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No. 3

Assessment of Body Composition and Total Energy Expenditure in Humans Using Stable Isotope Techniques

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ASSESSMENT OF BODY COMPOSITION AND TOTAL ENERGY EXPENDITURE IN HUMANS USING STABLE ISOTOPE TECHNIQUES

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FOREWORD

Improved nutrition represents a high priority area as part of the development agenda in many countries. The IAEA assists its Member States in their efforts to develop effective, evidence based interventions to combat malnutrition in all its forms using nuclear techniques, in particular stable (non-radioactive) isotope techniques. Stable isotope techniques have been used as research tools in nutrition for many years. However, the application of these techniques in programme development and evaluation is a relatively new approach, one in which the IAEA has a unique opportunity to contribute technical expertise. The use of stable isotope techniques adds value by increasing the sensitivity and specificity of measurements as compared with conventional techniques.

There is a wide range of stable isotope techniques used in nutrition. However, the scope of this report is limited to two of the most widely used techniques with particular relevance to the development and monitoring of nutritional interventions to combat both under and overnutrition globally. The IAEA has fostered the more widespread use of these techniques in Member States by supporting national and regional nutrition projects through its technical cooperation programme and through coordinated research projects addressing priority areas in nutrition over many years.

This publication was developed by an international group of experts as an integral part of the IAEA's efforts to contribute to the transfer of technology and knowledge in this field among nutritionists, analytical chemists and other professionals. It provides information on the theoretical background as well as the practical application of state of the art methodologies to monitor changes in body composition and total energy expenditure, and reflects recent advances in analytical techniques.

The major contributors to this report are gratefully acknowledged for generously sharing their technical expertise and extensive experience in stable isotope techniques in nutrition (in alphabetical order): L. Bluck (United Kingdom), A. Hills (Australia), T. Preston (United Kingdom), D. Schoeller (USA) and C. Slater (United Kingdom).

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

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1. INTRODUCTION

The world is currently experiencing a nutrition transition [1] in which energy undernutrition coexists with energy overnutrition as a consequence of significant lifestyle changes. Both industrialised and developing societies are suffering the burden of obesity related to inactivity and an excess of energy consumption and co-morbid conditions including hyperlipidaemias, insulin resistance, type 2 diabetes, cardiovascular disease and cancers [2, 3]. There is also increasing evidence of the link between undernutrition in utero and obesity and chronic disease in later life [4]. In either under- or overnutrition, there is an increasing need for nutritional status methodologies that provide reliable results for risk assessment and for the evaluation of nutrition and physical activity intervention programmes. Such measures of nutritional status require assessment of body composition and energy expenditure. The evidence from both developing and industrialized countries suggests a fundamental link between maternal and early child undernutrition, and an increased susceptibility in adult life to non-communicable diseases (NCDs) such as diabetes, heart disease and hypertension. Overweight and obesity are rapidly growing in all regions, affecting children and adults alike.

The measurement of body composition provides an objective means of nutritional assessment. The most common approach in body composition assessment is to utilize the two compartment model, dividing body mass into body fat (FM) and fat free mass (FFM). Despite the large number of body composition measurement techniques available, only three primary techniques are widely recognized: densitometry, elemental analysis and total body water (TBW) measurement. Densitometry, as the name suggests, involves the estimation of body density. Compared with fat free tissue, body fat has a distinct and lower density. Accurate measurement of body weight and volume provides an accurate estimate of FM and by difference, FFM. Body density has conventionally been measured by underwater weighing [5], however the availability of commercial apparatus based on air displacement plethysmography [6] provides a simpler alternative for the measurement of a wide range of ages and population sub-groups [7]. Both water and air displacement approaches require individuals to be measured within laboratory facilities and therefore are not suitable for use in the field. Elemental analysis includes techniques such as total body in vivo neutron activation analysis [8–10] and total body potassium analysis (TBK) [11]. TBK analysis of naturally radioactive ^{40}K is the oldest of these techniques and the least invasive. As intracellular potassium is at constant concentration, TBK analysis provides an accurate estimate of intracellular fluid (ICF) and body cell mass [12]. The ratio

of TBK to FFM varies slightly with body size and disease state, which reflects altered FFM composition (changes in ICF versus extracellular fluid (ECF) space; and changes in muscle mass). The combined use of TBK and TBW analysis has long been recognized as a powerful technique to describe body composition changes in different pathological states [13]. More complex multi-compartment models of body composition [8, 9, 13–15] are used to describe changes in FFM quality that are especially useful in studies of pathological alterations in cellular and extracellular components of FFM.

Analysis of TBW is the third primary method of body composition analysis. TBW comprises some 73.2% of the FFM [16]. When combined with knowledge of FFM hydration, analysis of TBW leads to accurate FFM estimates. Body fat is then calculated as the difference between body mass and FFM. TBW can best be measured with accuracy using isotope dilution [16–18]. As early as 1977, Halliday and Miller [17] reported reproducibility of 0.5% (200 g in 40 kg TBW; adult male subjects). Similar reproducibility (170 g in 31.4 kg TBW) was also reported in a more recent study [19]. Such excellent precision was achieved in both cases within controlled metabolic studies and with analysis by isotope ratio mass spectrometry (IRMS). The data reflect the optimum situation where both biological and analytical noise are minimized. Precision will be eroded as field protocols are developed, yet these figures serve to illustrate the inherent ability of isotope dilution. Unlike other primary methods of body composition analysis, field protocols can be developed using minimal equipment, with appropriate fluid samples (urine; saliva) being returned to the laboratory for analysis.

TBW analysis has overcome past difficulties as stable isotope dilution (^{2}H or ^{18}O) can be applied with greater safety, precision and accuracy than using radioisotopes (^{3}H). Slow and insensitive early instrumentation has given way to modern spectroscopy and IRMS. The latter in particular can be undertaken with minimal dose. The result is that TBW measurement, using stable isotope labels, is clearly the most appropriate primary method of body composition analysis suitable for field use. Depending on the size of study, a less exacting technique such as anthropometry [20] or bioelectrical impedance analysis [21–25] may be chosen to assess the whole population, with validation against TBW in a representative sample. Alternatively, TBW analysis may be applied for nutritional assessment of the whole population under study [26, 27] as it is the most accurate technique of body composition for use in the field.

The measurement of energy expenditure involves, in principle, the energy balance equation: total energy expenditure (TEE) = energy intake (EI) minus change in body energy stores. The measurement of TEE by the doubly labelled water (DLW) technique can solve one side of the equation and also provides a means of validating other methods used for solving the other side of the

equation (energy intake) [28–34]. DLW is a mixture of stable isotope labelled waters (^2H_2O and $H_2{}^{18}O$). TBW is measured by isotope dilution as part of the DLW procedure therefore an estimate of body composition can be made at the same time as TEE using the DLW technique. The study of changes in body composition can also be used to illustrate the quality of growth of children and adolescents and assist in the evaluation of nutrition and physical activity intervention programmes.

TEE is composed of three main components: resting energy expenditure (REE), thermic effect of food (TEF) and physical activity energy expenditure (PAEE). The best way to measure physical activity energy expenditure is to combine the use of stable isotopes in the DLW technique to determine total daily energy expenditure, with indirect calorimetry to measure basal or resting metabolic rate (RMR) [35]. The 'gold standard' approach for the estimation of total energy expenditure, including free-living physical activity, is the combination of both approaches in the same individuals [36].

The latest report on energy requirements [37] has used the DLW technique combined with appropriately validated heart rate methodology, to establish energy requirements in children 2–18 years of age in both developed and developing countries. This report referenced the DLW technique as the best option for the optimal measurement of TEE of individuals in normal daily living conditions. Further, the report suggested that other methods of energy expenditure measurement in children should be validated against the DLW technique.

The use of stable isotopes such as deuterium and ^{18}O will be crucial for the conduct of future studies regarding body composition and energy expenditure, especially in the determination of nutrition and physical activity recommendations compatible with healthy lifestyles for the global population [38, 39].

2. BACKGROUND INFORMATION INCLUDING SAFETY AND ETHICAL CONSIDERATIONS

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 of the nuclide is possible. The majority of chemical elements consist of a mixture of stable isotopes. Stable isotopes occupy the same position in the periodic table of elements due to the identical number of protons, and

hence undergo the same chemical reactions. Stable isotopes of several elements (carbon, hydrogen, oxygen and nitrogen) have been extensively used in biomedical research. This publication deals only with stable isotopes of hydrogen and oxygen, deuterium (^{2}H) and oxygen (^{18}O) because these are used to assess body composition and TEE by means of the DLW technique.

Hydrogen, the simplest element in the periodic table, consists of a nucleus with one proton (which is positively charged) and one electron (which is negatively charged). One proton represents mass one and thus the mass of hydrogen is 1. This isotope is also called protium. In deuterium, the heavier stable isotope of hydrogen, the nucleus also contains one neutron (which has no charge and represents mass 1). Thus, the mass of deuterium is 2. The nucleus of the most common isotope of oxygen contains 8 protons and 8 neutrons and, consequently, its mass is 16. The heavier stable isotope of oxygen used in the DLW technique has 8 protons and 10 neutrons in its nucleus which gives the mass 18. There is also a stable oxygen isotope with 8 protons and 9 neutrons having the mass 17. The mass of an element is often shown at the top left of the letter indicating the element. Thus, hydrogen is ^{1}H and deuterium ^{2}H. Deuterium is also commonly indicated as D. The most common isotope of oxygen is ^{16}O and the isotope used in the DLW technique is ^{18}O. These oxygen isotopes are also commonly described as oxygen-16 and oxygen-18.

Stable isotopes occur naturally and the natural abundance varies between isotopes of different elements. Only 0.015% of all hydrogen is deuterium, while approximately 0.20% of all oxygen is ^{18}O and 0.037% is ^{17}O. This means that an adult man weighing 70 kg with 40 kg of body water contains almost 80 g ^{18}O water, but only about 6 g deuterium labelled water. Consequently, mammalian cells are accustomed to molecules containing ^{2}H and ^{18}O at natural abundance levels. Indeed, when using IRMS, the stable isotope doses used are less than that naturally present.

Mass differences exist between stable isotopes and the predominant form of an element. Such differences may cause stable isotopes to exhibit so-called isotope effects in biological systems which may have an adverse impact at the cellular or whole organism level. Although isotope effects exist for all stable isotopes, the most significant would be expected when compounds are labelled with deuterium because of the greater mass difference (as a proportion of the atomic mass) between protium and deuterium, i.e. ^{2}H is twice as heavy as ^{1}H, whereas ^{18}O is only 1.125 times as heavy as ^{16}O.

This is consistent with experimental findings. Thus, tissues containing high concentrations (>15%) of deuterium labelled water may exhibit a number of effects including impaired protein and nucleic acid synthesis, altered conformation and stability of biopolymers, altered rates of enzymatic reactions due to the deuterium labelling of either the enzyme or substrate, impaired cell

division and morphological changes [40]. The overall effect of deuterium-labelling is a depression of tissue metabolism due to lower reaction rates of deuterium-labelled compounds in vivo. This is defined as a kinetic isotope effect because the rate of a chemical reaction is sensitive to atomic mass at a particular position in one of the reacting species [40]. High concentrations of deuterium can be problematic although harmful effects in mammals have not been detected in concentrations in body water below 15%. Levels of deuterium-labelling of at least 15% must be maintained by continual dosage in animal models before adverse effects become evident [41, 42]. The dose currently being promoted to measure lipogenesis is 100 g per 30–40 kg TBW, a maximum concentration of ~0.3%.

Lesser effects, such as transitory episodes of vertigo, have been reported in human adults consuming an amount of D_2O sufficient to enrich body water to 0.35–0.65% [42]. It has been suggested that the threshold for noticeable transitory side-effects exists when provided bolus doses of greater than 0.3% of TBW. The threshold of deuterium toxicity has been defined as 15% and is far in excess of concentrations conceivable for use in human studies [41, 42]. The method that currently uses the greatest quantity is to measure lipogenesis, raises the deuterium content of body water to only 1/50th of the threshold for toxicity [43].

Commonly used deuterium doses are 20–80 mg per kg body weight [44] resulting in a maximum concentration in body water at about 0.16%. The deuterium doses needed to assess TBW or TEE as described in this report will give a maximum concentration in body water of about 0.12%. Deuterium has been given to human participants until the deuterium concentration in body water reached 0.5% and this level was maintained for a period of time [41]. Similar studies have been conducted in pregnant women [45]. In summary, there is no evidence regarding disadvantageous long-term effects of deuterium [44] as deuterium is eliminated from the body in a relatively short period of time.

Less information is available regarding the toxicity of isotopes such as ^{18}O. This is probably a reflection of the fact that biological consequences will be small because of the smaller mass differences between the major and minor isotopes, and almost identical physical and chemical properties [41]. Replacement of up to 60% of body oxygen by $H_2{}^{18}O$ revealed an absence of adverse physiological and biochemical consequences [42]. Here ^{18}O replacement resulted from three generations of mice existing in an atmosphere of 90% $^{18}O_2$ with the third generation consuming 90% $H_2{}^{18}O$ [46]. Almost half a century of stable isotope usage in human metabolic studies has occurred without documented significant adverse effect. Consequently, deuterium and oxygen-18 are considered safe for use in

humans across the lifespan. Stable isotopes have been used in studies of pregnant and lactating women, in young infants (including premature babies), and children of all ages [41, 42].

Water labelled with ^{2}H or ^{18}O is produced at a range of international sites. The production of $^{2}H_2O$ can be based on enrichment of deuterium labelled molecules present in natural water by means of distillation, electrolysis or various chemical exchange processes exploiting a kinetic isotope effect [41]. However, production of pure $^{2}H_2O$ by distillation or electrolysis requires large amounts of power, so chemical methods are generally preferred. ^{18}O labelled water is used in biomedical research and diagnostic medicine. ^{18}O is manufactured from water using a process called fractional distillation, based on the slight difference in vapour pressure between $H_2{}^{18}O$ and $H_2{}^{16}O$ [41]. Because this difference is quite small and since the level of ^{18}O in water is only about 0.2%, production of ^{18}O labelled water also requires large amounts of power which makes such water very costly. An associated problem is that tritium, the radioactive isotope of hydrogen having a mass of 3, is concentrated during the production process. Therefore, before being used in human studies, ^{18}O labelled water should be normalised, that is, its tritium content is reduced to the level of that present in natural water.

3. ISOTOPE DILUTION AND THE ASSESSMENT OF BODY COMPOSITION

3.1. THE PRINCIPLE OF ISOTOPE DILUTION

The volume or mass of a large unknown quantity of water can be measured with accuracy by isotope dilution. Isotope dilution is the ‘gold standard’ technique in many areas of chemical and biochemical analysis. The principle of isotope dilution can be simply illustrated using the following equations, which describe a dose of enriched tracer being evenly distributed in a large pool of water (TBW):

$$F_1N_1 = F_2N_2 \tag{3.1}$$

$$N_2 = F_1N_1/F_2 \tag{3.2}$$

where N_1 is the pool size of the dose in moles and N_2 is the pool size of the distribution space in moles. F_1 is enrichment of the dose and F_2 is the enrichment of the distribution space.

The product, F_1N_1, equates to the moles of excess ^2H_2O in the dose. F can be given either in delta notation (delta over baseline in units of per mille (‰)), atom% excess or ppm excess, provided that both F_1 and F_2 are in the same unit. Units used to describe stable isotope enrichment (a measure of tracer concentration) are given in Appendix I, and their inter-conversion is described in Appendix V.

Isotope dilution is analogous to mixing a dye tracer in a beaker of water. For the same amount of added dye, a small beaker of water will have a more intense colour (higher concentration) than a large beaker because the dye is distributed across a smaller volume of water. It is assumed that the tracer dose is fully consumed and is fully mixed within the distribution space at the time of sampling.

3.2. DEVIATIONS FROM BASIC THEORY

The principle of isotope dilution can be applied to the measurement of TBW. Theoretically, TBW could be measured using tritium labelled water (^3H_2O), or with water labelled with either of the stable isotopes, ^2H or ^{18}O. Stable isotopes are preferred because of participant acceptance and minimal risk. Despite ^{18}O being an excellent tracer, the use of ^2H is far more economical as ^2H_2O is commercially available at ~1% of the cost of $H_2{}^{18}O$.

3.2.1. Correction for water turnover

Body water is not a simple closed system as described by Eq. (3.2) but is dynamic with a variety of inputs (drink, food and metabolic water) and outputs (urine, faeces, sweat, breath, etc). As measurement protocols extend until the tracer dose is fully equilibrated through body water, either, but not both, water gain or tracer loss has to be considered when calculating TBW. In practice, control of these effects depends upon the protocol and calculations used [47].

3.2.2. Correction for non-aqueous hydrogen exchange

Isotopes in body water enter other pools within the body and both H isotopes and O isotopes undergo non-aqueous exchange. Specifically, hydrogen exchanges with exchangeable atoms in body protein and other non-aqueous components, but most of the exchange is with protein [48, 49]. Hydrogen is also sequestered into fat and protein as these are synthesized. Oxygen undergoes exchange with organic matter, but to a lesser extent than hydrogen. A small quantity of oxygen also exchanges with bone minerals. The consensus is that

the ^{2}H space is approximately 1.041 times that of TBW, whereas the ^{18}O space is approximately 1.007 times that of TBW [50].

3.3. ISOTOPE DILUTION AND THE MEASUREMENT TBW

Two basic approaches are used to measure TBW, the intercept method and the equilibration method. The intercept method requires a longer protocol (7–14 days) and is relevant when studying water turnover such as in the DLW technique to measure TEE and when measuring maternal TBW within the protocol to measure human milk intake by breastfed babies [51, 52]. Briefly, the natural logarithm of the elimination of tracer from body water is plotted against time and the intercept gives the tracer dilution at the time of dosing. The only modifications to Eq. (3.2) required are to correct for non-aqueous hydrogen exchange (1.041) and to convert moles to kg (18.015):

$$\text{Intercept TBW (kg)} = \frac{(F_1 N_1 \times 18.015)}{(F_2 \times 1000 \times 1.041)} \tag{3.3}$$

where F_2 is the enrichment at the time of dosing given by the intercept of the tracer elimination plot.

The equilibration or plateau method to measure TBW is advantageous because it can be undertaken in a considerably shorter period than the intercept method. In adults, intracellular fluid and extracellular fluid come to equilibrium within ~3 hours. In infants and children the period is shorter and plasma or saliva samples will reflect this. Urine requires longer to equilibrate (4–8 hours) as early samples will include urine previously stored in the bladder [53]. In older adults, residual urine post-voiding will also delay equilibration. Oral dosing combined with either urine or saliva sampling means that the protocols are non-invasive. Saliva sampling may introduce a small error as water input has to be restricted prior to sampling and evaporative loss will cause isotopic fractionation. Urine sampling is the preferred option but requires IRMS for analysis. FTIR is only suitable for analysis of saliva and plasma samples. Different body fluids may be subject to different degrees of isotopic fractionation [17], therefore it is best not to combine saliva and urine sampling within the same protocol. In practice, a saliva sampling protocol is quicker. Fluid input during the equilibration period should be minimized or TBW data are corrected for fluid intake (Eq. (3.4)).

$$\text{Equilibrium (saliva) TBW (kg)} = \frac{(F_1N_1 \times 18.015)}{(F_2 \times 1000 \times 1.041)} \text{ water input} \quad (3.4)$$

where, F_2 is the enrichment given in saliva at 3 or 4 hours after dosing; water input (kg) after dosing is logged and subtracted.

When using urine sampling, TBW data are corrected for the dose lost in urine and other body fluids during the period of equilibration (Eq. (3.5)).

$$\text{Equilibrium (urine) TBW (kg)} = \frac{((F_1N_1 - \text{loss}) \times 18.015}{(F_2 \times 1000 \times 1.041)} \quad (3.5)$$

where, F_2 is the enrichment in urine in the second or third sample after dosing; urine volume is recorded and the dose (in moles) lost calculated as the cumulative product of urine volume and enrichment.

Unless otherwise known, the total water loss is assumed to be twice the urine volume under conditions that do not invoke sweating [54]. In practice, the calculation used to predict TBW makes no assumptions about the enrichment of the dose consumed, in that an aliquot of the dose is diluted and its isotopic abundance is measured with this data being used in the calculation (see Sections 5.1.1 and 5.2).

4. ISOTOPE DILUTION AND THE ASSESSMENT OF TOTAL ENERGY EXPENDITURE

4.1. INDIRECT CALORIMETRY

Calorimetry is the measurement of heat production or, more specifically, the rate at which heat is produced in the body as a consequence of the oxidation of the energy substrates to carbon dioxide and water. Indirect calorimetry represents a group of methods in which the heat is not measured directly, but rather calculated from the stochiometric relationship of the chemical reactions of energy substrates with oxygen producing carbon dioxide, water and heat. Commonly, these determinations are made by measuring the concentrations of these two gases in inspired and expired air using a mouth piece, flow-through (open) canopy, or a flow controlled room. In short, the broader definition is that the chemical reactant(s) or product(s) of oxidation be

measured and the heat production calculated. The DLW technique is a form of indirect calorimetry.

4.2. THEORY OF DOUBLY LABELLED WATER

In the DLW technique, the rate of carbon dioxide production is estimated. The technique, however, does not involve the measurement of respiratory gases directly, but rather the kinetics of water turnover in the body is measured. Although not intuitively obvious, the relationship between carbon dioxide production and the kinetics of body water is well founded in physiology [55, 56].

With the exception of extreme obesity, water represents the largest single compartment of the human body by weight. The body water compartment is normally an extremely well mixed compartment due to an extensive circulatory system that ensures that all cells and their surrounding media are constantly being flushed to restore nutrients and remove waste products. Therefore, the body water compartment can be described by single compartment, steady state kinetics. That is, when water molecules labelled with an isotope of either hydrogen or oxygen are introduced into the body water compartment, they quickly and evenly distribute. With the exception of growth, the TBW compartment remains relatively constant in size and changes rarely exceed a few per cent within or between days. Water molecules, however, are constantly entering and leaving the body. Every time we drink a beverage, consume a food containing moisture or produce a water molecule during energy substrate oxidation, these new molecules mix with the body water pool. At the same time, water is leaving the body as water vapour escaping from the lungs and skin or as part of urinary, faecal and other fluids. Over a normal day, the input of new water and the output are generally equal and the pool size stays relatively constant.

By labelling water molecules it is possible to measure the dynamic character of body water. If the water is labelled in one of the hydrogen positions with the stable isotope deuterium, the labelled water will distribute throughout the water pool and usually reach a near steady state concentration in about 3 hours. As described in Section 3.3, the deuterium oxide concentration in body water, along with knowledge of the exact dose of deuterium labelled water consumed provides a measure of the body water pool size, or more correctly, (as detailed later), the deuterium dilution space. The dynamic nature of body water now becomes apparent. With the introduction of each new molecule of water, the deuterium oxide is diluted and its concentration begins to fall. Moreover, as water leaves the body it carries with

it some of the deuterium oxide and the amount retained in the body decreases. The concentration decreases while the body water pool size stays almost the same.

The box below illustrates this process. Consider a large beaker of water with a drain at the bottom.

Fill the beaker with 1 L of water and add a dose of dye to colour the water. The amount of dye added and concentration of the dye provides a measure of the volume of water in the beaker. Now, add 100 mL of water, mix and then remove 100 mL from the beaker. The volume of water is the same, but the dye is diluted by 10% and the amount of dye removed from the beaker is 10% of the original dose of dye that was added. On the next day, again add 100 mL of water, mix and drain 100 mL of fluid from the bottom. The volume of water will still be the same and the concentration of dye will be 10% less than the day before, but the amount of dye removed from the beaker will only be 9% (0.10 × 90%) of the original dose added to the beaker and the amount remaining will be 81% of the dose. If this is repeated again, the amount of dye removed will be 10% of what was in the beaker (81% of the dose). Thus, 8.1% of the dose will be removed and 72.9% will remain. Because the amount of water in the beaker after each addition and removal remains the same, the volume of water in the beaker is referred to as being in steady state. The amount removed each day is 10% of the amount of dye in the beaker at the start of the day. However, the amount of the original dose removed is reduced each day because the amount of dose in the beaker at the start of the day is reduced with each round of adding and removing water.

