ____

Post-dose saliva samples:

3 hours: ____ : ____

4 hours: ____ : ____

5 hours: ____ : ____

Appendix IV

LIST OF EQUIPMENT

In the laboratory

- Deuterium oxide (99.8 or 99.9 at.% ^2H);
- Dose bottles (screw cap, leak proof, e.g. 60 mL wide mouth, polypropylene leak-proof, autoclavable bottles);
- Labels for dose bottles;
- Permanent ink pens for writing on labels;
- Glass measuring cylinder to transfer 30 mL $^2\text{H}_2\text{O}$ to dose bottles;
- Glass or plastic funnel;
- Electronic balance weighing to 0.01 g for weighing doses;
- Electronic balance weighing to 0.0001 g for making calibration standard;
- Refrigerator for storing doses;
- Freezer (-20°C) for storing saliva samples;
- Voltage stabilizers for all electronic equipment (electronic balances/FTIR instrument);
- Centrifuge with buckets to take sample vials, ideally refrigerated;
- FTIR instrument;
- Calcium fluoride cells for FTIR instrument;
- 1 mL disposable plastic syringes with Luer tip for filling the FTIR cell;
- Paper tissues/absorbent paper;
- Lens paper to clean window of FTIR cell;
- Volumetric flasks (1 L, 100 mL and 50 mL) for making calibration standards;
- Automatic pipettes plus tips (1mL, 200 μL , 20 μL) for making calibration standards;
- Wash bottle for filling volumetric flasks;
- Two borosilicate glass reagent bottles (1 L) with PTFE lined screw caps for storing calibration standard and sample of local drinking water used to make calibration standard;
- 100 or 250 mL borosilicate glass bottles with PTFE lined screw caps for the aliquots of the calibration standard and local drinking water used as ‘working standards’ on a daily basis.

In the field

- Doses (prepared in the laboratory);
- Drinking water;

- Drinking straws;
- Balance weighing to 0.1 kg for weighing participants;
- Stadiometer for measuring participant's height;
- Saliva sampling vials with screw cap (e.g. 4 mL internal thread self-standing cryovials);
- Labels for sample vials;
- Permanent ink pens for writing on labels;
- Zip-lock bags for storing saliva samples;
- Cotton wool balls for saliva sampling;
- 20 mL plastic syringes;
- Disposable gloves;
- Plastic bags/boxes for storing/transporting saliva samples;
- A watch (to note time of saliva sampling);
- A refrigerator for storing doses if working in the field for several days without returning to base;
- A cool box with an ice pack for storing samples in the field until they can be frozen.

Appendix V

ISOTOPIC FRACTIONATION

Deuterium oxide ($^2\text{H}_2\text{O}$) is not identical to water with respect to its physical properties.

When deuterium oxide mixes with body water, three isotopic forms are found (Fig. V.1). For example, in a water sample containing 1000 mg/kg (ppm) deuterium oxide, the probability of any particular H being ^2H is 0.001 and the probability of it being ^1H is 0.999.

For any molecule of water, the probability of both H being ^1H ($^1\text{H}-\text{O}-^1\text{H}$) is:

$$P(^1\text{H}-\text{O}-^1\text{H}) = 0.999 \times 0.999 = 0.998001 \text{ or } 99.8001\%.$$

The probability of both H being ^2H ($^2\text{H}-\text{O}-^2\text{H}$) is:

$$P(^2\text{H}-\text{O}-^2\text{H}) = 0.001 \times 0.001 = 0.000001 \text{ or } 0.0001\%.$$

The probability of any particular water molecule containing one ^1H and one ^2H is:

$$P(^1\text{H}^2\text{HO}) = 2 \times 0.999 \times 0.001 = 0.001998 \text{ or } 0.1998\%.$$

The factor of 2 arises because there are two possible arrangements, $^1\text{H}-\text{O}-^2\text{H}$ and $^2\text{H}-\text{O}-^1\text{H}$, which are equivalent.