When the dye, or deuterium oxide, concentration is plotted as a natural logarithm against time a straight line is observed. In a living animal, the line is not perfectly straight on an hour by hour basis because there is a diurnal rhythm to water turnover, which is highest during waking and in particular at meal times and lowest during sleep. On a day to day basis, however, the line is generally straight and the slope (in units of time^{-1}) is a measure of the fractional turnover of body water, that is, the fraction of the pool that is replaced within body water each day.

In the above beaker illustration, the value is 0.10 d^{-1} or 10% d^{-1}. The product of the slope and pool size is the absolute amount of deuterium oxide that is turned over, or replaced, each day. Finally, because the deuterium oxide

exits the body as water, the fractional deuterium elimination rate is equal to the water elimination rate (rH_2O). Stated mathematically, the deuterium dilution space (N_D) times the fractional deuterium oxide turnover rate (k_D) is equal to the water elimination rate:

$$rH_2O = N_D k_D \quad (4.1)$$

The next physiological observation is the key to the theory of the DLW technique. Some years ago, Lifson [55] observed that the oxygen in water is in rapid and complete isotopic equilibrium with the oxygen in carbon dioxide. Because of this, a water molecule labelled with ^{18}O will not only mix with and exit the body with water in the same way that a deuterium labelled molecule does, but it will mix with and exit the body with carbon dioxide. An oxygen isotope given as water will therefore be eliminated more rapidly than a deuterium isotope and the product of the oxygen hydride elimination rate and dilution space will not equal the water elimination rate. Rather, it will equal the sum of the water and carbon dioxide elimination rates. Stated mathematically:

$$rH_2O + 2rCO_2 = N_O k_O \quad (4.2)$$

where the factor of 2 in the second term of the left hand side of the equation adjusts for the fact that there are twice as many atoms of oxygen in each molecule of carbon dioxide as there are in each molecule of water.

Equations (4.1) and (4.2) are highly related and the difference in the hydrogen and oxygen water turnover rates is related to the rate of carbon dioxide production. This can be shown mathematically by substituting rH_2O in Eq. (4.2) from Eq. (4.1) and solving for the rate of carbon dioxide production:

$$rCO_2 = ½ (N_O k_O - N_D k_D) \quad (4.3)$$

4.3. DEVIATIONS FROM BASIC THEORY: ISOTOPE FRACTIONATION AND OTHER CORRECTIONS

Equation (4.3) was derived from the basic theory of the physiological chemistry of hydrogen and oxygen in water. The two tracers, deuterium oxide and ^{18}O, however, do not exactly mimic the physiological behaviour of the protium (^1H) and ^{16}O that make up more than 99% of water. This is because of isotope exchange and isotope fractionation.

Isotope exchange is the process in which the stable isotope label from the dose water, either deuterium or ^{18}O, exchanges with hydrogen or oxygen in a compound other than water. This non-aqueous exchange of the tracer increases the dilution space of the tracer compared to that of TBW alone. This is because the isotopic label is now distributed over the TBW plus the exchangeable hydrogen or ^{18}O pool and thus the isotope dilution space is larger than the TBW pool alone. In the case of deuterium, the major non-aqueous exchange is with acidic hydrogen in protein and other less abundant biochemicals with acidic hydrogens. This exchange increases the deuterium dilution space by 4.1% [50] compared with TBW alone. The overestimation also appears to vary during infancy [57]. In the case of ^{18}O, the major exchange is with inorganic compounds such as the oxygen in phosphate and to a very small degree, the carbonate in bone mineral. This exchange increases the ^{18}O dilution space by 0.7% compared with body water [58].

If these factors are incorporated into Eq. (4.3), it becomes:

$$rCO_2 = (TBW/2) \times (1.007k_O - 1.041k_D) \quad (4.4)$$

Isotope fractionation is the process in which the relative concentration or abundance of the isotopic label in a compound differs from that of the abundance in body water. This difference in abundance results from small differences in the bond energies of the two isotopes (deuterium versus protium or ^{18}O versus ^{16}O). With regard to DLW, the isotope fractionations of importance are those between body water and water vapour leaving the body, and between body water and carbon dioxide leaving the body [59]. There is no fractionation for urine, faecal water, or sweat. Sweat may appear to be an exception to the above statement on fractionation between body water and water vapour. However, sweat is excreted from the sweat glands as liquid water and the evaporation occurs after it leaves the body water and thus it is not fractionated as it leaves the body. The isotope fractionation for deuterium between water and water vapour has a value of 0.946 at 37°C, which means the deuterium abundance in water vapour is 94.6% of that in the liquid water that it equilibrates with in the body. As a consequence of this, the rate at which the deuterium label leaves the body as water vapour is only 0.946 times the true rate of water loss as water vapour. For ^{18}O in water vapour, the fractionation factor is 0.991. In the case of ^{18}O in carbon dioxide, the abundance of ^{18}O is actually higher than in body water, therefore the fraction factor is greater than 1 (1.038). As a consequence, the rate at which ^{18}O leaves the body as carbon dioxide is 1.038 times the true rate of carbon dioxide production. These fractionation factors need to be incorporated into Equation (4.4) to correct for

the small differences between the isotopic label losses and the water and carbon dioxide fluxes [50].

$$rCO_2 = (TBW/2)\ (1.037)\ (1.007k_O - 1.041k_D) - 0.0246\ (rH_2O_{FG}) \quad (4.5)$$

where rH_2O_{FG} is the rate of fractionated water vapour loss.

Although far less user-friendly than Eq. (4.3), Eq. (4.5) is more accurate. While the error varies depending on the ratio of water vapour loss to liquid water loss as well as the ratio of carbon dioxide production to water turnover, Eq. (4.3) overestimates carbon dioxide production by 10% [60]. In contrast, Eq. (4.5) is very accurate [50]. Validations have been performed in infants, healthy weight adults, obese adults, adults performing heavy exercise and others. In comparison with either carbon dioxide production measured by gas exchange or measured energy intake and change in body energy stores, DLW using Eq. (4.5) was accurate to 1% with a precision of 5% [58].

Almost always, Eq. (4.5) can be simplified. By assuming that breath is 96% saturated with water vapour and that the small amount of non-sweat skin water vapour loss is proportional to the exposed skin surface area, Eq. (4.5) can be simplified to:

$$rCO_2 = 0.455 \times TBW\ (1.007k_O - 1.041k_D) \quad (4.6)$$

This equation is equally as accurate as Eq. (4.5).

Finally, it is necessary to calculate heat production (energy expenditure) from the rate of CO_2 production [61]. This requires an estimate of the respiratory exchange ratio and then energy production can be calculated using standard indirect calorimetric equations [62]. The simplest equation to use is the modified Weir equation [63]:

$$TEE\ (kcal/d) = 22.4 \times rCO_2 \times (1.10 + 3.90/R) \quad (4.7)$$

where R is the assumed respiratory exchange ratio.

4.4. ASSUMPTIONS OF THE TECHNIQUE

The DLW technique as described above is dependent on four major assumptions. While these assumptions are generally robust, it is important to understand them from the perspective of study design as well as identifying the uncommon situations where a systematic error may result from a deviation from an assumption.

Assumption 1. Body water behaves as a steady state, single compartment pool with rapid equilibration throughout the pool.

From a data viewpoint, this assumption states that after a dose of DLW, the tracers reach their maximum enrichment quickly and thereafter fall on a straight line when the natural log of enrichment is plotted against time. This is generally true, but not perfectly. The enrichment in body water does not equilibrate instantaneously, but will take 3 h to reach a steady state, and will then decrease with time with a diurnal rhythm. The slight delay means that the protocol must include a delay in sampling, or at least dropping data that was collected before the equilibration was reached. This delay is up to 3 h, to be conservative for blood sampling and three voids and at least 4 h for urine specimens before the first data. Longer equilibration times may be required during pregnancy or as a result of conditions such as oedema, shock, and post-void residual volume in the bladder. The diurnal rhythm noted in the day to day decrease in isotope enrichment is a result of normal within-day variation of eating, drinking and exercising. During sleep, energy expenditure is lowest and isotope enrichments show little decrease. Consequently, sampling for calculation of isotope elimination rates should be performed at roughly the same time of day so that the influence of the diurnal rhythm is minimized.

Assumption 2. The isotopes only exit the body water as water or carbon dioxide.

Inspection of Eqs (4.1) and (4.2) indicates that the rates of water flux and the sum of water flux and carbon dioxide flux through the body are equated with the disappearance of the isotope from body water. If there is another route of exit of these isotopes from body water, then the rate of isotope disappearance will overestimate the rates of water flux and carbon dioxide flux and hence TEE. In humans, other routes of loss have been reported but they generally comprise less than 1% of total loss and can be ignored because the resultant effect on energy expenditure is minimal (less than 1%). These extraneous losses include incorporation into organic products excreted in the urine such as urea, exchange with hydroxyl groups in non-metabolized fibre in the faeces, or incorporation into deep body pools such as de novo fat synthesis. The former examples are generally minor in humans but can be more important in small animals or ruminants with high fibre excretion rates and these are dealt with by small modifications to the calculation of carbon dioxide production [64]. De novo fat synthesis is also generally too small to be a concern, but errors of 16% have been reported in rapidly growing pigs, which are noted for their high levels of fat synthesis [49]. Rapidly growing children however, do not display any influence of rate of growth on the accuracy of

DLW [65]. An adult would have to synthesize 50 g of fat per day (1883 kJ (450 kcal) overfeeding) before the error in a DLW measure of TEE would exceed 5% [49].

Assumption 3. Isotopes exit the body only in isotopic equilibrium with body water.

This assumption is very robust and has stood the test of time in human studies. However, it has resulted in many different forms of the equation being used to calculate carbon dioxide production. Most investigators now use Eq (4.6) as it results in the least person to person variation in validity.

Assumption 4. The water and carbon dioxide lost from the body does not reenter the body water pool.

This assumption is also very robust. Isotope reentry will artificially slow the elimination rate because the reentering isotope will sustain the levels in the body at high values. Reentry, however, is rare. Most DLW validations have been performed using metabolic chambers with limited airflow though the respiration chamber and these have not identified a reentry problem. A corollary of this assumption, however, that exogenous carbon dioxide influx will be measured by the DLW technique, is violated in individuals such as cigarette smokers. When a person inhales carbon dioxide, it undergoes isotope exchange with body water and thus alters isotope kinetics. Fortunately, the error is small. Assuming that the average smoker inhales about the half the carbon dioxide produced from a cigarette, consumption of a pack of cigarettes per day (approximately 24 g) will inflate the DLW measure of energy production by 207 $kJ.d^{-1}$ (50 $kcal.d^{-1}$) or about 2%.

5. APPLYING ISOTOPE DILUTION TO BODY COMPOSITION ANALYSIS

5.1. DEUTERIUM (^2H) DOSE TO MEASURE TBW

The quantity of ^2H_2O used depends on body size, the method used to measure its enrichment, and the precision of the analysis. The dose is designed to raise the enrichment to an optimal level for precise and accurate analysis. The enrichment of deuterium in urine, saliva or plasma can be measured using

isotope ratio mass spectrometry (IRMS). Fourier transform infrared spectrometry (FTIR) can be used to measure the enrichment of deuterium in saliva and plasma. FTIR is not as sensitive as IRMS. When using IRMS, the target enrichment can be 100 ppm excess ^{2}H and when using FTIR, 600 ppm excess ^{2}H in body water. IRMS will be able to measure samples at either dose levels but FTIR instruments are not sensitive enough to give accurate results at the lower dose. Laboratories using reduction to prepare hydrogen gas for dual inlet IRMS analysis as opposed to equilibration may use a lower target dose, such as 60 ppm excess ^{2}H. An enrichment of stable isotope that is 100 times the standard deviation for the analysis reduces the relative SD (CV) for the measurement of the TBW to 1.4%.

It is preferable to make up study doses in advance from a single large stock. It is recommended that the laboratory undertaking ^{2}H analysis prepares the study doses and calibrates sample analysis with reference to the study dose. If this is the case, the analytical laboratory would also prepare a gravimetric dilution of the dose for analysis. The ideal enrichment of this diluted dose is the same as that expected in TBW. TBW is crudely assumed to be 50% body weight. Thus, to obtain a target of ~100 ppm excess ^{2}H in body water, one assumes 0.05 $g.kg^{-1}$ body weight is needed. If a study is on adults of a similar size, a single dose would be appropriate. However, if the study population ranged from infants to adults, a range of doses would be more suitable. Table 1 illustrates the ideal dosage if IRMS is used for analysis.

TABLE 1. DOSES OF $^{2}H_{2}O$ BASED ON BODY WEIGHT

Body weight (kg)	~TBW (kg)[a]	$^{2}H_{2}O$ dose[b] (g)
120	60	6.0
96	48	4.8
72	36	3.6
48	24	2.4
24	12	1.2
12	6	0.6
4	2	0.2
2	1	0.1

[a] 50% body weight.
[b] IRMS TBW target: 650‰ (100 ppm excess ^{2}H).

TABLE 2. DOSES OF ^2H_2O FOR DIFFERENT AGE GROUPS BASED ON STRATIFIED BODY WEIGHTS

Category	Body weight range (kg)	Dose (g ^2H_2O)
Adult	>70	6
Adolescent/adult	35–70	3
Child	10–35	1
Infant	<10	0.3

If this 40 fold series of body weights is stratified into four groups, the dosage can be arranged so that most participants would receive between 75 and 150% of the ideal dose (Table 2).

It is recommended that sufficient doses be made up to accommodate the entire study. For studies involving more than 100 participants, doses can be made up in batches sufficient for 100 participants. For instance, if 50 doses of each of the four categories above were required, this would total 515 g ^2H_2O (with a ±10% margin). This is prepared gravimetrically and diluted tenfold with local tap water in a large clean glass bottle. The appropriate doses are aliquotted and weighed into individual dose bottles.

In practice, a clean, dry, 5 L bottle (such as a borosilicate glass bottle with a PTFE-lined screw top) is tared and 515 g 99.9 at.% ^2H_2O added and weighed to 4 significant places. Approximately 5 L tap water is added and the solution is mixed and weighed to four significant places (0.1 g). Pre-labelled and individually coded 125 mL polythene bottles with leak-proof screw caps are each tared and doses are aliquotted and then weighed into these, to four significant figures. In the above case 50+ marked 'adult doses' are prepared at 66 g weight, 50+ 'adolescent' doses are prepared at a target 36 g, 50+ 'child' doses at 11 g and 50+ 'infant' doses at 3.3 g. Using the exact weight of each coded dose, the amount of deuterium oxide in each dose can be calculated within a study spreadsheet.

5.1.1. Making up the diluted dose for analysis

It should be emphasized that these doses (A, Eqs (5.1) and (5.2)) contain a nominal tracer quantity and it is essential that an aliquot be mixed gravimetrically with local drinking water with this (the diluted dose; 'a', Eqs (5.1) and (5.2)) and a sample of local drinking water (W, Eqs (5.1) and (5.2)) being retained for analysis with the body water samples. The diluted dose should have a similar concentration of deuterium to the post-dose body water

specimens. The diluted dose is prepared by blending 0.1 g dose (weighed to four decimal places) with 100 g local drinking water (weighed to four decimal places) and analysed. In practice, a 100 mL volumetric flask (the opening minimizes evaporative loss) is tared on a balance capable of weighing to four decimal places. Approximately 50 mL of local drinking water is added. The weight is recorded and the balance tared. One hundred μL of the dose is pipetted into the flask and its weight recorded (to four decimal places). The balance is tared again. The volumetric flask is made up to 100 mL with local drinking water and its weight is noted. This dilution will produce an enrichment similar to the target in body water (100 ppm excess ^{2}H).

5.1.2. TBW protocol for urine sampling

The TBW content will be calculated correcting for water loss during the equilibration period. Body weight should be measured (to 0.1 kg in adults) after the participant has voided their bladder and bowels. At this time, a pre-dose urine sample is collected into a dry, graduated 1 L polythene measuring cylinder. Alternatively, a dry polythene jug is used and its contents are poured into a polythene measuring cylinder. For women, a dry, plastic toilet hat can be used. Two aliquots are poured into labelled, screw-capped, elastic seal 2 mL storage vials and the cap sealed securely. The cap can be double-sealed with parafilm. However, this is generally not required unless the specimen is less than 1 mL.

The dose code is recorded from the bottle. The dose is taken orally, either by drinking directly from the bottle or through a drinking straw. A small amount of drinking water or fruit juice is added to the bottle, which is mixed and the residue is consumed. Following the dose, food and drink can be taken ad libitum.

Three post-dose urine samples are collected. Samples prior to 4 hours are unlikely to have reached equilibrium (Fig. 1). Their analysis, however, is used to account for deuterium lost in the urine. The protocol need not continue past 8 hours if this is the time of the second post-dose sample. The total volume of urine passed at each void is noted and duplicate samples are transferred to labelled containers as above. The volume and time of sampling is recorded. The remaining sample is discarded. The sample bottle is tightly capped and stored away from direct sunlight or other heat sources (in a refrigerator if possible), until transported for analysis.

In the laboratory the ^{2}H enrichment of the samples is measured using isotope ratio mass spectrometry (IRMS).

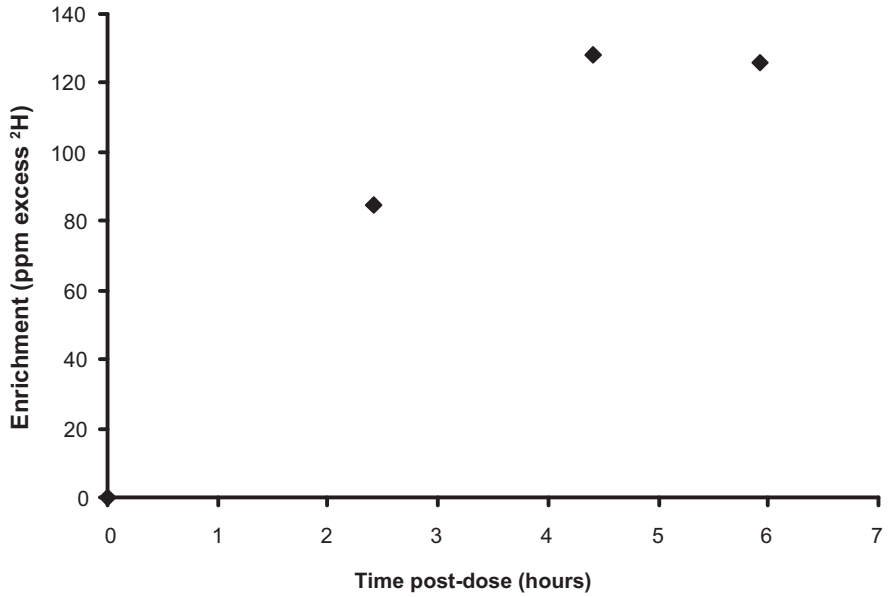


FIG. 1. Enrichment of deuterium in urine during a TBW protocol. Enrichment can be in ppm or delta notation. The first post-dose urine sample is collected before the dose has equilibrated with body water. ΔBW is based on the mean enrichment of the second and third post-dose samples.

5.1.3. TBW protocol for saliva and plasma sampling

Samples of 2 mL are required. It is often necessary to stimulate salivation to collect sufficient sample. Ensure that the participant did not eat or drink anything for at least 30 min before proceeding. If fluid is taken at any time during the protocol its volume should be recorded. Body weight is measured (to 0.1 kg in adults) after they have voided their bladder and bowels. Give the participants a dry cotton wool ball. Ask them to move it around their mouth for 2 min or until it is sodden. Remove the plunger from a new 20 mL plastic syringe. Ask the participants to move the cotton wool to the front of their mouth, and transfer it into the syringe barrel. Replace the plunger into the syringe barrel, and use it to squeeze the saliva out of the syringe into the appropriately labelled sample tube.

If insufficient sample is obtained the first time the participant can be given a second cotton wool ball and the process repeated. Never fill the sample tube entirely, always leave at least 10% of the tube volume for sample

expansion on freezing. Once adequate sample has been obtained cap the tube and store in the refrigerator (or preferably freezer) until analysis.

To sample saliva in very young children, a dry cotton wool swab with additional cotton wool wrapped around the swab can be used. The cotton wool is transferred to the barrel of a 20 mL syringe and the saliva is squeezed out as described above. Saliva samples may also be taken by spitting directly into a collection bottle however commercial saliva sampling systems are widely used for this purpose and are particularly helpful with children. Discard the syringe, cotton wool, and gloves (if worn). Never attempt to re-use a syringe. Two post-dose saliva samples are taken at 3 and 4 h. Each labelled sample bottle is tightly capped. All samples are stored away from direct sunlight or other heat sources (in a refrigerator if possible) until transported for analysis.

If plasma is used then 5 mL blood should be drawn from the participant into heparin-containing stoppered tubes. Do not use tubes with the heparin dissolved in water. Plasma should be separated by centrifugation as soon as possible before freezing until analysis.

Baseline (pre-dose) and two post-dose saliva samples are collected 3 and 4 hours after dose consumption.

5.2. CALCULATION OF TBW

5.2.1. Calculation of TBW from urine sampling

A similar approach and notation related to that used by the International Dietary Energy Consultancy Group (IDECG) [36] is used to calculate TBW. The IDECG report demonstrated how this equation relates to that used by Halliday and Miller [17]:

$$\text{TBW (kg)} = ((W \times A/a) \times (\Delta DD/\Delta BW)/(1000 \times 1.041)) - (2 \times \text{cumulative urine loss (kg)}) \quad (5.1)$$

where, W and ‘a’ refer to a portion of the dose that has been diluted for analysis, W is the amount (in g) of water used to make this dilution and ‘a’, the amount (in g) of the dose used in this dilution; ‘A’ is the dose (in g) taken by the participant; ΔDD is the enrichment measured in the diluted dose, which is the measured abundance in the diluted dose minus that in the local drinking water used to dilute it; ΔBW is the enrichment measured in body water, which is the measured abundance in a post-dose urine sample minus that in the pre-dose basal sample.

Units used to express isotope abundance can be ppm, atom % or in delta notation. Their interconversion is given in Appendix V. ΔDD and ΔBW should be expressed in the same units. The factor 1000 transforms TBW from g to kg. The constant 1.041 corrects for non-aqueous hydrogen exchange. Rather than adjusting the isotope dose by subtracting the small quantity of dose lost, TBW is corrected for the cumulative urine volume excreted adjusted by a factor to account for all routes of water loss. A factor of 2 can be used to correct for water vapour loss in temperate climates [45], but this should be adjusted if it is known that total water loss exceeds urinary loss by a different factor. The average water elimination rate (k_D) for the population under study, as calculated in the DLW technique, can be used with measured urine output to estimate this factor. (See Appendix III.)

5.2.2. Calculation of TBW from saliva sampling

The protocol for measuring TBW using saliva is shorter than that using urine. Some research groups choose to restrict fluid intake during the protocol and ignore the term for cumulative fluid intake in the following equation:

$$\text{TBW (kg)} = ((W \times A/a) \times (\Delta DD/\Delta BW)/(1000 \times 1.041)) - (\text{cumulative fluid intake (kg)}) \quad (5.2)$$

where W and 'a' refer to a portion of the dose that has been diluted for analysis, W is the amount (in g) of water used to make this dilution and 'a', the amount (in g) of the dose used in this dilution; 'A' is the dose (in g) taken by the participant; ΔDD is the enrichment measured in the diluted dose, which is the measured abundance in the diluted dose minus that in the local drinking water used to dilute it; ΔBW is the enrichment measured in body water, which is the measured abundance in a post-dose saliva sample minus that in the pre-dose basal sample.

5.2.3. Derivation of fat free mass and fat mass from TBW

The two compartment model divides the body weight into fat mass (FM) and fat-free mass (FFM) (Eq. (5.3)). Body weight is measured along with TBW, from which FFM is estimated. FFM is derived from TBW using a hydration coefficient, that is, the fraction of FFM comprised of water (Eq. (5.4)). Cellular hydration in all animals is controlled within strict limits. The classic work of Pace and Rathbun [16] is the source of the commonly used hydration coefficient, 0.732. A more recent study explored the constraints of this relationship [66].

$$\text{Weight (kg)} = \text{FM (kg)} + \text{FFM (kg)} \quad (5.3)$$

$$\text{FFM} = \text{TBW/hydration coefficient} \quad (5.4)$$

$$\text{Thus, FM (kg)} = \text{weight (kg)} - \text{FFM (kg)} \quad (5.5)$$

$$\text{and \% body fat} = 100 \times \text{FM/weight} \quad (5.6)$$

The hydration coefficient is known to vary as the composition of FFM matures during infancy and childhood [67, 68] and changes during pregnancy [69]. For individuals aged 21 years and above, the classic coefficient of 0.732 is used. A related effect is seen in maternal FFM hydration during pregnancy, which is temporarily increased as the foetus and amniotic fluid develops. Derivation of accurate FFM in these special cases requires the use of specific hydration coefficients. Once FFM has been estimated, FM and per cent body fat are calculated using Eqs (5.5) and (5.6), outlined above.

5.3. QUALITY CONTROL

All analyses are compared with natural abundance and enriched water standards. Analysis of replicate samples can be used to estimate analytical precision. When using IRMS precision (SD) of 1 ppm ^{2}H excess should be attainable. Comparison of TBW calculated from each post-dose sample can be used to estimate biological variation combined with analytical precision. In practice, the first post-dose urine sample is often omitted as urine may not have equilibrated with TBW by this time (that is, TBW is obviously overestimated). In the example given in Appendix III, the second and third post-dose urine samples give values that are within 2% of their mean. Measured TBW can be compared with a predicted value and the data flagged for checking or reanalysis if outside a normal range [27]. If no other prediction is available, the relationship with height^3 can be used: TBW (kg) = 7.4 × height^3 (m^3), validated in children and adults. If the measurements fall outside the 95% confidence intervals of this relationship (<5.7 × height^3 or >9.6 × height^3), the data and calculation should be checked and the samples re-analysed if necessary.

5.4. CONSIDERATIONS FOR SPECIAL POPULATIONS

5.4.1. Pregnancy and lactation

During pregnancy and lactation TBW can be assessed using the protocol described above. However, a few specific comments are relevant.

Dose calculation

During pregnancy women generally retain body fat and increase the amount of lean tissue. As a consequence the percent TBW in the pregnant body is, on average, not greatly affected. Although there is substantial water gained during pregnancy (40–50% of total weight gain), the per cent TBW does not change under normal circumstances. The same is true for post-partum women who generally lose, at least in part, the weight gained during pregnancy. Therefore, the per cent TBW in pregnant and lactating women can be assumed to be similar to that of non-pregnant, non-lactating women and, consequently, the isotope dose can be calculated as described above.

Equilibration time

The time required for the isotope to equilibrate in body water tends to be prolonged in individuals with expanded extracellular volumes such as women in the second and third trimesters of pregnancy. In such individuals the peak enrichment is reached after about 4 hours and it is suggested that samples are collected 4 and 5 h after dosing if saliva is used. If urine is used, samples should be collected for at least 8 hours. However, pregnancy is a dynamic state and a woman in the first trimester may not be different from a non-pregnant woman, and a woman at the end of pregnancy may require even longer equilibration periods. In studies of pregnant women it may be necessary to collect sufficient post-dose samples to detect the peak isotope enrichment in the body fluid sampled. Volume and isotope enrichment of urine produced during the equilibration period should be recorded together with fluid intake and taken into account when TBW is calculated. During lactation, the amount of isotope in the water fraction (about 87% by weight) of human milk leaving the mother's body during the equilibration period should be considered. This can be made by weighing the infant before and after each feed and assessing the isotope enrichment of a sample containing equal amounts of human milk, collected shortly before and after the feed. The intercept method is an alternative to assess TBW in pregnant and lactating women.

Calculating body fat from TBW

During pregnancy the water content of FFM, the so-called hydration factor, increases. Therefore the two compartment model needs modification when applied during this period. Hydration factors for the trimesters of pregnancy have been published by van Raaij et al. [70], Catalano et al. [71] and Löf and Forsum [69]. However, there is no consensus regarding the most appropriate hydration factors for different trimesters of pregnancy. Readers are referred to the following papers for more information [72–74]. The conventional hydration factor, 73.2%, is generally used in lactating women. However, Löf and Forsum [69] found that two weeks post-partum this factor is still higher than before pregnancy. Investigators studying body fat in lactating women are therefore recommended to use a multi-compartment body composition model.

5.4.2. Infants

In infants the procedure described above, based on the plateau procedure, can be used to assess TBW however the uniqueness of studying infants should be recognized. For example, volume and timing of urine samples cannot be controlled in infants. Due to difficulties in controlling the sampling procedure with respect to urine collection and intake of fluid, it is often preferable to use the back-extrapolation procedure to assess TBW in infants. When the DLW technique is applied to assess energy expenditure such an assessment can be obtained simultaneously (see Appendix III).

Sample fluid

Saliva or urine can be used and saliva collected as described above. Urine samples can be collected using special plastic bags which are pasted around the urethral opening. This is not difficult but requires some training. Another possibility is to squeeze urine from nappies (cotton diapers) used by babies at the appropriate time points. It is important to avoid the risk of evaporation of urine water with subsequent isotope fractionation.

Dosing and sampling

The appropriate amount of isotope for dosing can be calculated from the data given above. Administration of the dose to the infant is a critical point. This can be done by dropping the dose directly into the back of the mouth of the infant using a syringe or piece of tubing attached to a syringe. It is very

important that the complete dose is consumed by the infant therefore any spillage or amount not consumed should be accounted for. If the dose is administered via syringe or syringe and tubing, all should be weighed. Then the dose is administered and afterwards the tubing and syringe is weighed again. The difference in weight is equivalent to the amount of dose consumed by the infant corrected for any spilled dose. Mixing time in infants is more rapid than in adults and is probably complete within 2–3 h. All urine produced by the infant should ideally be collected for 3 h to allow for calculation of lost isotope. Then two or more consecutive urine samples should be collected and the time of their production noted. Fluid intake during the equilibration period should also be recorded and taken into account during calculation of TBW.

Calculating body fat from TBW

Data describing the hydration factor in full term infants are given by Fomon et al. [75] and Butte et al. [76]. Corresponding data for prematurely born infants are lacking, but some information regarding the hydration factor of a foetus at various stages of gestation is available [77]. These may be useful during the first days of life for prematurely born infants. However, during the first week of life there is a redistribution of body water [78] and therefore the intrauterine hydration factors probably become inaccurate quite quickly. Thus, until more information becomes available, any assessment of body fat in premature infants should use three or four compartment models of body composition.

5.4.3. Preschool children

The only special considerations here are dosing and perhaps specimen collection. All other procedures are identical to the average adult. Because preschool children may not wish to drink water, a small amount of a drink the child likes may be added to the dose so that the child is more likely to consume the dose over a short time period. Dosing preschool children can be quite difficult, due to very simple issues such as them objecting to anything other than their habitual bottle or cup. This can be readily overcome by asking the parent or guardian to bring that preferred bottle/cup to the study site. Straws are recommended for dose administration at this age. Care should be taken to avoid spillage of any dose clinging to the straw when the rinsing water is added. The dose can also be mixed with a fruit puree and given to the child with a spoon. The container can be rinsed with fruit juice which also is fed to the child. A dough scraper can be used to ensure that the dose puree mixture is

completely consumed by the child [79]. In summary, greater supervision during dose administration and specimen collection may be required in this age group.

5.4.4. Children and adolescents

No special considerations are required in this age group unless the child is unwilling to drink the dose. In that case, a drink may be used as indicated for preschool children. For FFM calculations, it is necessary to use age appropriate FFM hydration constants. [67, 68].

5.4.5. Elderly

One special consideration may be the use of a straw for dose administration. Elderly individuals with shaky hands should be asked to use a straw to avoid spillage. Care should be taken to avoid spillage of any dose clinging to the straw when the water for rinsing is added. In addition, many elderly experience post-void urine retention in the bladder. This slows the isotope equilibration process into the urine. This requires additional dose day urine collections to ensure that a plateau has been reached in urine isotope abundance.

In the elderly, residual urine volume after voiding delays equilibration. This has led to the development of an overnight TBW protocol [80]. In this protocol, a participant consumes the dose before bedtime and the equilibration occurs overnight. A second sample collection (of urine, saliva or blood) is made the following morning before breakfast providing a 10 h equilibration time [81, 82].

5.4.6. Extreme temperature or physical activity conditions

High temperature and/or high levels of physical activity (for example athletes during training), increase the risk of dehydration and thus a potential violation of the assumption of constant FFM hydration of 0.732. Routine measurements of body weight can help the individual to maintain fluid balance, particularly prior to TBW determination.

5.4.7. Medical study participants

Most medical conditions do not influence the procedures for measuring or calculations of TBW by isotope dilution. Doses are particularly easy in patients using a gastrostomy. Conditions that result in expanded extracellular fluid, however, may increase equilibration time. Oedema secondary to

congestive heart failure, burns, shock, severe obesity, some drugs, or pregnancy (as discussed above) can lead to a slower equilibration time. To deal with this, one can extend the time for the final specimen if the oedema is extreme. If concerns remain regarding equilibration time in a specific population, a pilot study may be performed in which specimens are collected hourly for up to 8 h, while all water intake is carefully measured. Time to equilibration as defined by the plateau value of calculated TBW corrected for water intake after the dose can then be calculated. It should also be noted that the excess extracellular water will invalidate the assumption of constant hydration of FFM. Consequently, this will invalidate the measurement of FFM by measurement of TBW, unless the excess hydration is stable and the new hydration factor is known.

Some medical conditions may influence specimen collection. Renal failure for example will require either saliva or blood sampling to determine the isotope dilution. Loss of participant cooperation due to coma or cognitive deficiency will require the same considerations as infants for urine collection, or use of saliva collected by mild suction or blood.

6. APPLYING ISOTOPE DILUTION TO THE ASSESSMENT OF TOTAL ENERGY EXPENDITURE

One of the advantages of the DLW technique is that it offers a great deal of flexibility in protocol design. However, the opposing view is that such flexibility can be confusing for new users. One of the first considerations in any project is the number of participants needed to address the study hypothesis. This requires a consideration of statistical power or the probability of detecting a given difference or effect in the outcome(s) of interest. Accordingly, the outcome(s) of interest must be identified along with the anticipated biological effect for that outcome, and the standard deviation for the measure. It is not possible to provide absolute answers regarding sample size that apply to all applications of the DLW technique, however some estimates can be provided.

As indicated in earlier sections, some of the specific outcomes of interest may include: the energy expenditures and hence energy requirements of specific populations; differences between the energy expenditures of different populations; changes in energy expenditure during an intervention; and, physical activity energy expenditure (AEE). Each of these outcomes dictates a different set of assumptions regarding the expected difference as well as the

standard deviation in the measurement. In some cases a reasonable estimate can be made for both of these parameters, in other cases it may be necessary to perform a pilot study to derive these estimates (see Appendix II for more detail).

6.1. GENERAL DLW PROTOCOL

There are two basic protocols for DLW, the two point and the multi-point approaches — each has advantages and disadvantages. The two point protocol in its minimal form requires three specimens, a pre-dose baseline, a post-dose specimen taken on the day of dosing after the isotopes have equilibrated throughout the body, and a final specimen taken at the end of the study (that is, at day 10–14). The multi-point protocol in its most extreme form generally involves taking a pre-dose baseline specimen and specimens every day after intake of the dose until the end of the sampling period.

An advantage of the two point protocol is that it requires fewer samples and provides the more exact estimate of TEE under conditions in which there is day to day variation in energy expenditure or water turnover. The disadvantage of the protocol is that it is most prone to analytical or sampling error and provides little data for assessing variance and internal consistency. In contrast, the multi-point protocol has the advantage of data averaging and thus minimizes analytical error. The protocol also provides an estimate of variation about the final TEE average, and also assures the investigator that the data will not be lost in participants who have high water turnover and may have reached unacceptably low levels of isotope enrichment by the planned last day of specimen collection.

In practice, the two approaches have been modified over time and are quite similar. For example, many users of the two point protocol obtain a pre-dose specimen and two specimens on the post-dose day and then two specimens on the final day for a total of five specimens. In contrast, many users of the multi-point protocol collect two pre-dose specimens, one specimen for each of first three days after dosing, and one specimen for each of the final three days of the measurement period, for a total of eight specimens. Example calculations from studies using both of these approaches are included in Appendix III.

6.1.1. Two point DLW protocol

The most common protocol for the modified two point approach uses morning dosing and sampling. Participants are asked to report to the clinical

site or collection point after an overnight fast (commonly from midnight). They may drink 100 to 250 mL of water during the fasting period if required. In situations where sweating and other forms of water loss during the night might be high, rehydration to restore body water levels is recommended.

A baseline urine sample is collected. Urine specimens should be collected in a urine cup or toilet hat, transferred to a storage device and capped. Urine specimens should be at least 50 mL and not left open to air for more than a few minutes to prevent evaporation. Specimens may be refrigerated or stored at room temperature for the remainder of the morning before processing. Great care should be taken to prevent contamination with tap water. If collection devices are rinsed and reused, be sure that they are completely dried between uses.

Shortly after collection of the baseline specimen, the participant should drink the DLW. The size of the dose relates to estimated TBW (see Table 3 on page 33). It is not necessary to be exact in dosing relative to body water, but care must be taken that the dose is carefully weighed with a relative accuracy of 0.3% and that the participant consumes the entire dose. After drinking the DLW, the container should be washed with about 50 mL of water and the rinsing water also consumed by the participant.

Participants should void 1 h after the dose to clear the bladder. This specimen need not be saved for analysis because it will not be in isotopic equilibrium with the body water. The time of the void, however should be recorded. The participant may be allowed a small meal for comfort, as well as a supply of fluid to maintain urine flow. The meal should be simple and less than 1250 kJ (300 kcal), or alternatively a liquid replacement meal. The time and volume of all liquid intakes should be recorded for correction of the TBW if the plateau method is used.

Two additional urine samples should be collected at 3 and 4 h after the dose if possible, but generally not more than 6 h after the dose for the final urine specimen. The participant should not drink and eat between the 3 and 4 h urine specimens to minimise any short term effect of water intake on urine enrichment. However, there are times that this is not possible because the individual needs some fluid to maintain sufficient urine flow to produce the final urine specimen.

On the final day, the participant should return to the specimen collection centre at about the same time of day as the 3 and 4 h specimens were collected on the dose day. Typical intervals between the dose and the final urine collections are 7, 10 or 14 days. Two voids should be collected with a 1 h interval between voids.

Urine specimens should be transferred to dry and tightly sealed tubes for storage and transport to the mass spectrometer centre. Vials should have screw

caps suitable for storage in freezers. The volume of vials may depend on the specific mass spectrometer centre, but 4–4.5 mL is generally sufficient. Some field centres prefer to save two aliquots just in case the first is lost in transit. Specimens should be transferred to labelled storage tubes with care taken not to contaminate the specimens with water or other urine specimens. A disposable pipette is an ideal transfer device and a new, clean and dry pipette can be used for each specimen. Specimens should be frozen (–10°C is sufficient) or refrigerated until ready for shipment.

An alternative approach used in some centres is to take the first post-dose sample on the day after the dose day and use the intercept method to calculate TBW. With a two point protocol this is inherently less accurate than the procedure outlined above.

6.1.2. Multi-point DLW protocol

Some investigators prefer to use more samples of body water than those collected on the first and last day of the metabolic period. However, the number of specimens is not a critical consideration with regard to the validity of the DLW technique. Rather, the choice of sampling frequency depends on the investigator's preference for precision of the method. The use of daily sampling along with the collection and analysis of two baseline samples allows the investigator to improve the precision of the measurement because the results are calculated from more specimens and the analytical variation is reduced by averaging [83]. The use of multi-point sampling allows the calculation of precision of the TEE [84]. Use of multi-point sampling may also allow the investigator to determine differences in energy expenditure for sub-periods within the metabolic period. For example, if the metabolic period is 14 days and the participants are being asked to exercise for 60 min on alternate days for three days each week, it is possible to calculate the energy expenditure for each day. These values are not very precise, because the change in enrichment over 24 h is small, however one can average the expenditure for the six exercise days and the eight non-exercise days and obtain an estimate of energy expenditure for these two conditions as well as the TEE over 14 days. Alternately, the investigator may elect to collect two pre-dose urines, urines on the mornings of days 1, 2 and 3 and urines in the mornings on days 12, 13 and 14 [36]. This provides for averaging to maximize precision while not inundating the analytical facility with the 16 samples expected for daily urine collection.

6.2. DLW DOSE TO MEASURE TEE

The dose of DLW is based on body size in an effort to match the body water enrichments to the IRMS precision. Dosing more than the prescribed amount increases the cost of the dose, but does protect against having too low a final enrichment for ^{18}O and ^{2}H. Dosing less than the prescribed amount will reduce the precision of the energy expenditure measurement and increase the likelihood of having a final enrichment that is too low to pass quality control tests (<60 times the measurement precision).

Assuming that the precision (± 1 SD) of analysis is about 1.5‰ (0.25 ppm) for deuterium and 0.15‰ (0.3 ppm) for ^{18}O, then the optimal doses for a typical DLW study in adults are 0.12 $g.kg^{-1}$ body water of 99 at.% deuterium labelled water and 1.8 $g.kg^{-1}$ body of 10 at.% ^{18}O water. The 99 at.% and 10 at.% are the most commonly used enrichments of the labelled water available on the market. In the event the more highly enriched ^{18}O water is used, then the dose should be reduced. For example, if the ^{18}O water were 95 at.%, then the dose would be 0.20 $g.kg^{-1}$ of total body water. These doses should produce post-dose enrichments of approximately 700‰ ^{2}H (110 ppm excess ^{2}H) and 90‰ ^{18}O (180 ppm excess ^{18}O). A 10% variance from one individual to the next in these initial enrichments is not considered to be significant. The ratio of the doses is chosen to minimize potential interference from slight variations in the natural abundance of fluids in the diet [36]. If the deuterium enrichment is to be measured by continuous flow IRMS after equilibration with hydrogen gas, the dose of deuterium labelled water may need to be increased to 0.5 $g \cdot kg^{-1}$ body water since the precision of analysis is less by this method [93]. See also Section 7.3.3.

Given that the dose is prescribed per unit of body water, an investigator must make estimates of TBW. This can be done assuming that 50–60% of the body weight in non-obese adults is body water. One can also estimate the body water for dosing purposes by using another body composition method. Overestimating or underestimating the dose by 10% is not considered a concern.

The dose can be pushed through a 0.22 μm filter prior to administration for sterilizing. The water or filtered water should be weighed to four significant digits. It should be in a bottle or other container that is easy to drink from. The container should be washed with 50 mL of tap water to ensure that the entire dose is administered because most containers will not deliver the final mL of the dose without washing.

Rather than preparing individual doses for each participant, in large studies it is easier to mix the required amount of 99 at.% deuterium-labelled water and 10 at.% ^{18}O labelled water in a large glass bottle, and then dispense

TABLE 3. DLW DOSES FOR ADULTS BASED ON BODY WEIGHT[a]

Adult males		Adult females	
Body weight (kg)	DLW dose (g)	Body weight (kg)	DLW dose (g)
<65	63	< 60	54
65.1–80	74	60.2–75	63
80.1–95	89	75.1–95	74
>95	100	>95	89

[a] Assuming samples will be analysed by dual inlet IRMS after reduction of water to hydrogen gas (see Section 7.3).

individual doses gravimetrically. A sample should be retained for mass spectrometric analysis. This procedure can be used to prepare enough doses to complete the whole study, unless more than 100 doses are required, then it is suggested that sufficient DLW for 100 doses be prepared. Assuming a study of 15 participants with two measures each is planned, a clean, dry, 2 L bottle (such as a borosilicate glass bottle with a PTFE-lined screw top) is tared and 1800 g of 10 at.% $H_2{}^{18}O$ is added and weighed to four significant places. Then 120 g of 99 at.% ^2H_2O is added, the solution is carefully mixed and weighed to four significant places. Pre-labelled and individually coded 125 mL polythene bottles with leak-proof screw caps are each tared and doses are aliquotted and then weighed into these, to four significant figures. The dose of this mixture based on adult body weight, is shown in Table 3.

6.3. CALCULATION OF TEE

For adults and adolescents, a similar approach and notation related to that used by the IDECG [36] is used to calculate TBW. This report demonstrated how this equation relates to that used by Halliday and Miller [17], except the individual isotope dilution spaces are calculated first

$$N_X\,(\text{kg}) = ((W \times A/a) \times (\Delta DD/\Delta BW)/(1000)) - (\text{cumulative fluid intake (kg)}) \quad (6.1)$$

where N_X is the isotope dilution space, W and 'a' refer to a portion of the dose that has been diluted for analysis, W is the amount (in g) of water used to make this dilution and 'a', the amount (in g) of the dose used in this dilution; 'A' is the dose (in g) taken by the participant; ΔDD is the enrichment measured in the diluted dose, which is the measured abundance in the diluted dose minus that

in the local drinking water used to dilute it; ΔBW is the enrichment measured in body water, which is the measured abundance in a post-dose saliva sample minus that in the pre-dose basal sample.

Units used to express isotopic abundance can be ppm, atom per cent or delta notation. The interconversion of these units is given in Appendix V. ΔDD and ΔBW should be expressed in the same units. The constant 1.041 corrects for non-aqueous hydrogen exchange. In older participants it may be possible to restrict fluid intake to a level where cumulative intake can be ignored.

In the procedure described in Section 6.1.1 and illustrated in Fig. 2 (two point protocol), ΔBW is the enrichment of the third post-dose specimen. In the multi-point protocol described in Section 6.1.2. and illustrated in Fig. 3, ΔBW is the y intercept of the isotope elimination curve (E_0).

TBW (g) values are calculated from N_x using the non-aqueous exchange values from the above

$$TBW_O = N_O/1.007 \quad (6.2)$$

$$TBW_D = N_D/1.041 \quad (6.3)$$

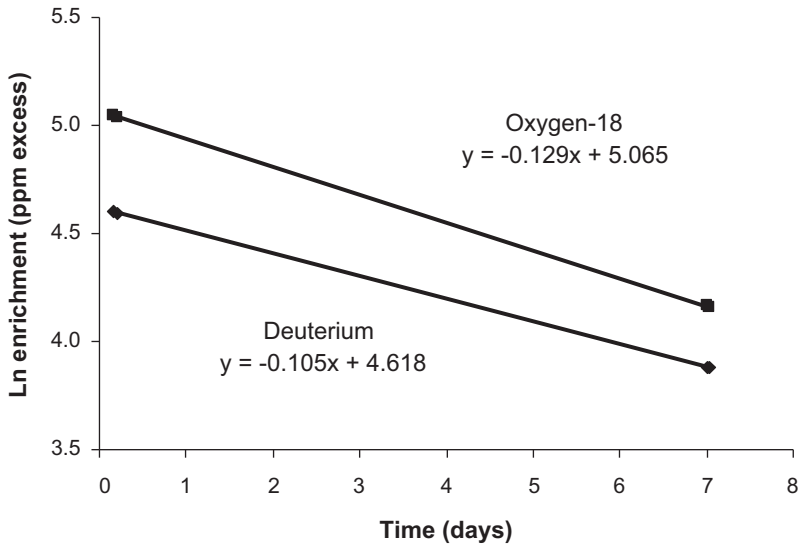


FIG. 2. Log-linear plot of isotope elimination in a two point DLW protocol. ln = natural logarithm; k_D = –0.105; k_O = –0.129. Enrichment can be in ppm or per mille units. ΔBW is the enrichment of the third post-dose specimen.