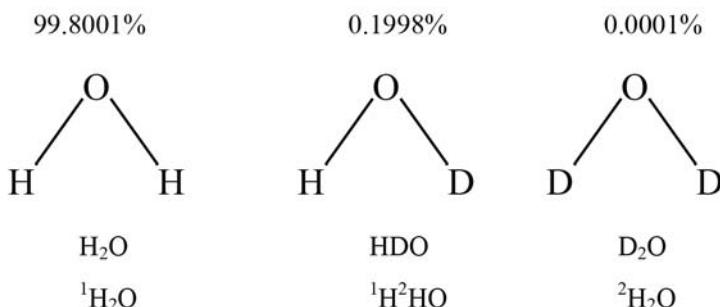


FIG. V.1. Abundance of different molecules in a water sample containing 1000 mg/kg D_2O per kg water.

The energy of the bond between deuterium (^2H or D) and oxygen (O) is slightly greater than the energy of the bond between hydrogen (^1H) and O. This can lead to isotopic fractionation when water undergoes a chemical or physical change. Isotopic fractionation of water occurs when water liquid becomes water vapour (gas).

There is less deuterium in water vapour than in the main volume of liquid water from which the vapour evaporated. The fractionation factor (f) for deuterium between water vapour (a gas) and liquid water is 0.941 at 25°C.

There is very little isotopic fractionation of water within the body. Plasma, urine, human milk and sweat show little fractionation. However, water leaving the body as water vapour in breath and transdermal evaporation contains less deuterium than body water. Transdermal evaporation is insensible water loss from the skin through routes other than the sweat glands. The effect of increased insensible water losses, which contain less deuterium than body water, is to concentrate the deuterium oxide left behind. This would lead to an underestimation of total body water and therefore an overestimation of body fat. For this reason, participants should not take part in excessive physical activity during the saliva sampling period.

Similarly, condensed water vapour on the caps of bottles used for storing doses, samples and standards contains less deuterium than the bulk of the liquid, and therefore bottles should be inverted or centrifuged to mix the contents before opening, and should not be left open to the atmosphere.

The following example (Fig. V.2) shows the effect of fractionation if 100 µL of condensation is clinging to the lid of a sample vial containing 4 mL saliva, which originally contained 1000 mg/kg D_2O .

The effect of fractionation is more pronounced when the volume of saliva is small. For example, if a vial containing 1 mL saliva is left open to the atmosphere and 100 µL evaporates, there will be only 900 µL (0.9 mL) left behind, which will contain 1006 mg/kg D_2O (Fig. V.3).

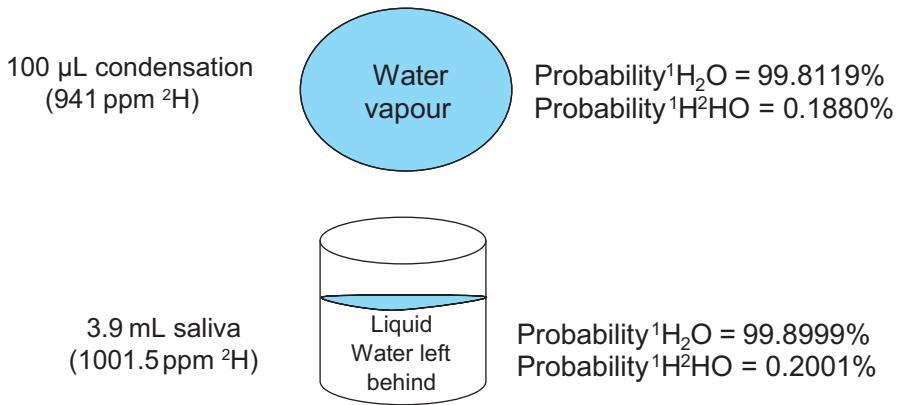


FIG. V.2. Effect of isotopic fractionation in 4 mL saliva sample originally containing 1000 mg/kg (ppm) D_2O .