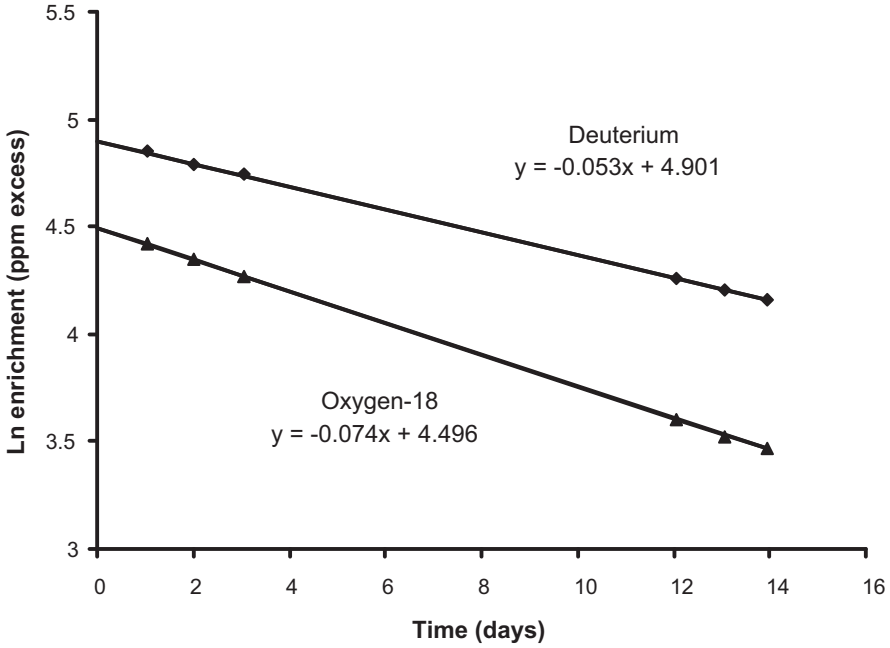


FIG. 3. Log-linear plot of isotope elimination in a multi-point DLW protocol. ln = natural logarithm; E_0 is the enrichment at time 0 = EXP(4.901) = 134.4 ppm excess for deuterium and EXP(4.496) = 89.7 ppm excess for ^{18}O; $k_D = –0.053$; $k_O = –0.074$. Enrichment can be in ppm or per mille units.

$$TBW_{AVG} = (TBW_O + TBW_D)/2 \quad (6.4)$$

The isotope elimination rates (k_x) are calculated from the gradient of the isotope elimination curve. In the two point protocol:

$$k_X = \ln(E_2/E_1)/(t_2-t_1) \quad (6.5)$$

where E is the enrichment calculated as $abundance_x - abundance_{baseline}$ and t is the time interval after the dose administration. The subscripts 1 and 2 refer to the specimen, where 1 is the post-dose specimen and 2 is the final specimen.

In the multi-point protocol, k_X is the gradient of the linear regression line through the isotope elimination data.

Carbon dioxide production and energy expenditure are calculated as follows:

$$rCO_2 = 0.455 \times TBW\ (1.007k_O - 1.041k_D) \quad (6.6)$$

To complete this calculation requires an estimate of the respiratory exchange ratio [61] and then energy production can be calculated using standard indirect calorimetric equations [62]. The easiest equation to utilize is the modified Weir equation [63]:

$$TEE\ (kcal/d) = 22.4 \times rCO_2 \times (1.10 + 3.90/R) \quad (6.7)$$

Finally, if desired, the rate of water production can be calculated. This is total water production (liquid and vapour) and is equal to total water input (fluids, food moisture, metabolic water, and inhaled water vapour). This calculation assumes that TBW has not changed by more than a few per cent from the start to the end of the protocol. TBW can change substantially during the course of a DLW study in infants. This can be addressed by assuming exponential increase, or linear increase, in body water proportional to body weight change during the same period

$$rH_2O = (N_D \times k_D)/(f) \quad (6.8)$$

where *f* is the fractionation correction for gaseous water loss, which is estimated in adults and adolescents as:

$$f = 1 - 1.45 \times TBW \times (1.007k_O - 1.041k_D)/N_D \times k_D \times 0.059.$$

6.4. QUALITY CONTROL

All of the considerations above assume that the laboratory performing the isotope analysis is providing accurate and precise isotope ratio analyses. Instrumentation, as described in other sections of this report, has dramatically improved over the past two decades. Nevertheless, IRMS analysis is significantly different from other mass spectrometric techniques and a lengthy learning period is not unusual. Because of the unusual nature of the mass spectrometry and because of the importance of precision in the power estimates, an internal check or study is highly recommended for all laboratories. Such a study should be performed using either mock urine water and urine specimens or actual specimens for which sufficient volumes are available for five analyses. Because the same specimens are used for the study, the only source of variation is analytical. A laboratory should be able to determine TBW with a relative standard deviation of 0.5–1.5%, the elimination rate constants for the two isotopes with relative standard deviation of 0.5–1.5%, and TEE with a relative standard deviation of 3–6%. Precisions worse than these will reduce the precision of any study and require

larger sample sizes and therefore reduce the scientific value of the study. Such precisions are attainable, but in an earlier cross-laboratory study performed by Roberts et al. [85], at least half of the 11 laboratories involved in the comparison failed to meet these requirements for urine specimens.

6.4.1. QC checks: Two point protocol

For each DLW analysis, there are generally five quality control estimates that can be used to check whether analyses are internally consistent. The first is to check the internal precision of the duplicate or triplicate analyses of each isotopic abundance analysis. If the ranges are greater than two times the internal precision one should be suspicious of the results.

The second is to check if post-dose urine samples collected on the dose day show signs of isotopic disequilibria. The isotopic enrichments of the two post-dose urines should agree to within 4%. If not, urine may not be isotopically-equilibrated with plasma and the results are suspect, and the protocol revised to include urine sampling on the dose day at 5 or 6 h.

The third quality control step is to compare the two isotope dilution spaces. The ratio of the deuterium space to the ^{18}O space should be between 1.000 and 1.070 with the ideal ratio for adults being 1.034. If the ratio is outside this limit, then the results are also suspect.

The fourth quality control step is to inspect the ^{18}O enrichment of the urines collected on the final day. These enrichments should exceed 60 times the measurement precision or about 8‰ or 16 ppm excess for a highest quality laboratory. Smaller enrichments generally provide imprecise DLW results because of natural variation in the fluids consumed by the participant as well as random error in the measurement of isotopic enrichment. DLW results should not be reported if the final enrichment is too low. If a urine specimen was collected at a time intermediate between the post-dose urines and the final urines, then calculation should be made based on the enrichments of the urines collected at the shorter time interval assuming it is at least long enough to have allowed the isotopic enrichments to fall to 70% of the post-dose enrichment.

The fifth and final quality control step is to compare the energy expenditure calculated using the 3 h post-dose urine and the first final day urine with that of the 4 h post-dose urine with the second final day urine. These should agree within 2 standard deviations for the analytical precision of the DLW procedure in one's laboratory. An agreement within 8% is the preferred value. If the two calculated energy expenditures differ by more than 8%, results are suspect.

If the DLW analysis passes all five quality control assessments, then results may be reported with confidence. If the results are suspect because of

one or more quality control failures the data input should be checked and if necessary the isotopic analyses should be repeated.

6.4.2. QC checks: Multi-point protocol

For each DLW analysis, there are seven quality control estimates that can be used to check whether analyses are internally consistent. The first is to check the internal precision of the duplicate or triplicate analyses of each isotopic abundance analysis. If the ranges are greater than two times the internal precision one should be suspicious of the results.

The second is to check R^2 of the regression line through the enrichment data (Fig. 4). It should be >0.99 for both ^{2}H and ^{18}O. If not, check the date and time of the samples and for errors in data input.

The third is to check for covariance of ^{2}H and ^{18}O enrichment (Fig. 5). This is done by plotting the residuals of the enrichment in post-dose specimens. The residual is the difference between the observed enrichment and that predicted from the linear regression line through the data.

The fourth quality control step is to compare the two isotope dilution spaces. The ratio of the deuterium space to the ^{18}O space should be between

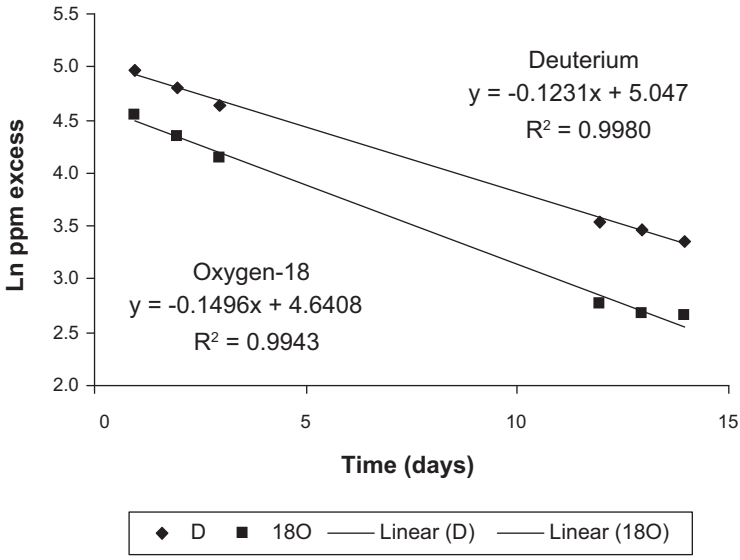


FIG. 4. Elimination of ^{2}H and ^{18}O with regression lines through the enrichment data.

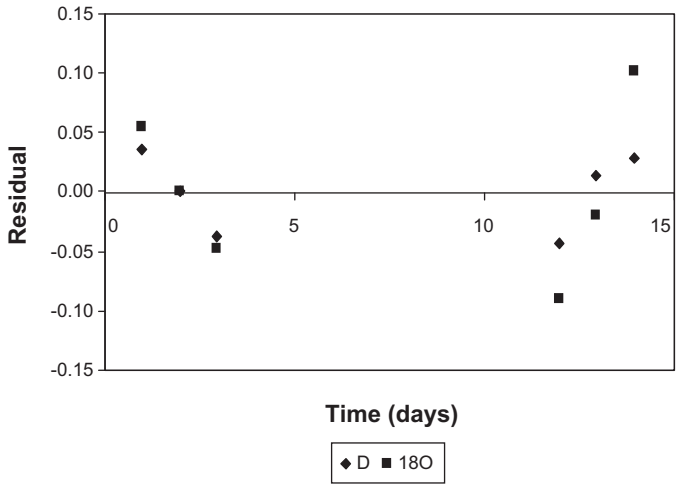


FIG. 5. Residual plot showing covariance of ^{2}H and ^{18}O.

1.000 and 1.070 with the ideal ratio for adults being 1.034. If the ratio is outside this limit, then the results are suspect.

The fifth quality control step is to inspect the enrichment of ^{2}H and ^{18}O of the specimens collected on the final day. The enrichment of ^{18}O should exceed 8‰ or 16 ppm excess. If the equilibration technique is used to prepare hydrogen samples for analysis, the final enrichment should be greater than 128‰ or 20 ppm excess. Smaller enrichments generally provide imprecise DLW results because of natural variation in the fluids consumed by the participant as well as random error in the measurement of isotopic enrichment. Specimens with enrichments below this level should not be included in the calculation of k_X and ΔBW. An advantage of the multipoint approach is that urine specimens collected on an earlier day can be utilized and the calculations based on the enrichment of urines collected over a shorter time interval, assuming it is at least long enough to have allowed the isotopic enrichments to fall to 70% of the enrichment on the first day after dosing.

The sixth quality control step is to consider the ratio of the isotope elimination rates (k_O:k_D). This should be between 1.1 and 1.7.

The seventh and final quality control step is to compare the isotope elimination rates calculated by dropping one data point at a time, i.e. using 5 out of 6 of the data points in the above example. This approach is described by Wolfe [86]. The coefficient of variation should agree within 2 standard deviations for the analytical precision of the DLW procedure in one's

laboratory. An agreement within 8% is the preferred value. If the calculated isotope elimination rates differ by more than 8%, results are suspect.

If the DLW analysis passes all seven quality control assessments, then results may be reported with confidence. If the results are suspect because of one or more quality control failures the data input should be checked and if necessary the isotopic analyses should be repeated.

6.5. CONSIDERATION FOR SPECIAL POPULATIONS

6.5.1. Infants

Infants have a higher water turnover rate than adults and therefore the dosing and/or sampling protocol should be different. In practice, sampling for one week is often appropriate. As noted above, in infants, assessing TBW by means of the back-extrapolation approach is an alternative, especially in a DLW experiment where the protocol will provide the appropriate information. The DLW technique can be applied both in breastfed and bottle fed infants. Parents of bottle fed infants should be asked to use one defined source of water before and during the experiment when preparing formula. In countries where formula is typically purchased in the ready to consume liquid form, the parents should be asked to use only one manufacturer's infant formula product. If weaning the infant from the breast coincides with the DLW experiment, errors will be introduced, but these can be reduced by manipulating the protocol [87].

Because infants do not void on request, urine collection requires sampling as available. Children should be fitted with either a urine collection bag or a cloth nappy or diaper. Paper diapers with fluid absorption gels must not be used because it very hard to extract any urine and because they generally absorb moisture from the air before use and this dilutes the stable isotope in the urine causing an overestimate in TBW and TEE. To simplify specimen extraction, a small cotton insert should be placed inside the diaper along with a small plastic sheet to isolate the small cotton insert from the diaper. When the insert is very wet, the diaper should be changed and the insert placed in a sealed plastic bag. The urine is extracted by placing the cotton insert into a 50 mL syringe and squeezing out 3–5 mL of urine by compressive force. Care should be used to ensure that the diaper and insert are dry before use and that the insert is fully wet before removal to minimize specimen dilution by absorbed atmospheric moisture. It is also important that appropriate action is taken to avoid the risk of evaporation of urine water with subsequent isotope fractionation. This can be minimized by placing the diaper in a plastic bag quickly after removal and chilling before urine extraction.

Because infants in the first six months of life have a larger body surface area to weight than older children and adults, the equation for calculating rCO_2 needs to be modified with regard to isotope fraction of water lost from the body as vapour. The revised equation is:

$$rCO_2 = 0.445 \times TBW\ (1.007k_O - 1.041k_D) \quad (6.9)$$

Equation (6.6) is used by most laboratories for all other age groups. For FFM calculation (see Section 5.4.2).

6.5.2. Preschool children

The only special consideration here is dosing and perhaps specimen collection. All other procedures are identical to the average adult. Because preschool children may not wish to drink the water, a small amount of sweetened drink mix may be added to the dose and wash so that the preschool child is more likely to consume the dose and wash over a short time period. Straws are recommended for dose administration at this age. Care should be taken to avoid spillage of any dose clinging to the straw when the wash water is added. Greater supervision of specimen collection may be required in this age group. As with studies in infants, a sampling interval of one week is appropriate.

6.5.3. Children and adolescents

No special considerations are required for this age group unless the child is unwilling to drink the dose. In that case, a drink may be used as indicated for preschool children. For FFM calculation see Section 5.4.4.

6.5.4. Elderly

One special consideration may be the use of a straw for dose administration. Elderly individuals with shaky hands should be asked to use a straw to avoid spillage. Care should be taken to avoid spillage of any dose clinging to the straw when the rinsing water is added. In addition, many elderly experience post-void urine retention in the bladder. This slows the isotope equilibration process in the urine. This may require later dose day urine collections, if the two-point approach is used.

6.5.5. Extreme temperature or physical activity conditions

High temperature and/or high levels of physical activity (for example, athletes during heavy training) increase the biological elimination rates of the isotopes (k_O and k_D). Because of this, a two-week elimination period for TEE may result in isotope abundance that is too near to baseline for accurate assessment of the elimination rates and hence the calculation of TEE [83]. Under these circumstances, the period between dosing and final urine collection should be shortened. A one week period is generally recommended. A minimal final ^{18}O enrichment that is accepted is 8‰ (16 ppm) above baseline, or approximately 50 times the precision of the instrument.

6.5.6. Medical issues

Intravenous (IV) fluids

IV fluids are made from distilled water and have an isotopic composition that is different from ground water and meteoritic water. This can cause a shift in the background isotopic abundances that can result in an error in the DLW method. If patients are receiving or are expected to shortly begin receiving IV fluids daily, then the DLW testing period should be delayed until the fourth day of IV administration at the earliest, but the seventh day is preferred. In this way, the patient has had sufficient time for the baseline ^{18}O and deuterium abundances to respond to the change in background isotopic abundance to minimize the potential isotopic artifact. For patients receiving a single IV fluid or several days of IV fluid administration, the DLW dose should not be given until five days after the last IV administration. If the IV fluid administration occurs during the metabolic period of the DLW test, then the multi-point method should not be used. The two point method, however, can be used, but the period of IV administration should be limited to after the final specimen collection of the dose day and not on or after the 5 days before the final specimen collection [88].

Abnormal body fluid

Oedema or reduced blood perfusion secondary to shock or very poor vascular function can increase the time for the DLW dose to equilibrate with body fluids. In a healthy individual, equilibration generally occurs within 3 h after an oral dose of DLW. Expansion of the extracellular water pool or reduced blood flow may extend this to 5 h. If these are concerns, then there are two options for the investigator. One is to use the multi-point method with the

first post-dose specimen not collected until 24 h after the dose to ensure equilibration. The two point method can be used, but additional specimens should be collected at 5 and possibly 6 h after the dose to check for a post-dose plateau in the isotope abundance.

Altered renal and urinary function

Reduced urine production or post-void urine retention in the bladder will lengthen the time to reach isotope equilibration into urine. The increase in equilibration time may be quite long. The incidence of post-void urinary retention is believed by some to be most common in persons over 65 years of age, but others believe it can also be significant in younger individuals. This problem can have a major influence on the post-dose urine specimens, but minor influence on later urine specimens. The investigator again has several options. One can substitute a plasma or serum specimen for the 3 h post-dose urine specimen for the two-point DLW protocol as these conditions do not alter the isotope equilibration with body water, but only with the urine. The investigator may also use the multi-point approach with the first specimen delayed until 24 h after the DLW dose. Ongoing treatment with diuretics should have little to no effect on the DLW technique.

Surgery or trauma

These conditions alone do not have a major impact on the DLW method except that they may expose an individual to IV fluids. The impact of these fluids has been discussed above.

Conditions that cause change in TBW

Novel diuretic treatment of oedematous patients, treatment for severe dehydration, rapid growth or other conditions that change TBW by more than 5% can introduce an error in the DLW technique. This is because the calculations presented above assume steady-state with respect to TBW. If the TBW is within 5% at the start and end of the DLW period, there should be little influence of the change in TBW on the DLW technique [89]. However, if there is a change between the TBW at the start and at the end of a DLW metabolic period, then a change in the calculations is required. This can generally be accomplished by measuring or estimating TBW at the end of the DLW period and using the average of the TBW measured at the start and end of the DLW period in Equation 6.6 or 6.9 depending on the age of the participant. TBW can be measured at the end of the DLW with a second dose of deuterium (less

expensive then ^{18}O). However, it should be remembered that the baseline for this second TBW determination is the specimen collected just before the second dose. Fluid intake between this second deuterium dose and the following specimens for the measurement of plateau enrichment should be limited as the fluid will cause a decrease in the deuterium enrichment of the baseline or background enrichment and cause the TBW to appear smaller than is the case. The measurement of TBW at the end of the DLW period does, however, lengthen the study and increase participant burden. Measurement of weight change from beginning to end of the DLW metabolic period can usually be substituted for a second TBW measure. The change in TBW can be estimated from change in weight assuming the hydration percentage of weight measured at the start of the DLW period or simply assuming a central hydration value (50%) to minimize potential error from changes in hydration. Failure to accommodate a systematic change in TBW with the appropriate calculation results in a relative error in the DLW measured TEE equal to one-half the relative change in TBW. Thus, a 5% change is usually considered negligible as the error would only be 2.5%. For studies in which all participants undergo the same change in body water such as in a neonatal growth study, however, correction for even a 5% change in TBW may be desired.

7. SAMPLE STORAGE AND ANALYSIS

7.1. SAMPLE HANDLING AND STORAGE FOR TOTAL ENERGY EXPENDITURE AND TBW STUDIES

The considerations for DLW and TBW are different than for most biological specimens. This is because water is resistant to breakdown and therefore refrigeration and freezing are not required. However, refrigeration is recommended to minimize bacterial and mould growth which would produce metabolic water that would dilute the isotopes in the specimen. Refrigeration also minimizes the accompanying odours that make specimens less pleasant to work with. The major concern during handling and use is the minimization of evaporation and contamination. Both processes will alter the isotopic abundance of the specimen and introduce error into the calculation of TBW or TEE.

Contamination can occur from water, humidity in the air, and other specimens. To minimize the contamination from water, and dilution of the

specimen, all specimen containers or handling equipment should be dry before use. For example, if a urine collection "hat" is reused by a participant, great care should be taken to rinse the container after the first use and then towel dry the container. Air drying is generally incomplete and should be avoided. To avoid contamination with humidity, do not leave specimens exposed to air for more than a few minutes and do not store small specimens (<1 mL) in containers with large head spaces (>50 mL). If blood plasma is used as the specimen, avoid the use of blood collection tubes that contain water such as those containing aqueous heparin. To avoid cross-contamination of specimens, always use fresh, dry pipettes when transferring specimens.

Evaporation can cause the specimens to concentrate the heavy isotopes and become slightly enriched. Evaporation can be avoided by not leaving the specimen exposed to air for more than a few minutes. This is particularly important when the specimens are warm (body temperature).

Another way of avoiding contamination by humidity and evaporation is by not working with small specimens. Keeping specimens sealed for all but a few minutes may work for specimens that are 1 mL or larger, but even this exposure is too long for smaller specimens. Investigators working in humid, tropical climates may find that specimens of 2 or 3 mL are preferred and that head spaces in the containers should be limited to 1 or 2 mL.

If specimens are frozen before aliquotting for analysis, be sure to fully thaw the specimen and gently mix to avoid isotope fractionation between the liquid and solid forms of water.

Specimens should be collected in containers that can be capped until they are aliquotted into long-term storage containers. Plastic containers (1, 3, 5, or 10 mL depending on laboratory requirements) with airtight elastic-sealed screw caps are recommended. Containers recommended for storage down to –70° C are ideal as the O-ring seal remains elastic even in the freezer.

A final precaution is to never store or ship specimens in the same device or box as the DLW dose water or the 10 at.% $H_2{}^{18}O$ or 99 at.% deuterium oxide. Even a few μL of such highly enriched materials transferred as vapour can seriously contaminate a specimen.

7.2. SAMPLE ANALYSIS

A mass spectrometer is the most versatile analytical instrument for measuring stable isotopes in terms of sample throughput, sensitivity and selectivity. A variety of less sensitive spectrometry techniques exist, such as Fourier transform infrared (FTIR) spectrometry, which can be used as an alternative to mass spectrometry for deuterium analysis in studies of body

composition and human milk output. As analysis of ^{18}O currently requires mass spectrometry and TEE studies using the DLW technique use both ^{2}H and ^{18}O, DLW samples are analysed using isotope ratio mass spectrometry (IRMS).

A mass spectrometer is an instrument that separates ions in a high vacuum according to their mass to charge ratio (m/z). The major components of a mass spectrometer are the inlet system, high vacuum, ion source, mass analyser and detector. Modern mass spectrometers are computer controlled and have sophisticated data processing software. The abundance of the stable isotopes of C, H, O and N are measured in simple gases using IRMS with individual detectors for each isotope.

7.3. ISOTOPE RATIO MASS SPECTROMETRY

An IRMS is a low resolution, magnetic sector mass spectrometer with multiple detectors. The sample must be introduced to the mass spectrometer ion source in the form of pure gas molecules (CO_2, H_2 or N_2). Sample gas is ionised by electron impact from electrons emitted from a hot filament within a high vacuum. The ions are separated in a magnetic field. The current of each ion beam is measured as the charge generated by ions impacting a detector for each isotopic species, known as Faraday Cups. Each sample is compared with a reference gas of known composition. IRMS can accurately measure very low enrichments, down to natural abundance.

There are two kinds of IRMS in terms of their inlet systems: dual viscous inlet gas IRMS (DI-IRMS) and continuous flow IRMS (CF-IRMS) systems. DI-IRMS is the classical technique. It requires pure gas samples (H_2 or CO_2) which are prepared off-line. Samples of pure hydrogen gas from body water specimens are prepared either using reduction or equilibration. For ^{18}O analysis, excess fluid samples are equilibrated with a minimal volume of CO_2 to produce $C^{16}O^{18}O$ for analysis. In a continuous flow IRMS, gas samples are purified on-line and are swept into the IRMS in a stream of helium carrier gas. Although DI-IRMS can have superior precision, CF-IRMS has the advantage in terms of automation, throughput and reduced sample size.

The details of isotope analysis are generally instrument-specific and therefore cannot be fully detailed in this report. Therefore, this commentary is limited to a general description of the analysis and several important, but general cautions.

IRMS is used in most laboratories for the analysis of the isotope abundances of deuterium and ^{18}O in specimens. IRMS has a high degree of isotope specificity and low (sensitive) detection limits. The preferred sample forms for the mass spectrometer are hydrogen gas for deuterium and carbon

dioxide for ^{18}O, because these molecules present little isotopic interference and are somewhat inert in the instrument and subject to a minimum of memory on absorptive surfaces. However, some instruments have been designed for water or other molecules in order to minimize analysis time or sample size requirements.

When performing IRMS analyses, several approaches can maximize the precision of the TBW or TEE measurement. One of the most important is to include all of the specimens from a single participant in the same batch of specimens to be analysed. This is because within-day precision is two to three times better than day to day precision and therefore propagates a greater precision in TBW and TEE. A memory of 2–3% between specimens can be included in automated deuterium analysis. Additionally, the order of analyses may be arranged to analyse baseline specimens directly after the unlabelled water secondary standard, followed by the 14 d (least enriched) specimens, and then the post-dose specimens. Analyses can also be performed in duplicate or triplicate to allow for washout of the memory effect. Finally, diluted dose water and the diluting water should be analysed in the same batch as these values are required for calculation of dilution space, TBW and TEE. Natural abundance and enriched water standards should be included in each batch to correct for any day to day variation in the isotopic scale.

7.3.1. Standards (and dose sample)

Good laboratory practice requires standards to be traceable and analytical instruments to be monitored for accuracy and reproducibility. It is impractical (and often expensive) to continually use a primary standard as one's reference material. Hence, laboratory working standards need to be prepared and calibrated.

The following guidelines will help in the preparation of a gravimetric standard curve of ^{2}H enriched water standards. Two sets of standards are described. One is a large set of standards to test instrument linearity over the entire working range. These are generally needed only when the instrument is new, for problem solving when the instrument is not functioning properly, or testing linearity after major repairs. The second set is a smaller set of working standards that should be used on a daily basis.

Starting material

$^{2}H_2O$ is usually purchased at about 99–99.9 at.% ^{2}H, that is, at least 990 000 ppm ^{2}H. When undertaking a TBW or DLW measurement sufficient tracer should be used to raise TBW enrichment to a measurable level for the

duration of the experiment. This is usually 100 ppm excess ^{2}H (640‰) in body fluids (more if FTIR is to be used for analysis). For an adult male of 80 kg with 40 kg TBW, this would be a dose of 4 g 99 at.% $^{2}H_2O$. Further, the IRMS should be calibrated through the likely measurement range, say 0 to 250 ppm excess ^{2}H.

$H_2{}^{18}O$ is usually purchased at 10 at.% ^{18}O because the cost per unit of isotope is lower than more highly enriched material, although when dose volume is limited, the more highly enriched material may be preferred. Even 10 at.% ^{18}O hydride (water) is 100 000 ppm ^{18}O and thus well outside the range encountered in typical specimens for TBW or TEE and therefore must be diluted to make standards. For TBW determinations, specimens are generally in the range of 80 ppm excess ^{18}O (40‰). For TEE measurements a larger dose is used so that excess ^{18}O can be followed for one to two weeks after the dose. Thus, specimens range from 200 ppm excess ^{18}O (100‰) down to zero excess (baseline). Therefore, IRMS calibration standards must be prepared from 0 to 250 ppm excess to ensure that the IRMS is calibrated through the likely range of use.

Preparing a range of standards

Both FTIR and IRMS require a full range of standards to confirm that the instrument is linear over the range of use. After this is done, two working standards are required for TBW (natural abundance and an enriched standard, for example 150 ppm excess ^{2}H for IRMS or 1000 $mg.kg^{-1}$ for FTIR). For DLW measurements, ^{18}O and ^{2}H standards are required at natural abundance, 150 ppm excess (or the maximum target enrichment) and a standard that approximates the end urine isotopic enrichment.

It should be noted that ppm for IRMS is traditionally calculated using molar ratios and not weight ratios. Thus, 150 ppm for deuterium means that there are 150 atoms of deuterium and 1 000 000 atoms of protium.

Plan to make a range of dilutions between 0 and 150 ppm excess ^{2}H and 0 and 250 ppm excess ^{18}O. Make large volumes (50–250 mL) of each stock solution that can be reanalysed later. Store in the dark in airtight bottles. Storage upside down in boxes is recommended as a leaking seal will not cause fractionation of liquid whereas evaporative loss of vapour will. Ensure that there are at least two working standards for routine use that are available in very large volume, say 1 to 2.5 L at 0 and 150 ppm excess. Plan to have the following series: 0, 10, 25, 50, 75, 100, 150, 200, 250 ppm excess ^{2}H and a separate set of standards made up for the same nominal ppm excess ^{18}O to span the likely ranges from the unknown samples. The two isotopes are not mixed for these linearity standards because it is far more difficult to calculate ppm

from the gravimetric data when both isotopes are mixed. One should use a large volume of local drinking water as the zero enrichment and for dilutions. Each sample should be weighed independently to avoid compounding weighing errors. Pipetted volumes are not sufficiently accurate. A dilution series that aliquots a previous dilution simply compounds any error. If using a pipette, be sure to weigh each aliquot. Therefore, the series may be as illustrated in Table 4.

The approximate ppm and delta values assume that the local tap water used is 155.7 ppm or 0 ‰ δ^2H VSMOW for deuterium. The water may range, however, from 130 ppm or –150‰ δ^2H VSMOW to 156 ppm or 0‰ δ^2H VSMOW, depending upon latitude. The approximate ppm values assume that the local tap water is 2005 ppm or 0‰ δ^{18}O VSMOW for ^{18}O. This may range from 2005 ppm down to 1965 ppm or -20 to 0 δ^{18}O VSMOW, depending upon latitude.

Each item must be weighed accurately (4 decimal points for deuterium) and using the ^{2}H supplier's nominal abundance, recalculate the approximate ppm accordingly. This calculation uses the molecular weight of each isotopic form of water. To calculate fractional abundance, each should be converted from weight to moles. In practice, this calculation is coded into a spreadsheet. For example, the apparent enrichment of the nominal 150 ppm excess ^{2}H sample can be calculated as follows:

TABLE 4. STANDARDS OF ^{2}H AND ^{18}O

ppm ^{2}H excess (Nominal)	Tap water (g)	99.9% ^{2}H (g)	Approximate ppm* (δ^2H)	ppm ^{18}O excess (Nominal)	10% $H_2{}^{18}O$ (g)	Approximate ppm* (δ^{18}O)
0	2500.0	0.0000	156 (0)	0	0.0000	2005 (0)
10	250.00	0.0030	166 (64)	10	0.0250	2015 (5)
25	250.00	0.0070	181 (160)	25	0.0630	2030 (12)
50	250.00	0.0140	206 (321)	50	0.1300	2056 (26)
100	250.00	0.0300	264 (694)	100	0.2500	2104 (49)
150	2500.0	0.4200	307 (970)	150	3.8000	2155 (75)
200	250.00	0.0560	358 (1297)	200	0.5000	2203 (99)
250	250.00	0.0700	408 (1618)	250	0.6300	2254 (124)

* Approximate ppm (per mille (‰)) is not accurate until compared with the international standard, Vienna Standard Mean Ocean Water (VSMOW).

Molecular weight water = 18.0153
Molecular weight ^2H_2O = 20.0254
Abundance of isotope supply = 99.99 at.% ^2H
Natural abundance of ^2H in water = 0.015 at.% ^2H
Enrichment of isotope supply = 99.99 – 0.015 = 99.885 at.% excess ^2H
Weight of local drinking water = 2500 g
Moles of local drinking water = 2500/18.0153 = 138.77
Weight of deuterium stock = 0.4200 g
Moles excess deuterium in stock = 0.4200/20.025 × 99.885/100 = 0.02095
Enrichment = $10^6 \times 0.02095/(138.77 + 0.02095)$ = 150.95 ppm excess ^2H

By adding natural abundance, the enrichments can then be converted into ppm units of abundance and then to delta notation as required, using:

$$\delta = ((\text{ppm} - \text{ppm}_{\text{VSMOW}})/\text{ppm}_{\text{VSMOW}}) \times 1000 \quad (7.1)$$

where VSMOW is 155.7 ppm for D and 2005 ppm for the ^{18}O of CO_2 equilibrated with VSMOW. In the above calculations, it was assumed that the natural abundance of local drinking water = 155.7 ppm ^2H or 0‰ δ^2H VSMOW and 2005 ppm or 0‰ $\delta^{18}O$ VSMOW.

Hence for D:

Abundance = Enrichment + 155.7 = 306.6 ppm ^2H

δ^2H (‰) = 1000 × ((306.6/155.7) – 1) = 969 δ^2H VSMOW
or in the form of the equations given in Appendix V (V-4 and V-7)

δ^2H (‰) = 1000 × ((R/0.000 155 76) – 1)
where R is (ppm ^2H/(1 000 000 – ppm ^2H))

δ^2H (‰) = 1000 × (((306.6/(10^6 – 306.6))/0.000 155 76) – 1) = 969 δ^2H VSMOW

If the enrichment of the ^2H_2O supply is not the same as this example, enter the new value in the formulas above and recalculate the enrichments. Finally, the true enrichment of this series should be measured by comparison with an independent international standard during analysis of this standards curve. If desired, the true abundance of the isotope source can then be derived by back calculation.

Daily working standards

For day to day analysis, isotope analysis laboratories should prepare a minimum of two secondary or working standards and possibly three depending on whether the goal is TBW or TEE. These should be prepared by adding carefully weighed amounts of deuterium and ^{18}O to the water. The water standard is isotopically similar to a participant's baseline specimen. The second working standard is an enriched standard made by weighing 0.014 g of 99% deuterium oxide with 0.2 g of 10% ^{18}O hydride for each 100 g of water. If an intermediate standard is also desired, it should mimic a 14 day specimen. For this, weigh 0.003 g of deuterium oxide and 0.03 g of 10% ^{18}O hydride with each 100 g of water. Laboratories should make enough working standard to last ten years and package the standard in small, well sealed containers so that any container is not used for more than 6–12 months.

7.3.2. Sample preparation by chemical reduction of water

Sample preparation for dual inlet IRMS is often by chemical reduction of water to prepare pure hydrogen gas. Matrix effects can be encountered when variations in sample matrix (between samples and/or between samples and standards) cause systematic differences in measured isotope ratio. These are more likely when chemical reactions are undertaken during sample preparation. Probably the most significant report has been that of an effect of urine solids when using zinc reduction to produce hydrogen from water [90]. Early work took the sample preparation technique from hydrology and used depleted uranium to reduce water to hydrogen gas for analysis. Use of cryogenic traps to recycle unreacted water through a vacuum line, ensured the original off-line reduction was quantitative. Off-line batch zinc reduction was introduced in an attempt to avoid use of depleted uranium as reagent. More recent introduction of chromium as a reducing agent has proven successful for pure waters on-line. The ease of preparing large numbers of samples of biological fluids by equilibration, without incurring matrix effects, has made this technique more popular. It is ideally suited to modern, fully-automated, continuous flow IRMS instruments.

The amount of sample preparation for physiologic specimens is generally small because the matrix effects for the isotope abundance measurement is usually small. Roberts et al. [85] however, showed the matrix effects do increase measurement errors in urine specimens more so than those for water and should not be taken lightly. Specifically, they reported results from a multi-laboratory study in which the accuracy and precision of mass spectrometric

isotope analyses were significantly degraded for urine specimens compared to water.

The matrix effect and hence the amount of sample preparation depends on the specific method in the analysis. Laboratory managers can detect these by comparing standards made up in water with those made up in the specimen under study. Differences in precision are easily detected by comparing the relative standard deviation for analyses of standards with similar isotope abundances but made up in different matrices. Accuracy can be investigated by making up carefully weighed specimens that have been spiked with weighed amounts of isotopic water. This requires great care and also recognition of the fact that urine is usually greater than 99% water by weight with only minor amounts of solids. Saliva is about 99% water and 1% solids by weight, plasma about 94% water and 6% solids by weight. The water percentage can be determined by freeze drying two to three aliquots of pre-weighed specimen and then determining the weight of solid residue after drying.

Some examples of the matrix effects and the required sample preparation to obviate the effect are provided below. The analysis of blood products for deuterium using either chromium or uranium reduction requires removal of the most organic solids [91]. This is because the organic hydrogen is also converted to hydrogen gas and this will dilute the hydrogen from water and make the specimen appear to have a lower enrichment than that of the water portion of the specimen. The preparation of urine is minor and only requires the addition of 200–400 mg of dry carbon black followed by filtration using a syringe filter to remove interferences. Blood products require the removal of protein. The amounts of residual solids are only minor problems in most laboratories. The matrix effect can generally be obviated by passing the specimen through a 50 000 Dalton filter using centrifugation. The filter assembly should be covered with parafilm with only a small pin hole to allow air into the specimen during centrifugation to prevent evaporation. This process usually produces 0.5–0.9 mL of filtrate from a 1.5 mL specimen.

7.3.3. Sample preparation by equilibration with hydrogen or carbon dioxide gas

The majority of laboratories undertaking IRMS analysis of ^{2}H abundance for TBW and DLW are now using equilibration to transfer ^{2}H from sample (water vapour) to hydrogen gas [92]. Equilibration is very suitable for large sample sizes. It is performed with 0.5 mL samples or reference waters held in septum capped tubes (12 mL Exetainers are typical) overlaid with hydrogen gas either using the IRMS autosampler or a dedicated manifold, in the presence of a platinum catalyst. Different catalysts can be used, from re-usable high surface area platinum impregnated rods or beads to disposable powders

containing much less platinum. The former achieve equilibration much more rapidly (4 h versus 72 h) but they are costly and need to be cleaned and dried prior to reuse. Deuterium analysis by equilibration with hydrogen gas can be subject to problems if a platinum catalyst is used in contact with the sample because many of the normal materials present in urine and blood poison the catalyst and slow the equilibration process. This is why some laboratories prefer to use the disposable catalyst in a small 'insert' tube which is not in contact with the sample. Constant temperature during equilibration and sample analysis is very important due to a strong temperature dependence of the equilibrium constant. Very good temperature control is particularly important when using the rapid equilibration techniques. An equilibrium constant of ~3.6 results in the enrichment scale being compressed in the gas phase (at equilibrium, the ^{2}H abundance in the gas is less than that of the water). This is an advantage in that a wide range of enrichments can be measured, but precision near natural abundance, although acceptable for TBW and DLW applications, is poorer than the best achieved using reduction (which does not introduce scale compression). Typical precision at natural abundance is 0.5 ppm ^{2}H (3‰ ^{2}H) using equilibration as opposed to 0.16 ppm ^{2}H (1‰ ^{2}H) using reduction. Reference waters must be prepared with each batch of samples, treated in exactly the same way as the samples and analysed in the same batch.

The exact approach to sample preparation will be instrument and procedure specific and should be described in full in the operating procedures of the analytical laboratory. This effect on precision may result in the choice of IRMS sample preparation procedure influencing tracer dosage, especially in DLW studies [93].

The majority of users of ^{18}O have employed the equilibration reaction between water and carbon dioxide to produce CO_2 for IRMS analysis [94–96]. CO_2 is an excellent gas for IRMS analysis. There is temperature dependence of the equilibrium constant so temperature control is important but unlike the case with ^{2}H analysis, the abundance scale is not significantly compressed. The analysis of the ^{18}O can be subject to matrix effects when analysed by equilibration with CO_2. For IRMS, this includes both air and organic compounds. Both the O_2 and N_2 interfere with the ion chemistry in the ion source and reduce accuracy, sometimes dramatically [96]. This matrix effect can be eliminated by either equilibrating the water sample with CO_2 in helium or another inert gas or by separating the O_2 and N_2 from the CO_2 using a 20 cm on-line gas-solid chromatographic column packed with chromosorb T [94, 96–98].

Other approaches to ^{18}O analysis exist. These include pyrolysis to CO and direct analysis of H_2O. Neither has any obvious advantage over equilibration

with CO_2. The former is used to analyse ^{18}O enrichment in organic oxygen. The latter was the approach used in one design of commercial IRMS that is no longer in production [99].

7.3.4. Isotopic calibration

Although it is possible to consider the isotopic accuracy of the analysis, absolute accuracy for any method is rare. This is because the isotopic scale may be reduced due to some aspect of analysis. The prime example is the analysis of deuterium by equilibration with hydrogen gas. The equilibration is subject to large isotope effect that reduces the deuterium abundance of the hydrogen gas by about 28% compared to the specimen itself. Thus, a specimen with 200 ppm ^2H will yield equilibrated hydrogen with 144 ppm deuterium. This in itself is not a major problem as long as the isotope effect is reproducible, that is, the standard deviation for replicate analyses is small as long as a diluted dose and tap water standard are run with the samples. As with all these techniques, it is essential to prepare and analyse reference waters in parallel with all unknown samples.

7.3.5. Corrections for scale compression

The greatest requirement for a correction for scale compression results from the use of equilibration to prepare hydrogen samples for analysis. The measured difference between any two isotopic samples or standards is often smaller than the actual or theoretical value. Additionally, the oxygen or hydrogen in the specimen is usually contaminated with a small amount of extraneous oxygen or hydrogen. This may come from exchange with materials in the analysis system or from extra material that contaminates the sample. For example, assume that you have a urine specimen that is –10‰ ^2H VSMOW (154.2 ppm) and a second that is 500‰ ^2H VSMOW (233.6 ppm), and that when these samples are analysed they exchange 5% of the hydrogen in the standard with the hydrogen (–60‰ or 146.4 ppm) in the walls of the quartz tube used in the hydrogen reduction reactor. If this happens, the natural abundance urine will measure at –12.5‰ (154 ppm) and the enriched urine at 472 per mil (229.3 ppm). The difference will be 484.5 per mil.

This scale compression if constant will not affect the calculation of TBW or TEE if a diluted dose is analysed with the specimens and is subject to exactly the same exchange. The compression, however, can be corrected by analysing a pair of known standards as follows:

$$\delta_{true} = (\delta_{obs} - \delta_{\text{low std obs}})\,(\delta_{\text{high std theor}} - \delta_{\text{low std theor}})/(\delta_{\text{hi std obs}} - \delta_{\text{low std obs}}) + \delta_{\text{low std obs}} \quad (7.2)$$

where the subscripts refer to the natural abundance working standard, the enriched working standard, and the observed or measured value, and the theoretical or true value. Abundance can be expressed as δ per mille (‰), or ppm.

7.3.6. Corrections for drift

In addition to scale compression, measured isotopic abundances are also subject to instrument drift and memory between successive specimens. Instrument drift refers to the systematic shift in the measured isotopic abundance between the first and last analysis during a daily batch of specimens. This may be due to changes in the instrument analyses caused by changes in room temperature, changes in source focusing due to power supply drift. In dual inlet IRMS, changes in the working standard in the instrument reservoir also need the application of a drift correction. This drift can be characterized by analysing a full batch of one specimen or working standard and measuring the change in the apparent isotopic abundance from the start to the end of the analysis. If this change is linear with time, then it can be measured by simply measuring a working standard at the start and end of the batch. All specimens can then be corrected for drift using the following equation:

$$\delta_{true} = \delta_{obs} + (\delta_{\text{stdt = end}} - \delta_{\text{std t = start}})\,(t_{specimen})/(t_{end} - t_{start}) \quad (7.3)$$

where δ_{obs} is the observed isotope abundance with drift, $\delta_{stdt=end}$ is the isotopic abundance of the working standard at the end of batch, $\delta_{\text{std t=start}}$ is the isotopic abundance at the start of the batch, $t_{specimen}$ is the time after the start of the batch at which the specimen is analysed, t_{end} is the time the working standard is measured at the end of the batch, and t_{start} is 0, the time the working standard is measured at the start of the batch. This can be expressed in per mille (‰) units or ppm.

If the calibration drift is small, then this correction is not necessary. For example, if the specimens from one set of samples collected from an individual participant are measured within 1 to 3 h and the drift is less than one third of the analytical standard deviation per hour, then the drift is comparable to the uncertainty in the measurement and will not have a large effect on the results for the dose range specified above.

7.3.7. Corrections for memory

A small amount of specimen can adhere to the analytical system and exchange with the next or next few specimens. This is referred to as specimen-to-specimen memory. It can be measured by analysing three–six replicates of a natural abundance (natabn) specimen followed by the same number of replicates of an enriched specimen, followed again by replicates of the natural abundance specimen. When this is done, the first specimen in each series will usually be a little different from the second and then the replicates should approach a plateau or constant value. The amount of memory can be calculated as:

$$\text{memory } \% = 100 \times (\delta_{\text{last replicate}} - \delta_{\text{first replicate}})/(\delta_{\text{enriched plateau}} - \delta_{\text{natabn plateau}}) \quad (7.4)$$

where δ is the measured abundance of each specimen as indicated by the subscript. The units can be per mil or ppm as long as the units are the same for all variables.

Sometimes, the memory observed in the second replicate after the step change in isotopic abundance is further from the plateau value than would be predicted by memory from the first specimen in the series of replicates. The memory for the second specimen can then be calculated using Eq. (7.4) above, but substituting $\delta_{\text{first replicate}}$ and $\delta_{\text{second replicate}}$ for $\delta_{\text{last replicate}}$ and $\delta_{\text{first replicate}}$, respectively.

If the memory for the first replicate is less than 4%, then memory correction of each specimen is suggested using Eq. (7.5) below:

$$\delta_{\text{true}} = \delta_{\text{n}} + (\text{memory}/100)(\delta_{\text{n}} - \delta_{\text{n}-1}) \quad (7.5)$$

This may require a two step correction should the second replicate memory be more than a few percent different from the first replicate memory. Also, note that the sign of the correction is vital as it may increase or decrease the observed value depending on the sign of the step change between specimens.

If the memory is larger than 4% or the step change between standards is more than 300–400‰, then it may be prudent to discard the first measured isotope abundance from any calculations and simply include an extra replicate. Memory effects in continuous flow IRMS systems can often be reduced by identifying and eliminating the cause (such as, when fitted, by changing chemical water traps regularly).

7.3.8. Calibration against international standards

It is not a requirement that working standards are calibrated against international standards, but it is useful as this is the only way to compare isotopic abundances between laboratories. This can be done by measuring the working standards in the same batch as two or more international standards. After correction for drift and memory, the working standards can be calibrated against international standards using Eq. (7.2). All measured isotopic abundances can then be converted to the international scale using Eq. (7.2) and the newly calibrated working standards.