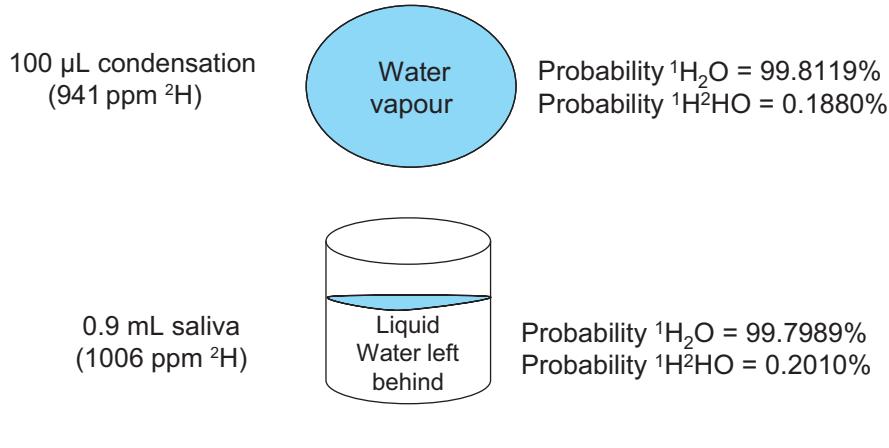


FIG. V.3. Effect of isotopic fractionation in 1 mL saliva sample originally containing 1000 mg/kg (ppm) D_2O .

GLOSSARY

atom per cent (at.%). The number of atoms of the stable isotope of interest expressed as a proportion of the total number of atoms of that element, e.g.

$$\text{atom \% } {}^2\text{H} = \frac{[{}^2\text{H}]}{[{}^1\text{H}] + [{}^2\text{H}] + [{}^3\text{H}]} \times 100.$$

In practice, the number of ${}^3\text{H}$ atoms is negligible, and is therefore ignored.

deuterium. The stable isotope of hydrogen with the symbol ${}^2\text{H}$, also abbreviated as D.

deuterium oxide. Water in which 99.8 or 99.9% of the hydrogen atoms are in the form of deuterium (${}^2\text{H}_2\text{O}$ or D_2O).

deuterium oxide dilution method of measuring TBW. A well established technique to measure total body water (TBW) from which body composition is estimated using a two compartment model, assuming the body is composed of fat and fat free mass (FFM). FFM is 73.2% water in healthy adults. $\text{TBW} \text{ (kg)} / 0.732 = \text{FFM} \text{ (kg)}$. Fat mass is calculated as the difference between FFM and body weight.

deuterium oxide dose to mother technique. A method of assessing human milk intake by breastfed infants, which involves giving a dose of deuterium oxide to the mother and measuring the rate of elimination in the mother and the rate of appearance in the baby. The amount of water consumed from sources other than human milk can also be estimated using this technique.

enrichment. Because stable isotopes are naturally present, baseline samples must be taken before administration of the labelled compound. Enrichment is the concentration of the isotope above the baseline level. The concentration of deuterium in body water (above the baseline level) can be measured by FTIR. As the background is automatically subtracted when deuterium concentration is analysed by FTIR, the output is enrichment in units of mg/kg.

equilibration. The hydrogen atoms on water molecules in the body are not permanently attached to the oxygen atoms, but are constantly exchanging: they are in a state of constant flux. When a person drinks a dose of deuterium oxide, it is not a simple matter of the deuterium oxide (${}^2\text{H}_2\text{O}$) mixing with water in the body. The deuterium atoms in ${}^2\text{H}_2\text{O}$ exchange with

hydrogen atoms in water molecules, so that after a few hours, the probability of finding a molecule of $^2\text{H}_2\text{O}$ is very low. Most water molecules are still in the form of $^1\text{H}_2\text{O}$, but a few are in the form of $^1\text{H}^2\text{HO}$ after exchange of ^1H with ^2H . This is the process of equilibration.

fat free mass. The term used in body composition studies to refer to the part of the body that is not fat. Fat free mass (FFM) includes water, protein, bone minerals and non-bone minerals. FFM contains 73.2% water in healthy adults [9], but the hydration of FFM is higher in children, the latter stages of pregnancy and certain clinical conditions.