When using per mille units, the preferred international scale for hydrogen and oxygen measured in water is Vienna Standard Mean Ocean Water (VSMOW), which is defined as 0‰ (155.76 ppm and 2005 ppm for deuterium and oxygen, respectively). Note that this actually ignores the natural fractionation that may occur between water and gas. In this case however, the value for oxygen is defined as the oxygen in CO_2 that has been equilibrated against VSMOW at 25°C, but this fractionation induced difference is the same for all specimens and thus it falls out of all calculations as long as all specimens are equilibrated in the same manner and temperature (±0.5°C). If a standardized CO_2 or H_2 is purchased for calibration, then these gases will often be calibrated without ignoring the fractionation and will be on a different scale [95].

There are six international standards for water analysis. These and their isotopic abundances relative to VSMOW are (Table 5):

TABLE 5. INTERNATIONAL STANDARDS FOR WATER ANALYSIS AND THEIR ISOTOPIC ABUNDANCES RELATIVE TO VSMOW

	Deuterium	^{18}O
IAEA SLAP	–428 (89.1 ppm)	–55.5‰ (1893.9 ppm)
IAEA GISP	–189.5 (185.3 ppm)	–27.8‰ (1995.5 ppm)
IAEA VSMOW	0‰ (155.76 ppm)	0‰ (2005.2 ppm)
IAEA-OH-1	–3.9‰ (155.2 ppm)	–0.1‰ (2005.0 ppm)
IAEA-302-A	508.4‰ (234.9 ppm)	
IAEA-302-B	996 ‰ (310.9 ppm)	
IAEA-304-A	502.5 ‰ (3012.8 ppm)	
IAEA-304-B	251.7 ‰ (2509.9 ppm)	

Aliquots of these international standards can be obtained from:

— IAEA: http://www.iaea.org
— NIST: http://www.nist.gov

7.4. FOURIER TRANSFORM INFRARED SPECTROMETRY (FTIR)

FTIR is a spectroscopic technique that uses the absorption of infrared light to determine the concentration of deuterium in a sample. FTIR can be used to measure the concentration of deuterium in saliva or plasma samples in studies of body composition using the deuterium dilution method to measure TBW. Absorption in the infrared region of the electromagnetic spectrum is associated with the energy of vibration of O–H and O–D bonds. The absorption band at 2504 cm^{-1} is used to determine the deuterium content of an aqueous sample. The intensity of this band is linearly related to the concentration of deuterium in the saliva sample.

7.4.1. Doses when saliva samples are analysed by FTIR

The signal to noise ratio in FTIR measurements is such that for accurate TBW determination the concentration of deuterium in water needs to be raised by about least 750 ppm. For adult women, TBW ranges from 26 to 33 L, while for men the range is usually between 38 to 46 L [100], and therefore this implies an adult dose of 20 to 35 g of ^2H_2O. On this basis a standardised dose of 30 g ^2H_2O is proposed for adults: For children calculation of the dose should be performed on an ad hoc basis: Rough estimates of the expected size of the body water pool may be obtained from predictive equations [101].

7.4.2. Preparation of the FTIR standard

The FTIR must be calibrated using a standard solution of known composition. This is prepared by weighing ^2H_2O into a volumetric flask, and then making it up with local drinking water. The maximum body water enrichment expected is 1000 ppm excess, and so weighing a nominal 1 g (accurate to 0.0001 g) into a 1 L volumetric flask conveniently makes the standard. This will give a solution that is 1000 ppm by weight. This solution can be stoppered and stored at 4°C for prolonged periods. It is a good idea to send aliquots of the standard and the water used in its preparation to an IRMS laboratory. The enrichment of the standard should be reported in mg/kg ^2H.

7.4.3. Infrared liquid cells

When performing infrared analysis the cells used to hold the sample must be transparent to the electromagnetic radiation. For investigation of aqueous samples calcium fluoride, CaF_2, is the material of choice but this material is attacked by urine, causing it to become opaque (therefore the recommendation that saliva is used). If it is not possible to get saliva samples of sufficient volume (for example in critically ill patients) then plasma is the next best choice. The cell is comprised of two transparent windows separated by a thin spacer. The sample is introduced so that a bubble-free aliquot lies between the two windows.

7.4.4. Filling the cell

It is of great importance that, after filling, the water in the cell contains no air bubbles, and that none of the sample is spilt on the outside face of the cell. The recommended procedure for achieving this is to place the cell on a slightly inclined surface so that one of the filling ports is higher than the other. Remove the bugs from both filling ports. Use a standard 1 mL disposable syringe to suck out any residual water in the cell via the bottom port.

Take up the sample in a new 1 mL syringe. The cell volume is approximately 150 μL and with practice filling can be successfully achieved with as little as 300 μL of sample. Place a tissue loosely around the top filling port, and fill the cell from the syringe via the bottom port, depressing the plunger smoothly. Stopper both top and bottom ports. If desired the cell windows can be wiped with a lint free cloth.

7.4.5. Setting up the FTIR

The FTIR should be set to report measurements in the absorbance mode. It is recommended that 32 scans covering the range 2300 cm^{-1} to 2900 cm^{-1} be averaged. Since the peak is broad there is no great advantage to running at high resolution, and 2 cm^{-1} is adequate. For the same reason the Box Car apodisation function is best avoided, and triangular apodization is recommended (see Appendix VI).

7.4.6. Obtaining the spectra

The standard and samples should each be measured against their own baselines. This means that the standard, made up as described in Section 7.4.2 should be read with a sample of the drinking water from the region used in its

preparation as the baseline, while samples of biological fluid should be measured with the basal (pre-dose) sample as the baseline.

The spectrum of the standard is usually obtained first in the following manner. If the equipment is not fitted with a shuttle then firstly the cell should be filled with drinking water from the region, and placed in the sample holder. The spectrum of this should be recorded as a background. When this is complete the cell should be removed from the sample compartment and refilled with the standard. This should be returned to the sample holder and the spectrum recorded as a sample.

If a sample shuttle is being used then this procedure is more automated. One of the matched cells is filled with drinking water from the region and the other with the made up standard. The drinking water from the region is placed in the reference position of the shuttle (usually to the rear of the sample compartment) and the standard is placed in the sample position (usually to the front). The FTIR should now be set to measure. The sample shuttle should move to position the drinking water from the region in the beam. Its spectrum will be acquired, and then the shuttle will move so that the beam passes through the standard. The acquisition will start again, and when complete a spectrum of the standard corrected for basal will be displayed.

In either case the spectrum should now be exported as a text file ready for use in the fitting procedure described in Section 7.4.7.

Samples are run in an exactly analogous fashion, except that the background used is the pre-dose sample. Therefore, if a sample shuttle is not being used the cell is first filled with the pre-dose, and a background spectrum is acquired. The cell is then filled with a post-dose sample, and read as a normal sample. If there are several samples to be analysed for the participant (say those collected at 3, 4 and 5 h post-dosing) then they can be done consecutively without recording a new background.

If the sample shuttle is available then the pre-dose sample can be loaded into the background position and the post-dose into the sample position for automated background correction. In the case of multiple samples only the sample cell needs to be refilled, and each sample will have an individually recorded background subtracted from it.

After each sample has been analysed its spectrum should be exported as a text file ready for further processing.

7.4.7. Exporting data

Most FTIR instruments have the capability to export data as a text file. However the file format produced is not the same from every instrument, and often parameters defining the format need to be set up in the instrument's software.

If the Medical Research Centre (MRC) programme 'isotope.exe.'* is to be used for the isotope determination then the required file format is either tab or comma delimited text files. The first field of each record should be the wave number (correct to at least 1 decimal place), and the second field should be the absorbance (correct to at least 3 but preferably six decimal places. Either standard or scientific notation is acceptable. Examples of the first few records of suitable exported files are

2000.044 478 0.050 611
2000.526 649 0.050 465
2001.008 820 0.050 203
2001.490 991 0.050 004
2001.973 162 0.050 139
2002.455 332 0.050 220

(tab delimited) or

2000.0 0.050 611
2000.5 0.050 465
2001.0 0.050 203
2001.4 0.050 004
2001.9 0.050 139
2002.4 0.050 220

(tab delimited) or

2.0000E+3 5.061E-2
2.0005E+3 5.047E-2
2.0010E+3 5.020E-2
2.0014E+3 5.000E-2
2.0019E+3 5.014E-2
2.0024E+3 5.022E-2
2.0029E+3 5.036E-2

(comma delimited)

(*isotope.exe can be obtained from Medical Research Council Collaborative Centre for Human Nutrition Research, Elsie Widdowson Laboratory, Fulbourn Road, Cambridge CB1 9NL, United Kingdom; telephone (+44 1223) 426356; web site www.mrc-hnr.cam.ac.uk)

7.4.8. FTIR maintenance

Modern FTIR instruments are designed to be operated by comparatively non-specialist users and are designed to be relatively maintenance-free. They can tolerate a wide range of environmental conditions. However for the best performance, and to extend instrument lifetime some maintenance operations are required to be conducted routinely.

Optics humidity

Many of the FTIR optical components are susceptible to damage by water vapour. To prolong their life the majority of the optical system is situated in a sealed unit that also contains a desiccant. Usually a humidity indicator is provided, and this should be inspected daily. If the humidity level in the chamber is too high then the desiccators should be replaced.

Cleaning the FTIR

Keeping the FTIR generally dust-free is advisable. Use a water dampened cloth to wipe the exterior of the instrument weekly. It is not advisable to wipe inside the sample compartment. In the event of spillages from the cells inside the sample compartment clean up with an absorbent lint free cloth immediately.

Care of the cells

When not in use store the cells in their original packaging. Wipe only with a lint-free cloth. Slight scratches and other imperfections can be removed from cell windows using a commercially available polishing kit. To test for flatness after the polishing an optical flat (usually provided with the kit) can be used.

7.4.9. Derivation of body composition from FTIR data

Once the data has been exported and the excess concentration of deuterium in the body fluids, C calculated, the deuterium space V_D is estimated by

$$V_D(\text{kg}) = \frac{\text{dose (mg)}}{\text{C (mg/kg)}} \tag{7.6}$$

The usual assumptions concerning the relationship between deuterium space and TBW, and the hydration factor of lean tissue are made.

$$\text{Total body water} = \frac{V_D}{1.041}$$
$$\text{Fat free mass} = \frac{\text{Total body water}}{0.732} \quad (7.7)$$
$$\text{Fat mass} = \text{Total mass} - \text{fat free mass}$$

Mass ^2H_2O taken = 1.086 g, made up to 1 L with water
Mass of ^2H_2O plus water = 10001.086 g
Mass of water added = 1000 g
Therefore, standard concentration = 1086 mg/kg (ppm weight)

Weight of participant = 92.7 kg
Dose given = 30 g = 30,000 mg
Recorded ^2H_2O concentration in body water = 585 mg/kg (ppm weight)

Calculation of deuterium space
V_D = 30,000/585 = 51.2 kg
TBW = 51.2 / 1.041 = 49.2 kg
FFM = 49.2 / 0.732 = 67.2 kg
Fat mass = 92.7 – 67.2 = 25.5 kg

8. FUTURE DEVELOPMENTS INCLUDING CAVITY-RING-DOWN SPECTROSCOPY

IRMS is an excellent analytical technique for both ^2H and ^{18}O analysis however it is complex and expensive. The simpler but robust spectroscopic instrumentation (FTIR) is available for stable isotope analysis in TBW studies. Both ^2H and ^{18}O can be analysed through their absorption spectra however traditionally, spectroscopic techniques have not had the sensitivity to analyse the low enrichments necessary when considering the cost of stable isotope tracers. However, FTIR has proven that spectroscopy can provide a suitable alternative to IRMS with an acceptable (~10 fold) increase in dosage. Recent development in hydrology (along with ^{13}C breath tests and atmospheric gas

analysis) has included instrumentation capable of ^{2}H and ^{18}O analysis at natural abundance. The advance has been made possible with the introduction of IR diode lasers working at room temperature, combined with techniques to dramatically increase the path length of spectroscopic instruments (cavity-ring-down spectroscopy: www.lgrinc.com).

This technology utilizes a novel shape in the optical cell that provides absorption pathways measured in kilometres. The long optical path along with stable electronics provides a degree of precision for isotopic analysis that is approaching that of IRMS for biological specimens. This has been demonstrated for ^{13}C where precisions of 0.3‰ (3 ppm) have been reported [102]. Instrument companies are developing similar instruments for the analysis of ^{18}O and D in water with precisions for isotope analysis that are adequate for the analysis of DLW. The major advantage that would be derived from such developments would be a reduction in costs and simplified operator training. Such a development would potentially expand the number of laboratories that could undertake biological isotope dilution studies and reduce the cost for such studies.

9. RECOMMENDATIONS FOR PRESENTATION OF RESULTS IN PEER REVIEWED JOURNALS

Reviewers require evidence that all procedures have been carried out according to good clinical and laboratory practice. The methods section should include details of the dosing procedure, sample preparation and analysis, and calculations.

The description of the dosing procedure should include the physiological state of the participants, amount and timing of dose, the equilibration period and timing of post-dose samples, and precautions taken to ensure that the complete dose was consumed or to account for losses where ingestion was not complete.

The description of analytical procedures should include: instrumentation used, standards, estimates of analytical precision for ^{2}H and ^{18}O within the ranges measured, and an assurance that the dose enrichment has been measured, and not just assumed from the manufacturer's value.

The description of calculations should include details of the formula used, any corrections made for stable isotope losses, description of how the plateau enrichment, slope and intercept were derived, and the method of deriving a

single pool space (usually the mean of N_O and N_D). Details should be given of any cut-offs used to screen out unacceptable estimates. Details of fractionation corrections should be provided together with assumptions regarding RQ including details of derivation from dietary assessment if this was done, or justification for using group mean estimates of RQ.

The results section should include the precision of analysis of ^{2}H and ^{18}O, the mean plus 95% confidence interval, (which is the mean ± 2 SD) of the dilution space ratio (N_D/N_O), as well as summary statistics for body composition and total energy expenditure. The mean ratio of the deuterium space to the ^{18}O space (N_D/N_O) should be close to 1.034 in adults.

Additional details may be included, depending on the space requirements of the journal.

Appendix I

GLOSSARY

abundance. The proportion of an isotope as compared with the total number of atoms of that element (a fraction, as in atom fraction or atom percent) or that compared with the number of atoms of the major isotope of that element (a ratio, as used in delta notation).

accuracy. Describes the correctness of a result, that is, how close the measured value is to the true value. Accuracy is expressed in terms of either absolute error or relative error. The absolute error of the mean of a small set of replicate analyses is = (measured – true) where true is an accepted value of the quantity being measured. It is often useful to express accuracy in terms of relative error where relative error = (measured – true)/true × 100%. Both absolute and relative errors bear a sign: a positive sign indicates that the measured result is greater than its true value and a negative sign the reverse.

atom per cent (at.%). The number of heavy atoms expressed as a proportion of the total number of atoms of that element
e.g. atom% $^{13}C = (^{13}C/(^{12}C+^{13}C)) \times 100$.

atom per cent excess (atom% excess, ape). The SI unit of enrichment, i.e. the abundance of a sample above the baseline level. For low enrichments it is more convenient to use parts per million excess (ppm excess; see below).

chromatography. Chromatographic techniques separate mixtures of compounds into their individual components by flowing a mobile phase over a stationary phase. A chromatogram is a time resolved series of peaks, reflecting the composition of the mixture. Unknown samples are compared with standards of known composition.

continuous flow techniques. Most of the sample preparation and purification occurs on-line in carrier gas (helium), for example combustion-IRMS, which involves oxidizing a crude sample to produce CO_2, water (and nitrogen/nitrogen oxides). The water is removed and the other gases are separated on-line using a GC column, so that pure CO_2 or N_2 enters the mass spectrometer in a stream of helium. Other continuous flow techniques involve separation of partially purified mixtures of compounds by gas chromatography or liquid chromatography (GC or

HPLC), before allowing them to enter the mass spectrometer. If the mixture is separated using a GC column, the components will enter the ion source of the mass spectrometer in a stream of helium. If the mixture is separated by HPLC, the components will be in a solvent, which must be removed prior to the solute entering the ion source of the mass spectrometer.

delta units. Parts per mille (thousand), ‰, or δ. The notation introduced by geochemists to study natural abundance variations, compared to an international standard, e.g. δSMOW, δPDB.

$\delta = ((R_R + R_S)/R_R) \times 1000‰$ (per mille)
where R_S is the ratio of the minor (heavy) to major (light) isotope for the sample, and R_R is the same ratio for the reference standard. The international standards have accepted isotope ratios, therefore, delta units can be converted to the SI unit, at.%.

delta above baseline (Δ(DOB)). A unit of enrichment. The delta value of a sample above the delta value of the baseline sample. This is not an SI unit and therefore it is not recommended for use in studies involving enriched tracers.

dual viscous inlet gas isotope ratio mass spectrometer. The classical technique. Requires pure gas samples (H_2, CO_2, N_2), which are prepared off-line usually by labour intensive techniques. This has largely been replaced by continuous-flow techniques in biomedical and environmental research.

enrichment. The abundance of a sample above the baseline level. Enrichment should be expressed in units of at.% excess or parts per million (ppm) excess, but sometimes the non-SI unit Δ(DOB) is used.

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, but the hydration of FFM is higher in children, pregnant women and certain clinical conditions.

Fourier transform infrared spectroscopy (FTIR). A technique that can be used to measure deuterium concentration in saliva samples from studies of body composition and human milk intake.

fractionation. The presence of a heavy atom in a molecule shortens covalent bonds making them stronger and more resistant to breakage. An isotope effect is the term used to describe the fact that molecules containing different isotopes display slightly different reaction rates. Fractionation is the term used when an isotope effect results in an observed change in isotopic signature. This can occur during chemical or enzyme-catalysed reactions, exchange reactions or during physical changes such as evaporation. Kinetic isotope effects and equilibrium effects describe these phenomena.

fractionation factor (f). The fractionation factor is the correction used to account for the fact that evaporative losses from body water concentrate the heavier isotopes of hydrogen and oxygen, so that deuterium and ^{18}O elimination is slower than water turnover ($k(^2H_2O) = fk(H_2O)$ and $k(H_2{}^{18}O) = fk(H_2O)$, where f is the fractionation factor: 0.946 for ^2H and 0.991 for ^{18}O, and k is the elimination rate). With regard to doubly labelled water, the isotope fractionations of importance are those between body water and water vapour leaving the body and between body water and carbon dioxide leaving the body.

gas chromatography (GC). A gas chromatograph is composed of a heated oven with a heated inlet (the injector) and a detector. A capillary column, typically 0.25 mm internal diameter and 30 m long is inserted into the injector at one end and a detector (for example, flame ionization detector (FID) or mass spectrometer). The column is coated with a phase, most usually bonded to the silica column, which is capable of retarding some components of a mixture more than others. Samples are injected into a stream of helium, known as the carrier gas, and are carried into the column. Components partition between the carrier gas and the column phase. They are differentially retained in the phase and eventually reach the detector. The output from a gas chromatograph is known as a gas chromatogram. Only volatile compounds (liquids or gases) can be analysed by gas chromatography. It is often necessary to make a volatile derivative from a non-volatile compound, for example long chain fatty acids and amino acids prior to analysis by gas chromatography. The original gas chromatographs had glass columns containing an inert support coated with a liquid phase. This type of instrument is called a gas-liquid chromatograph (GLC).

insensible water loss. Water lost from the body in sweat, 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 or oxygen-18 than body water. A correction should be made for insensible water losses other than sweat in the calculation of the pool space in isotope dilution techniques such as the measurement of TEE by the DLW technique, and the estimation of non-milk water intake in breast fed infants using the deuterium oxide dose-to-mother technique.

isotope. Isotopes of an element have the same atomic number (the number of protons in the nucleus) but different atomic masses (the sum of the number of protons and the number of neutrons).

isotope dilution technique. If a known amount of tracer is added to a biological system, then the dilution of the tracer by endogenous 'tracee' will give a measure of the size of the pool.

isotope effect (kinetic isotope effect or equilibrium isotope effect). The difference in rate constants between a reaction involving a molecule containing a heavy isotope and that containing a light isotope. Note that if a reaction goes to completion, fractionation will not be observed.

isotope exchange. Non-aqueous isotopic exchange occurs when the tracer is sequestered into compounds other than water, mainly lipids and proteins. For this reason, the measured isotope distribution space, also known as the pool space (N), in studies of TBW by deuterium dilution is larger than total body water. It is therefore necessary to correct the measured isotope distribution space (N) by a factor known as the non-aqueous exchange factor. Non-aqueous isotopic exchange is assumed to be 4.1% of the pool space for ^{2}H.

isotope ratio. The output from an IRMS, which is the derived ratio of the amount of the minor isotope compared to the amount of the major isotope with certain instrumental corrections applied during data reduction. The IAEA standards have accepted values of their isotope ratios.

isotope ratio mass spectrometer (IRMS). A low resolution magnetic sector mass spectrometer. The sample must be introduced to the mass spectrometer ion source in the form of a pure gas (CO_2, N_2 or H_2). The gas is ionized by electron impact from a hot filament. The ions are separated in a magnetic field. The detectors are Faraday Cups. There are

usually three detectors, which are designed to monitor masses m, m+1, m+2. For example, m/z 44, 45, 46 can be from CO_2 as follows: m/z 44 = $^{12}C^{16}O^{16}O$, m/z 45 = $^{13}C^{16}O^{16}O$ or $^{12}C^{16}O^{17}O$, m/z 46 = $^{12}C^{16}O^{18}O$. A correction is made for the presence of $^{12}C^{16}O^{17}O$ in samples where the intention is to measure $^{13}C^{16}O_2$ abundance. m/z 28, 29, 30 can be from N_2, CO or NO as follows: m/z 28 = $^{14}N^{14}N$ or $^{12}C^{16}O$, m/z 29 = $^{14}N^{15}N$ or $^{13}C^{16}O$ or $^{12}C^{17}O$, m/z 30 = $^{15}N^{15}N$ or $^{14}N^{16}O$. Precautions must be taken to avoid isobaric interference. IRMS can measure very low enrichments, down to natural abundance, very accurately.

linearity. The term used to describe the constancy of measured ratio with sample pressure (ion current) in a mass spectrometer. Linearity is a term commonly used when describing the performance of IRMS instruments. However, instruments often show strong changes in measured peak ratio with ion current, necessitating strict control of sample and standard quantity for accurate isotope ratio analysis. Alternatively, calibration procedures can be used to reduce such effects. The theoretical natural abundance ratio of molecules and molecular fragments can be calculated.

major isotope. The stable isotope of greatest natural abundance is sometimes referred to as the major isotope.

mass spectrometer. An instrument which separates ions in a vacuum according to their mass to charge ratio (m/z). The major components of a mass spectrometer are an inlet system, ion source, mass analyser, detector and a vacuum system. Modern mass spectrometers are computer controlled, have sophisticated data processors and a large variety of sample introduction systems.

minor isotope. The stable isotope of lower natural abundance is sometimes referred to as the minor isotope (or the heavy isotope).

natural abundance. The abundance of a stable isotope naturally present in (unenriched) samples.

non-aqueous exchange. The process whereby isotopes in body water enter components of the body, other than water, is known as non-aqueous exchange. Deuterium trades places with exchangeable hydrogen atoms (mainly –NH and –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, of the tracer is

slightly greater than TBW. The ^{2}H space (N_2 or N_D) is 1.041 times that of TBW. This is accounted for by dividing the calculated dilution space (N) by 1.041 to achieve TBW (kg).

precision. The reproducibility of a method, usually measured by making repeated analyses of the same sample. Measurements of precision include standard deviation, variance and coefficient of variation.

stable isotope. Stable isotopes are non-radioactive isotopes, for example ^{2}H (deuterium), ^{13}C (carbon-13), ^{15}N (nitrogen-15), ^{18}O (oxygen-18).

total body water (TBW). The term used to refer to the water content of the body, which makes up 70–75% of body weight at birth, but decreases to approximately 60% of body weight in lean adults and less than 40% in obese adults. Water is contained exclusively within the fat-free mass, which is approximately 73.2% water in adults. Measuring TBW establishes the amount of fat-free mass, from which body fat can be derived by difference from body weight. TBW includes both intracellular fluid (ICF) and extracellular fluid (ECF).

tracee. The unlabelled form of the tracer compound.

tracer. A stable isotope labelled compound used to follow metabolic pathways, but given at a concentration which is not sufficient to change the rate of the process being measured. The ideal tracer is chemically identical to the compound of interest (tracee).

volume of distribution. The volume through which the isotope is distributed, also known as the pool space (N). In studies of total body water by deuterium dilution, the volume of distribution is larger than total body water.

Appendix II

SAMPLE SIZE AND POWER CALCULATIONS

A power calculation is used to provide reasonable insurance that a study will be able to detect a specified difference in body composition or TEE by including a sufficient number of participants to average out normal physiologic variation without including an excessive number of participants and thus expending excess financial or personal resources. It is always recommended that a power analysis be undertaken in advance of any new study. Indeed, good practice is to request help from a statistician during planning of any new study. A number of publications illustrate the general approach, which is also available from commercial suppliers of statistical software. The simple approach presented here is based on statistical distributions.

In general, the investigator needs two pieces of information:

(1) An estimate of the difference between for example, measures of body composition specified by study aim;
(2) An estimate of the standard deviation for the measure, for example, of body composition under the experimental conditions.

The final piece of information is the power that one is willing to accept. This power is usually expressed as the percentage of the time that study will provide a statistically significant result. More often than not, a power of 80% is selected, which means, if the investigator were to perform the experiment 100 times, 80 would provide a statistically significant result for the measure while 20 would not. Note that both the projected difference in the measure and the standard deviation are estimates and that power analysis, while extremely important, is still an educated guess unless the investigator has previous data from a similar study.

The method for power estimates is to calculate sample size based on the theory of the Student's t distribution. As sample sizes in stable isotope studies can be small (<30), it is preferable to use a more exacting method based on the Student's t distribution rather than from the theory of standard errors. Sample number can be calculated by substituting the interval, 1.96, by the appropriate t-statistic:

$$n_t = (((\mathbf{t}_\alpha + \mathbf{z}_\beta) \times SD)/(D))^2 \quad \text{(I.1)}$$

where n_t = sample number in each treatment; t_α = the t-statistic for a given probability, α and n_t-1 degrees of freedom (α is usually set to 0.05); $\mathbf{z}_\beta$ is the

adjustment to $\mathbf{t}_{\alpha}$ to allow for uncertainty in the small sample **SD** compared with the population **s,** SD is the sample standard deviation, D is the projected difference or effect size for the experiment.

To facilitate analysis of the result of iterative calculations of key parameters, this approach to power analysis can be set out in a proprietary spreadsheet, such as Microsoft Excel. Here, the function 'TINV' is helpful as it reports the t-statistic given probability level (α) and degrees of freedom ($n_t - 1$). This function can be used to provide the appropriate one or two-tailed t-statistic as desired. The degrees of freedom can be minimized by adjusting the parameters α, SD and D, as desired. For more experienced users of Excel, the 'Solver' function can be used to semi-automate sample number minimization for each given parameter set.

If no data are available to allow such an estimate of D and/or SD, it is recommended that a pilot study be conducted to determine the necessary sample size. This is particularly important for new laboratories or established laboratories undertaking TBW measurements for the first time, because the sd may be larger than that of an established laboratory that has had sufficient time and experience to minimize measurement error.

Power calculation example — TBW

As an example, we may wish to conduct a 10 kg weight loss intervention in obese women that may result in a decrease in TBW. The mean at the start may be approximately 40 kg TBW. The expected effect size (change in TBW with weight loss) is likely to be small, about 2 kg (10 × 0.2 kg). Using the equation above, we can tabulate values of n for selected powers. A typical weight loss will be 80% fat (8 kg), 14% water (1.4 kg), and 6% FFM solids (0.6). Thus, the effect size is 2 kg (TBW) and a typical SD of change in TBW during controlled weight loss is 1.4 kg. For an α of 0.05, the value of t_{α} is 1.96.

β	$1 - \beta$	power	z_{β}	n_t
0.01	0.99	99%	2.32	38
0.05	0.95	95%	1.64	27
0.10	0.90	90%	1.28	22
0.20	0.80	80%	0.82	16
0.30	0.70	70%	0.52	13

One can see the need to propose an increasingly larger group size as the power increases. Because of this most investigators select a power of 80% as this provides 8 out of 10 chances that the difference or effect will be significant for the proposed study.

Power calculation example – TEE

Power calculations are best performed with the involvement of an expert biostatistician, however, several examples are included here to provide users with an estimate of the number of participants that may be needed for different studies. As indicated above, power calculations are usually made for an 80% probability to detect a difference with a p value of 0.05. This calculation will provide an estimate of the number of participants to be studied in order to detect a difference 80 times out of 100 that would only occur due to random variation in one out of 20 studies.

Using data collected from multiple sources, we can provide some crude estimated TEE mean values and the accompanying between-participant SDs that can be used in selective power calculations.

Participant group	Male (kcal/d)	Female (kcal/d)
Infants (1–2 a)	950±150	850±160
Children (3–8 a), BMI = 15 kg/m^2	1600±200	1600±200
Children (3–8 a), BMI >17.5 kg/m^2	1800±250	1700±250
Children (3–8 a), BMI = All	1650±275	1650±275
Children (14–18 a), BMI = 18.5-24.9 kg/m^2	2900±300	2200±300
Children (14–18 a), BMI >25 kg/m^2	3400±600	3000±500
Children (14–18 a), BMI = All	3000±400	2500±400
Adults (31–50 a), BMI = 18.5–24.9 kg/m^2	2600±350	2100±300
Adults (31–50 a), BMI >25 kg/m^2	3000±400	2400±350
Adults (>71 a), BMI = 18.5–24.9 kg/m^2	2200±300	1600±300
Adults (>71 a), BMI >25 kg/m^2	2500±350	1800±350

As an example, one can calculate the sample size for detecting a difference in TEE between adult men with a BMI between 18.5 and 24.9 kg/m^2, and those with a BMI >25 kg/m^2 an effect size of 400 kcal/d. The standard deviation is not identical for both groups, but is roughly = 400). For an 80% power to detect a difference with an α of 0.05, z_β from the TBW section above

is 0.82. Using Eq. (I.1), the number of participants in each group is estimated to be 8, or 16 total participants. Assuming that 85% of participants will complete a simple protocol, the investigator should recruit 20 participants for the study.

Appendix III

SAMPLE CALCULATIONS

III.1. TOTAL BODY WATER (IRMS)

$$TBW\ (kg) = ((W \times A/a) \times (\Delta DD/\Delta BW)/(1000 \times 1.041)) - (2 \times \text{cumulative urine loss (L)})$$

III.1.1. Inputs from the field study

Study name;
Study date;
Participant number;
Measurement number
Participant name;
Date of birth;
Participant weight, height and sex;
Code of dose bottle taken;
Time of baseline urine sample and time and volume (mL) of three urine samples post-dose (up to 8 h).

III.1.2. Inputs from the laboratory

Weight of dose (assuming this was prepared in the analytical laboratory);
Weight of the diluted dose and the tap water used for its dilution;
Isotopic abundance of the diluted dose and the tap water;
Isotopic abundance of each body water sample.

III.1.3. Outputs

Total body water (kg);
Lean body mass (kg);
Fat (kg);
Fat (%).

Example:

Weight =	80.0 kg
Height =	183 cm
Age =	50 years
Sex =	Male

Time of dose =	07:00
Weight of dose (A) =	39.9846 g
Time of first urine sample =	09:10
Volume of first urine sample =	135 mL
Time of second urine sample =	10:05
Volume of second urine sample =	60 mL
Time of third urine sample =	11:15
Volume of third urine sample =	70 mL

Weight of diluted dose (a) =	0.1032 g
Weight of tap water (W) =	49.7426 g
^{2}H abundance of tap water =	154.3 ppm ^{2}H
^{2}H abundance of diluted dose =	345.7 ppm ^{2}H
^{2}H abundance of basal sample =	155.6 ppm ^{2}H
^{2}H abundance of first urine sample =	213.8 ppm ^{2}H
^{2}H abundance of second urine sample =	240.5 ppm ^{2}H
^{2}H abundance of third urine sample =	243.1 ppm ^{2}H

III.1.4. Intermediate calculations

^{2}H enrichment of diluted dose (ΔDD) =	191.4 ppm excess ^{2}H
^{2}H enrichment of first urine sample (ΔBW_1) =	58.2 ppm excess ^{2}H
^{2}H enrichment of second urine sample (ΔBW_2) =	84.9 ppm excess ^{2}H
^{2}H enrichment of third urine sample (ΔBW_3) =	87.5 ppm excess ^{2}H

$$TBW\ (kg) = ((W \times A/a) \times (\Delta DD/\Delta BW)/(1000 \times 1.042))$$

Apparent TBW calculated using first urine sample =	60.78 kg
TBW calculated using second urine sample =	41.69 kg
TBW calculated using third urine sample =	40.45 kg
Mean TBW calculated using second and third sample =	41.07 kg

$$TBW\ (kg) = ((W \times A/a) \times (\Delta DD/\Delta BW)/(1000 \times 1.041)) - (2 \times \text{cumulative urine loss (L)})$$

Loss corrected apparent TBW calculated using first urine sample =	60.85 kg
Loss corrected TBW calculated using second urine sample =	41.56 kg
Loss corrected TBW calculated using third urine sample =	40.08 kg
Mean TBW calculated using second and third sample =	40.77 kg

The first sample obviously overestimated TBW as the urine sample had not equilibrated with body water after only ~2 h. TBW was calculated using the mean of the second and third samples. In this example, TBW has been corrected by subtracting ~0.5 kg water (1% TBW) lost over the ~4 h measurement period. Some laboratories deem that the relatively small volume of water lost over the protocol can be ignored to simplify the procedure. Whatever is decided, the same approach should be followed for every participant under study.

III.1.5. Body composition outputs

TBW =	40.8 kg
LBM = 40.77/0.732 =	55.7 kg
Fat = weight – FFM =	24.3 kg
% body fat =	30.4%

III.2. TOTAL ENERGY EXPENDITURE — MODIFIED TWO POINT PROTOCOL: EXAMPLE CALCULATION

In this example, a baseline urine sample was collected and further samples 4 and 5 h after the DLW dose were collected, and then two more samples were collected 7 d after dosing. Calculations should be performed in a spreadsheet, thus avoiding errors due to rounding up or down of numbers. Note that this laboratory has better precision on the deuterium analysis (by dual inlet IRMS following reduction to hydrogen gas) than ^{18}O analysis and therefore the dose of ^{18}O was higher than the dose of deuterium.

III.2.1. Inputs from the field study

Study name;
Study date;
Participant number;
Measurement number;
Participant name;
Date of birth;
Participant weight, height and sex;

Code of dose bottle taken;
Time the dose was taken and date and time of post-dose urine samples;
Weight of fluids consumed during the first 3 h after intake of the dose or cumulative urine volume of the 3 h and 4 h specimens.

III.2.2. Inputs from the laboratory

Weight of dose (assuming this was prepared in the analytical laboratory);
Weight of the diluted dose and the local drinking water used for its dilution;
Isotopic abundance of the diluted dose and the local drinking water;
Isotopic abundance of each body water sample.

Example

Participant and study	*Input*
Study	ABC
Department	Department of Nutrition
Institute	Anywhere University
Participant number	HABC-1
Notes	Healthy control
Date of birth (Excel format)	09-Sep-54
Age (years)	52
Gender	Female
Study date (Excel format)	06-Jul-07
Time of dose	09:40
Weight, kg	57.7
Height, cm	
BMI (kg/m^2)	
Measured REE (kcal/day)	
Schofield predicted REE (kcal/day)	1740
Dose	
Dose number	2
Weight of DLW dose given to participant (A, g)	60.52
Diluted dose	
Weight of diluted dose (a, g)	0.1525
Weight of local drinking water used for dilution (W, g)	80.1723
^{2}H abundance in tap water (ppm)	146.9
^{18}O abundance in tap water (ppm)	1984.1
^{2}H abundance in diluted dose (ppm)	258.8
^{18}O abundance in diluted dose (ppm)	2155.8

Example

Modified two point protocol: Summary of stable isotope analysis and intermediate calculations

Sample Dose Time	Date	Time	Time since dose (days)	Abundance ppm ^{2}H	Enrichment ppm xs ^{2}H	Abundance ppm ^{18}O	Enrichment ppm xs ^{18}O	Ln ppm xs ^{2}H	Ln ppm xs ^{18}O
	06-Jul-07	09:40	0	149.3		1993.6			
4 h	06-Jul-07	12:40	0.17	249.2	99.9	2149.1	155.5	4.604	5.047
5 h	06-Jul-07	13:40	0.21	248.1	98.8	2147.4	153.8	4.594	5.036
7 d	13-Jul-07	09:40	7.00	197.9	48.6	2058.1	64.6	3.884	4.168
7 d	13-Jul-07	10:40	7.04	197.6	48.3	2057.3	63.7	3.878	4.154

xs: excess.

The isotope dilution space (N_X) is calculated from the plateau enrichment in body water on the day the dose was consumed (the mean of 4 h and 5 h enrichment), the dose taken by the participant, and the analysis of the diluted dose.

H plateau enrichment = 99.36 ppm excess ^{2}H

O plateau enrichment = 154.65 ppm excess ^{18}O

$$N_X\ (kg) = ((W \times A/a) \times (\Delta DD/\Delta BW))/1000 - 2 \times \text{cumulative fluid loss (kg)} \quad \text{(III.1)}$$

where W is the amount of water used to make the diluted dose (80.1723 g); a is the amount of the dose used in this dilution (0.1525 g); A is the dose taken by the participant (60.52 g); ΔDD is the enrichment measured in the diluted dose (258.8 – 146.9 = 111.9 ppm excess ^{2}H; 2155.8 – 1984.1 = 171.7 ppm excess ^{18}O); ΔBW is the plateau enrichment in body water. Cumulative fluid loss is 0.240 kg

$$N_D\ (kg) = ((80.1723 \times 60.52/0.1525) \times ((111.9/99.36))/1000) - (2 \times 0.240)$$
$$= 35.352$$

$$N_O\ (kg) = ((80.1723 \times 60.52/0.1525) \times ((171.7/154.65))/1000) - (2 \times 0.240)$$
$$= 34.844$$

Observed pool space ratio $N_D/N_O = 1.015$

N_D/N_O should be between 1.000 and 1.070 with a mean of approximately 1.034 in adults.

TBW (kg) is calculated from the isotope dilution space (N_X) by dividing by the appropriate non-aqueous exchange factor (1.007 for ^{18}O and 1.041 for ^{2}H).

$$TBW_O = N_O/1.007 \quad \text{(III.2)}$$

$$TBW_O = 34.844/1.007 = 34.60\ kg$$

$$TBW_D = N_D/1.041 \quad \text{(III.3)}$$

$$TBW_D = 35.352/1.041 = 33.96\ kg$$

$$TBW_{AVG} = (TBW_O + TBW_D)/2 \quad \text{(III.4)}$$

TBW_{AVG} = (34.60 + 33.96)/2 = 34.28 kg

TBW_{AVG} (kg) must be converted to TBW_{AVG} (mol)

TBW_{AVG} (mol) = TBW_{AVG} (kg) × 1000/18.0153 (III.5)

where 18.0153 is the molecular weight of water.

TBW_{AVG} (mol) = 34.28 × 1000/18.0153 = 1902.9 mol

The isotope elimination rates (k_O and k_D) are the gradient of the plot of the natural logarithm (ln) of the enrichment in body water versus time since the dose was consumed (Fig. III.1):

$k_D = -0.1050\ d^{-1}$

$k_O = -0.1288\ d^{-1}$

k_O/k_D = 1.2267 (k_O/k_D should lie between 1.1 and 1.7)

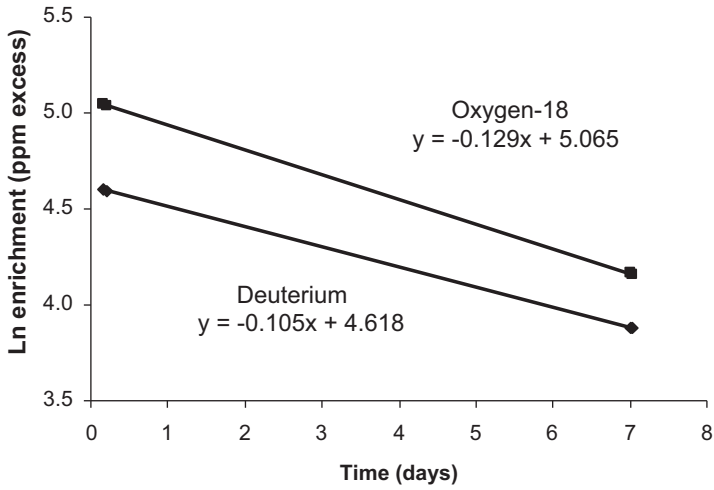


FIG. III.1. Log-linear plot of isotope elimination in a two point DLW protocol. ln = natural logarithm; k_D = –0.105; k_O = –0.129. Enrichment can be in ppm or per mille units. ΔBW is the enrichment of the third post-dose specimen.

Carbon dioxide production (rCO_2) and energy expenditure are calculated as follows:

$$rCO_2\ (mol.d^{-1}) = 0.455 \times TBW\ (mol) \times ((1.007 \times -k_O) - (1.041 \times -k_D)) \quad (III.6)$$

$$\begin{aligned} rCO_2\ (mol.d^{-1}) &= 0.455 \times TBW\ (mol) \times ((1.007 \times -k_O) - (1.041 \times -k_D)) \\ &= 0.455 \times 1902.9 \times ((1.007 \times 0.1288) - (1.041 \times 0.1050)) \\ &= 17.66 \end{aligned}$$

$$rCO_2\ (L.d^{-1}) = 22.414 \times 17.66 = 395.8$$

The factor 22.414 is the constant that converts moles of a gas to L (Charles Law).

Finally, calculation of TEE requires an estimate of the respiratory exchange ratio (R) and then energy production can be calculated using standard indirect calorimetric equations. The easiest equation to utilize is the modified Weir equation [63].

$$TEE\ (kcal.d^{-1}) = rCO_2\ (L.d^{-1}) \times (1.10 + 3.90/RQ) \quad (III.7)$$

In developed countries with Western diets that provide 30–35% of energy from fat, RQ is usually assumed to be 0.86 or 0.85, but it is recommended that a value representing the local diet is used. RQ can be estimated from the food quotient (FQ) of the diet [36, 61].

$$TEE\ (kcal/d) = 395.8 \times (1.10 + (3.90/0.85)) = 2251.5$$

$$\begin{aligned} TEE\ (kJ.d^{-1}) &= TEE\ (kcal/d) \times 4.184 \\ &= 2251.5 \times 4.184 = 9339 \end{aligned}$$

$$TEE\ (MJ.d^{-1}) = 9.34$$

III.3. MULTI-POINT PROTOCOL: EXAMPLE CALCULATION

In this example, a baseline urine sample was collected and further samples 1, 2, 3, 12, 13 and 14 days after the DLW dose was consumed. Calculations should be performed in a spreadsheet, thus avoiding errors due to rounding up or down of numbers.

III.3.1. Inputs from the field study

Study name;
Study date;
Participant number;
Measurement number;
Participant name;
Date of birth;
Participant weight, height and sex;
Code of dose bottle taken;
Time the dose was taken and date and time of post-dose urine samples.

III.3.2. Inputs from the laboratory

Weight of dose (assuming this was prepared in the analytical laboratory);
Weight of the diluted dose and the local drinking water used for its dilution;
Isotopic abundance of the diluted dose and the local drinking water;
Isotopic abundance of each body water sample.

Example

Participant and study	*Input*
Study	XYZ
Department	Department of Nutrition
Institute	Anywhere University
Participant number	000
Participant initials	ABC
Notes	Healthy control
Date of birth (Excel format)	01-Sep-42
Age (years)	62
Gender	Male
Study date (Excel format)	02-Mar-05
Time of dose	12:21
Weight, kg	92.2
Height, cm	181.5
BMI (kg/m^2)	28.0
Measured REE (kcal/day)	1770
Schofield predicted REE (kcal/day)	1667

Dose	
Dose number	2005-SIBL-0009
Weight of DLW dose given to participant (A, g)	47.868
Diluted dose	
Weight of diluted dose (a, g)	0.1034
Weight of local drinking water used for dilution (W, g)	49.7507
^{2}H abundance in tap water (ppm)	151.3
^{18}O abundance in tap water (ppm)	1999.8
^{2}H abundance in diluted dose (ppm)	418.1
^{18}O abundance in diluted dose (ppm)	2170.4

Example
Summary of isotope analysis

Sample dose time	Date	Time	Time since dose (days)	Abundance ppm ^{2}H	Enrichment ppm xs ^{2}H	Abundance ppm ^{18}O	Enrichment ppm xs ^{18}O	ln ppm xs ^{2}H	ln ppm xs ^{18}O
	02-Mar-05	12:21	0	144.1		2009.0			
1	03-Mar-05	13:00	1.0	271.8	127.7	2092.2	83.2	4.85	4.42
2	04-Mar-05	13:00	2.0	264.0	119.9	2086.2	77.1	4.79	4.35
3	05-Mar-05	14:00	3.1	258.6	114.5	2080.5	71.5	4.74	4.27
12	14-Mar-05	14:00	12.1	215.0	70.9	2045.6	36.6	4.26	3.60
13	15-Mar-05	14:15	13.1	210.9	66.8	2043.0	33.9	4.20	3.52
14	16-Mar-05	10:45	13.9	208.0	63.9	2041.2	32.1	4.16	3.47

xs: excess.

The isotope dilution space (N_X) is calculated from the y-intercept of the plot of the natural logarithm (ln) of the enrichment in body water versus time (days) since the dose was consumed (Fig III.2), the dose taken by the participant, and the analysis of the diluted dose.

H intercept = antilog 4.901 = 134.4 ppm excess ^{2}H

O intercept = antilog 4.496 = 89.7 ppm excess ^{18}O

$$N_X\,(kg) = ((W \times A/a) \times (\Delta DD/\Delta BW))/1000 \qquad (III.8)$$

where W is the amount of water used to make the diluted dose (49.7507 g); a is the amount of the dose used in this dilution (0.1034 g); A is the dose taken by the participant (47.868 g); ΔDD is the enrichment measured in the diluted dose (418.1–151.3 = 226.8 ppm excess ^{2}H; 2170.4–1999.8 = 170.6 ppm excess ^{18}O); ΔBW is the antilog of the y intercept of the graph of ln enrichment in body water versus time. There is no need to subtract cumulative fluid loss when N_X is calculated by the intercept method.

$$N_D\,(kg) = ((49.7507 \times 47.868/0.1034) \times (226.8/134.4))/1000 = 45.711$$

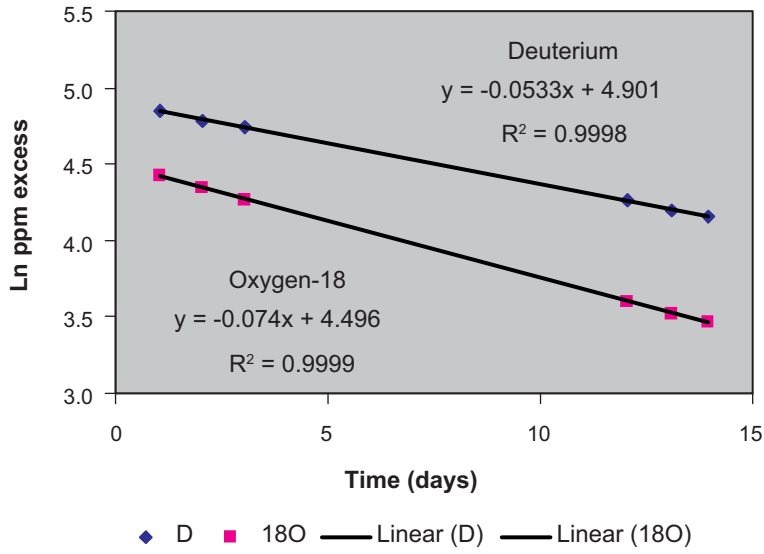


FIG. III.2. Elimination of ^{2}H and ^{18}O with regression lines through the enrichment data.