Fourier transform infrared (FTIR) spectrometry. A technique used to measure deuterium enrichment in saliva samples from studies of body composition and human milk intake.

fractionation. Isotopic fractionation is the term used to describe the fact that molecules containing different isotopes display slightly different reaction rates. This can occur during physical changes such as evaporation. Water leaving the body as water vapour in breath contains less deuterium than body water. Similarly, condensed water vapour on the caps of bottles used for storing doses, samples and standards contains less deuterium than the bulk of the liquid. Therefore, bottles should be inverted to mix the contents before opening.

insensible water loss. Insensible water loss refers to water lost from the body in breath and transdermal evaporation, which is water lost from the skin by routes other than the sweat glands. Water leaving the body as water vapour contains less deuterium than liquid body water, due to fractionation. A correction is made for insensible water losses when water intake from sources other than human milk is estimated in breastfed infants using the deuterium oxide dose to mother technique.

isotopes. Elements with the same number of protons and different number of neutrons.

- **Hydrogen** has one proton in the nucleus ^1H (stable isotope);
- If one neutron is present in the nucleus, this is **deuterium** ^2H (stable isotope);
- If two neutrons are present in the nucleus, this is **tritium** ^3H (radioactive isotope).

isotope dilution. A known amount of a labelled compound is added to a biological system and mixes fully with that pool. The dilution of the labelled compound by endogenous unlabelled compound will give a measure of the size of the pool. This principle is the basis of the deuterium dilution method of measuring total body water.

isotope exchange. Deuterium (^2H) can exchange with hydrogen (^1H) atoms in water molecules and in other compounds. This is known as isotope exchange.

non-aqueous exchange: The process whereby isotopes in body water enter components of the body other than water is known as non-aqueous exchange. For example, deuterium exchanges with exchangeable hydrogen atoms (mainly $-\text{NH}$ and $-\text{OH}$) in body protein. Hydrogen isotopes are also sequestered into fat and protein as these are synthesized. Therefore, the volume of distribution, also known as the dilution space or pool space, of the tracer is slightly greater than TBW. The ^2H dilution space (V_D) is 1.041 times that of TBW. This is accounted for by dividing the calculated volume of distribution (V_D) by 1.041 to achieve TBW (kg).

radioactive isotope: Radioactive isotopes have unstable nuclei which emit ionizing radiation in the form of particles or waves. Radioactive decay is the process by which a nucleus releases energy and transforms to a lower energy state. Tritium is the radioactive nuclide of hydrogen. Tritium has a half-life of 12.35 years.

stable isotope. Stable isotopes are not radioactive, and are present naturally in the environment, including the human body, in concentrations known as the ‘natural abundance’ of the isotope. Hydrogen has two stable isotopes: ^1H or protium, the major stable isotope of hydrogen and ^2H or deuterium the minor stable isotope of hydrogen. Approximately 0.015% of hydrogen atoms in natural water are in the form of deuterium (^2H).

total body water (TBW). The term used to refer to the total water content of the body, which makes up 70–75% of body weight at birth, but decreases to 50–60% of body weight in lean adults and less than 40% in obese adults. The fat free mass is approximately 73.2% water in adults. Measuring total body water (TBW) establishes the amount of FFM. Fat mass is calculated as the difference between FFM and body weight. TBW includes both intracellular fluid (ICF) and extracellular fluid (ECF).

volume of distribution. The volume through which the isotope is distributed, also known as the pool space or dilution space. In studies of total body water by deuterium dilution, the volume of distribution (V_D) is larger than TBW due to non-aqueous exchange.

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