N_O (kg) = ((49.7507 × 47.868/0.1034) × (170.6/89.7))/1000 = 43.825

Observed pool space ratio N_D/N_O = 1.0430

N_D/N_O should be between 1.000 and 1.070 with a mean of approximately 1.034 in adults.

TBW (kg) is calculated from the isotope dilution space (N_X) by dividing by the appropriate non-aqueous exchange factor (1.007 for ^{18}O and 1.041 for ^{2}H).

$$TBW_O = N_O/1.007 \quad \text{(III.2)}$$

TBW_O = 43.825/1.007 = 43.52 kg

$$TBW_D = N_D/1.041 \quad \text{(III.3)}$$

TBW_D = 45.711/1.041 = 43.91 kg

$$TBW_{avg} = (TBW_O + TBW_D)/2 \quad \text{(III.4)}$$

TBW_{avg} = (43.52 + 43.91)/2 = 43.72 kg

TBW_{avg} (kg) must be converted to TBW_{avg} (mol).

$$TBW_{avg}\ (mol) = TBW_{avg}\ (kg) \times 1000/18.0153 \quad \text{(III.5)}$$

where 18.0153 is the molecular weight of water.

TBW_{avg} (mol) = 43.72 x 1000/18.0153 = 2426.8 mol.

The isotope elimination rates (k_O and k_D) are the gradient of the plot of the natural logarithm (ln) of the enrichment in body water versus time since the dose was consumed (Fig. III.2).

$k_D = -0.0533\ d^{-1}$

$k_O = -0.0740\ d^{-1}$

k_O/k_D = 1.2155 (k_O/k_D should lie between 1.1 and 1.7)

Carbon dioxide production (rCO_2) and energy expenditure are calculated as follows:

$$rCO_2\ (mol.d^{-1}) = 0.455 \times TBW\ (mol) \times ((1.007 \times -k_O) - (1.041 \times -k_D)) \quad (III.6)$$

$$\begin{aligned} rCO_2\ (mol.d^{-1}) &= 0.455 \times TBW\ (mol) \times ((1.007 \times -k_O) - (1.041 \times -k_D)) \\ &= 0.455 \times 2426.8 \times ((1.007 \times 0.0740) - (1.041 \times 0.0533)) \\ &= 21.02 \end{aligned}$$

$$rCO_2\ (L.d^{-1}) = 22.414 \times 21.02 = 471.05$$

The factor 22.414 is the constant that converts moles of a gas to L (Charles Law).

Finally, calculation of TEE requires an estimate of the respiratory exchange ratio (R) and then energy production can be calculated using standard indirect calorimetric equations. The easiest equation to utilize is the modified Weir equation [63].

$$TEE\ (kcal.d^{-1}) = rCO_2\ (L.d^{-1}) \times (1.10 + 3.90/R) \quad (III.7)$$

In developed countries with Western diets that provide 30–35% of energy from fat, RQ is usually assumed to be 0.86 or 0.85, but it is recommended that a value representing the local diet is used. RQ can be estimated from the food quotient (FQ) of the diet [61, 103].

$$TEE\ (kcal/d) = 471.05 \times (1.10 + (3.90/0.85)) = 2679.5$$

$$\begin{aligned} TEE\ (kJ.d^{-1}) &= TEE\ (kcal/d) \times 4.184 \\ &= 2679.5 \times 4.184 = 11211 \end{aligned}$$

$$TEE\ (MJ.d^{-1}) = 11.21$$

Outputs:

Body composition	
TBW =	43.7 kg
FFM = 43.72/0.732 =	59.7 kg
Fat = weight – FFM = 92.2-59.72	32.5 kg
% body fat =	35.2%
BMI = $92.2/(181.5/100)^2$	28.0 $kg.m^{-2}$

$k_O = -0.0740$
$k_D = -0.0533$

k_O/k_D = 1.2155 (k_O/k_D should lie between 1.1 and 1.7)
N_D/N_O = 1.0430 (should be between 1.000 and 1.070, close to 1.034 in adults)
rCO_2 ($L.d^{-1}$) = 471.05
TEE ($MJ.d^{-1}$) = 11.21
TEE ($kcal.d^{-1}$) = 2679
PAL = TEE/REE = 2679/1770 = 1.51

Quality control checks:

(1) R^2 of the regression line through the enrichment data should be >0.99 for both ^2H and ^{18}O. If not, check the date and time of the samples and for errors in data input.

(2) The residual plot is a means of checking for covariance of ^2H and ^{18}O. The residual is the difference between the measured value of ln enrichment and the value predicted by the regression equation. If the residuals do not co-vary (both should be on the same side of the zero line or very close to the line), check for errors in data input. If there are none, there may be a problem with the analysis of either ^2H or ^{18}O. The residual plot also provides a pictorial image of the magnitude of the errors. In this example, (Fig. III.3) the errors are small. Figures III.4 and III.5 show an example of a data set with larger, but acceptable, errors.

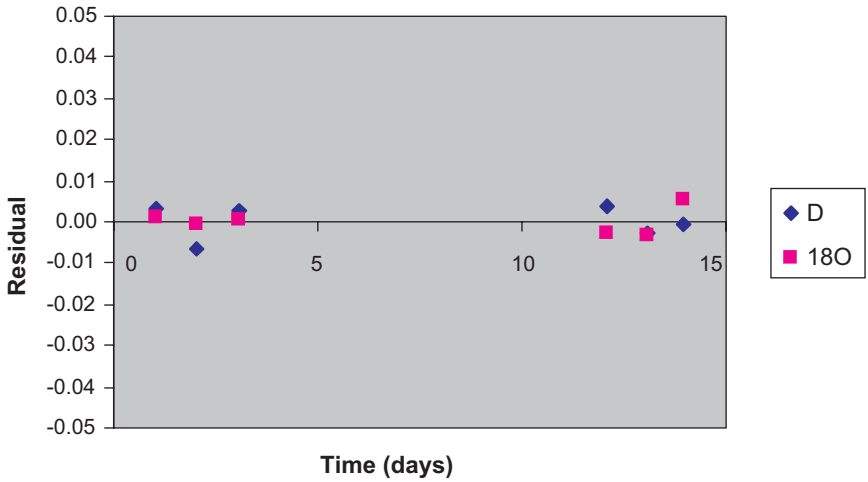


FIG. III.3. Residual plot showing covariance of ^2H and ^{18}O.

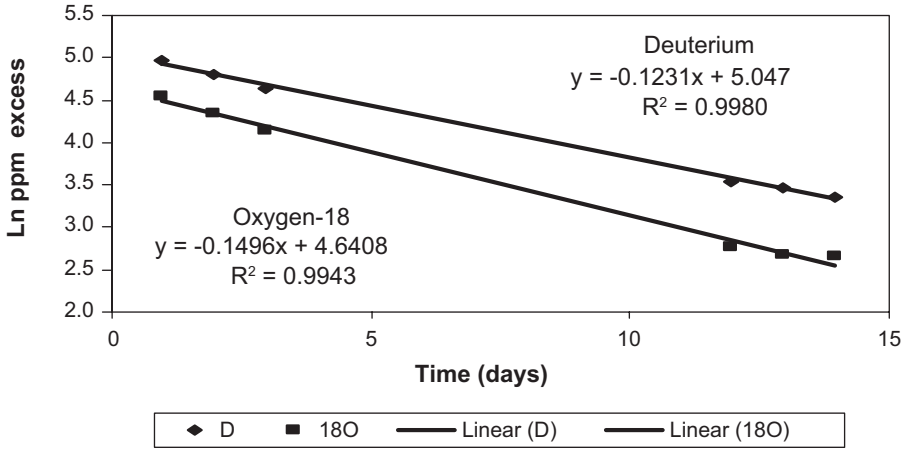


FIG. III.4. Elimination of ^{2}H and ^{18}O.

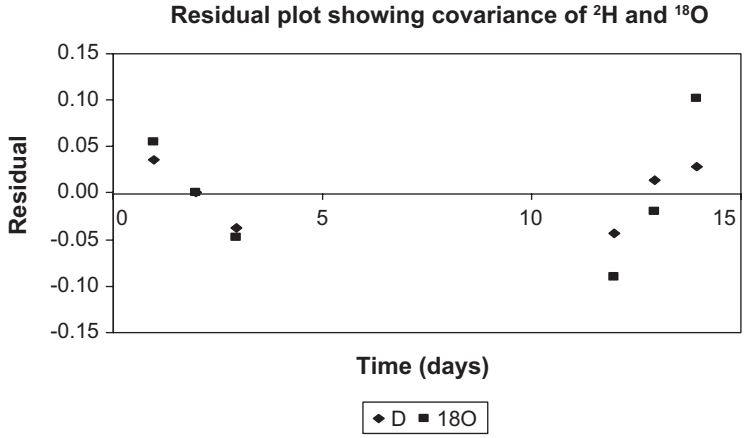


FIG. III.5. Residual plot showing covariance of ^{2}H and ^{18}O.

Appendix IV

SAMPLE DATA SHEETS

IV.1. FIELD DATA

Field and laboratory data should be available as paper copies and in spreadsheet format. A pro forma data sheet is taken into the field. Once filled, the data are coded into a spreadsheet as soon as possible. Such input data might form the first worksheet in a workbook. Specimens should be labelled clearly, including a unique numerical code that is also written into the paper data sheet and coded into the spreadsheet. Suitable labels can be computer-generated but it is imperative that the quality of the ink and adhesion of the label is suitable for long-term freezer storage. If the workbook has been designed as a template, it is straightforward to set it up to receive raw MS data, to convert this into calibrated enrichments, to calculate TBW (and TEE, as appropriate) and to produce a comprehensive report in an agreed format. Suggested content of these is given below:

The field data sheet should include:

— Study name.
— Study date.
— Participant number.
— Measurement number.
— Participant name, initials or identification number (depending on ethic requirements). (Local data security issues may make it preferable to record the participant's initials or ID number rather than their full name on samples that may be transported to research laboratories that are outside the hospital environment.)
— Date of birth.
— Hospital number.
— Participant weight, height and sex.
— Code of dose bottle taken.
— Time of baseline urine sample(s).
— Time and volume (mL) of three urine samples post dose (up to 8 h).
— Diet and fluid intake details as required by protocol.

The specimen label should include:

— Study name.

— Study date.
— Participant initials (if allowed).
— A unique code may be used to record the study code.
— Participant number.
— Measurement number.
— Time of sample.
— Replicate number. (This should be clearly described and be transparent to all field and laboratory workers. If samples are to be posted or sent by courier, a postal address or contact details should either be printed in the label or should accompany each batch of samples.)

IV.2. ANALYTICAL REPORT

The analytical report may have a short form to report the essential details of the study:

— Participant name;
— Date of birth;
— Date of study;
— Hospital number;
— Study name;
— Participant number;
— Measurement number;
— Weight;
— Height;
— Sex;
— BMI;
— TBW;
— FFM;
— FM;
— Per cent body fat;
— DLW;
— All of the above plus;
— TEE;
— Pool space ratio (N_D/N_O);
— k_O/k_H.

Appendix V

INTERCONVERSION OF COMMON UNITS USED TO EXPRESS STABLE ISOTOPE ABUNDANCE

There are two notations in common use. By convention, the delta notation has been used to express variations in stable isotope natural abundance, whereas notation based on absolute atom fractions has been applied when enriched tracers are used.

V.1. CONVERSION OF DELTA NOTATION INTO ATOM FRACTIONAL ABUNDANCE, ATOM % AND PPM

$$^2\text{H fractional abundance} = (1/(1 + (1/(((\delta^2\text{H}/1000) + 1) \times 0.000\,155\,76)))) \quad \text{(V-1)}$$

$$\text{Atom\% } ^2\text{H} = (100/(1 + (1/(((\delta^2\text{H}/1000) + 1) \times 0.000\,155\,76)))) \quad \text{(V-2)}$$

$$\text{ppm } ^2\text{H} = (1\,000\,000/(1 + (1/(((\delta^2\text{H}/1000) + 1) \times 0.000\,155\,76)))) \quad \text{(V-3)}$$

where δ^2H is the per mille ^{2}H with respect to the international reference, VSMOW. The factor, 0.000 155 76, is the accepted ^{2}H/^{1}H ratio of VSMOW.

V.2. CONVERSION OF ATOM FRACTION, ATOM% AND PPM TO DELTA NOTATION

$$\delta^2\text{H (per mille)} = 1000 \times ((\text{R}/0.000\,155\,76) - 1) \quad \text{(V-4)}$$

where R is the ^{2}H/^{1}H ratio in the sample and 0.00015576 is the ^{2}H/^{1}H ratio in VSMOW;

$$\text{R} = \text{atom fraction } ^2\text{H}/(1 - \text{atom fraction } ^2\text{H}) \quad \text{(V-5)}$$

$$\text{R} = \text{atom\% } ^2\text{H}/(100 - \text{atom\% } ^2\text{H}) \quad \text{(V-6)}$$

$$\text{R} = \text{ppm } ^2\text{H}/(1000000 - \text{ppm } ^2\text{H}) \quad \text{(V-7)}$$

V. 3. ISOTOPIC ENRICHMENT

When abundance has been expressed in delta notation, the enrichment of a post-dose sample is given by subtracting the measured abundance of a pre-dose basal sample from the measured abundance of the post-dose sample (sometimes referred to as delta over baseline, DOB). The symbol Δ is used to denote enrichment in delta notation. When abundance has been measured in atom %, the appropriate unit of enrichment is atom% excess (atom% sample – atom% baseline). Similarly for ppm, ppm excess (ppm sample – ppm baseline) is the appropriate unit of enrichment.

V.4. UNITS USED IN FTIR STUDIES

When measuring deuterium concentration by FTIR, the output is automatically corrected for the background concentration, so all measures are given as enrichment. Some descriptions of FTIR use ppm units (wt/wt) which differ from the molar units of ppm excess ^{2}H given above. To avoid confusion of using different ppm units, use of units of deuterium concentration, in mg/kg, is recommended for FTIR. If the FTIR instrument is calibrated in mg/kg using a gravimetric dilution of the deuterium stock, then ΔBW is now in mg/kg.

Appendix VI

MEASUREMENT OF DEUTERIUM ENRICHMENT USING FTIR

VI.1. INFRARED RADIATION

The infrared region of the electromagnetic spectrum lies in between the visible and microwave regions. The precise position of radiation in the electromagnetic spectrum can be defined either by the wavelength of the radiation (λ) or its frequency (ν), the two being directly related by the wave equation:

$$\lambda\nu = c \tag{VI-1}$$

where c is the velocity of light (2.998×10^8 ms^{-1}). In addition the radiation can be categorized by the energy of the photons. Plank's law indicates that

$$E = h\nu = h\frac{c}{\lambda} \tag{VI-2}$$

where h is Plank's constant (6.626×10^{-34} Js^{-1}). Usually, the two fundamental constants are combined into a single constant of proportionality, and the photon energy expressed in units of inverse length. Therefore there are three equivalent ways of defining where radiation lies in the electromagnetic spectrum, related by Eqs (VI-1) and (VI-2), and on this basis the infrared region extends from 1000 to 0.75 μm (wavelength), which is equivalent to 0.3–400 Thz (frequency) and 10–13300 cm^{-1} (energy).

VI.1.1 Infrared spectroscopy and isotope effects

The middle of the infrared region (approximately 25–2.5 μm, 12–120 THz, 400–4000 cm^{-1}) corresponds to the energy range of molecular vibrations. The exact energy required to excite these vibrations depends on the entire molecular structure, however it is common to model each bond in the molecule by a light stiff spring. The approximation is then made that the vibrational behaviour of the springs are mutually independent. Therefore the energy of the vibrational modes depends only upon the bond strength and the masses of the atoms between which the bond is formed. On this basis a particular bond type is expected to have a vibrational energy at a characteristic frequency whatever the overall molecular structure.

In the case of the hydroxyl bond (that is, the sigma bond between oxygen and hydrogen) where the hydrogen nucleus is much lighter than the oxygen, substituting deuterium for hydrogen will not change the strength of the bond, or the force constant of the spring. However the increased mass of the deuterium atom will change the frequency of vibration, reducing it by the square root of the relative masses of the two isotopes. It is therefore predicted that the characteristic frequency of a particular vibration of the O–D bond will be approximately 70% of that of the corresponding O–H bond.

VI.1.2. Infrared spectra of the isotopomers of liquid water

While the approximations discussed in the previous section are useful for understanding the elementary concepts of the physical processes being observed by infrared spectroscopy, an in-depth study of the vibrational energies of a particular molecule necessarily require the complete theory to be developed, and this is outside the scope of the document. Instead the focus will be to report on the experimental data for water and briefly consider its interpretation.

Initially restricting attention to the lightest water molecule (H_2O) there are three possible bond deformation modes of vibration (symmetric stretch, asymmetric stretch and scissoring), and a further three vibrational (rocking) motions. In general the spectral features observed do not correspond to a single vibrational mode, but to some composite motion. In the infra-red spectrum of liquid water the most intense feature is a broad absorption band covering the range 2800–3700 cm^{-1} with a band maximum at about 3404 cm^{-1}, with a second similar band to lower energy centred on 2128 cm^{-1} [104]. Similarly, pure ^2H_2O has two peaks with maxima at 2504 cm^{-1}, and 1555 cm^{-1}. Note that the ratio of energies of equivalent bands for the two isomeric species (73.6% and 73.1%) is in agreement with the simple model proposed in the previous section.

Any mixture of liquid H_2O and liquid ^2H_2O (including water at natural abundance) actually comprises three species since the H and D atoms are not permanently bonded to a particular oxygen, but are free to exchange giving rise to the hybrid HDO [105]. The spectrum of HDO is extremely difficult to obtain (since the species cannot be isolated) [106]. However, the examination of a series of mixtures of ^2H_2O in H_2O reported [106] indicates that the intense broad absorption line with a maximum near 3400 cm^{-1} observed in pure H_2O shifts by only 70 cm^{-1} over the entire concentration range of the mixture, and can therefore be associated with O–H bond stretching. Similarly, the band observed at 2504 cm^{-1} in pure ^2H_2O, and therefore associated with the O–D bond stretching, is observed throughout the solution composition range with a shift of less than 35 cm^{-1}. More importantly the intensities of these bands are

linearly related to the mole fraction of deuterium in the mix, and therefore not only is the assumption that the two bond species behave independently is confirmed as valid, but there is experimental evidence for the validity of using the intensities of these peaks as measures of sample composition.

VI.1.3. The Beer–Lambert Law

The observation that the intensity of the O–D absorption line can be used to measure the amount of that species present is an example of the Beer–Lambert Law, which states that "for a parallel beam of monochromatic radiation passing through a homogeneous solution the amount of radiation absorbed is proportional to the product of the concentration (c) and path length (l)". The constant of proportionality is a property of the particular molecular vibration concerned, and is known as the extinction coefficient, ε. The Beer–Lambert Law can therefore be summarized by:

$$A = \varepsilon cl \tag{VI-3}$$

VI.1.4. Infrared spectroscopy instrumentation

The original infrared spectrometers (often referred to as spectrophotometers) used a dispersive element (a prism or grating) to separate the radiation from an infrared source into a continuous range of frequencies. A slit was then used to select a narrow range of radiation and pass it through the sample to a detector. The total spectrum was obtained by rotating the prism or grating so that each radiation frequency successively traversed the slit, thereby scanning the sample with the whole range of infrared energies available. In the 1960s an alternative technique based on an interferometer was introduced. This had a number of advantages in speed of scanning and stability, but the complexity of the computer needed for the numerical computations required to generate the spectra precluded their use in routine analysis. Since the 1980s adequate computing power has been obtainable at affordable prices, and now these so-called FTIR instruments are standard equipment for modern analytical laboratories.

VI.1.5. Fourier transform infrared (FTIR) spectrometers

An FTIR comprises a source of infrared radiation, a beam splitter, two mirrors and a detector. The source is a material that can be heated (usually electrically) so that it emits infra-red radiation. The amount of infrared energy emitted increases with the source temperature (in accordance with the black

body radiation law); consequently, most are operated at a sufficiently high temperature for them to emit visible light as well, making them glow.

The beam splitter and mirrors make up the interferometer. One of the mirrors is rigidly fixed (the stationary mirror), whilst the other is mounted on an assembly, which is designed to move the mirror back and forth at constant velocity (the moving mirror). Radiation from the source is directed towards the beam splitter (see Fig. VI.1). This is a semi-transparent/semi-reflective material. Consequently half the incident radiation is reflected to a stationary mirror, and half is transmitted to the moving mirror. After reflection from the two mirrors the two beams are recombined at the beam splitter, and their sum passed through the sample and focussed on the detector. At any given time there will be a difference in the path lengths travelled by the radiation hitting the mirror. Suppose the distance between the beam splitter and the fixed mirror is l_f. Radiation passing through the beam splitter will have travelled a

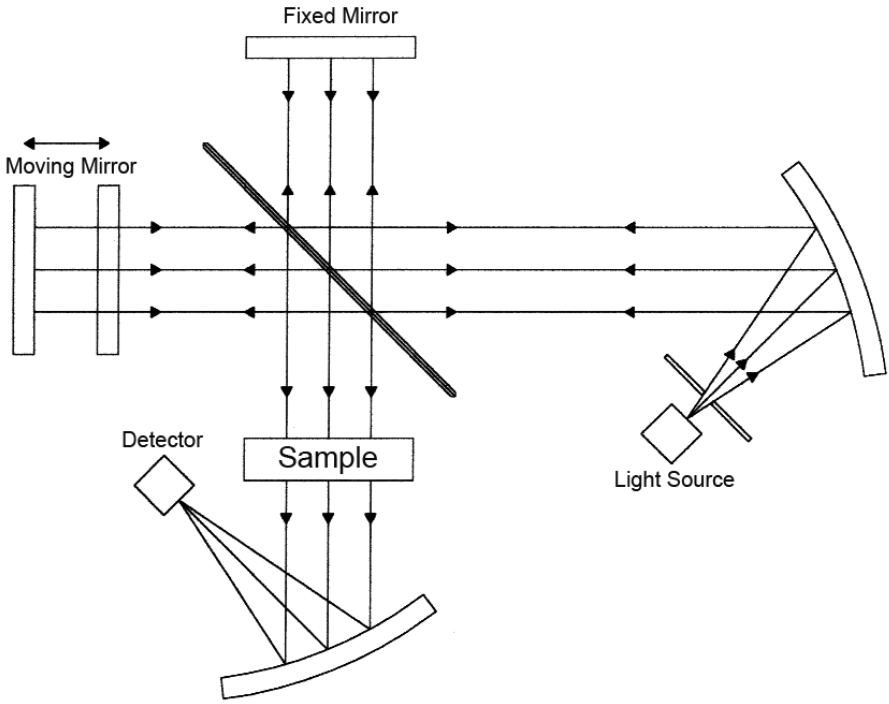


FIG. VI-1. A schematic representation of an FTIR instrument. The beam splitter is represented by the sloped heavy line.

distance equal to $2l_f$ before arriving back at the beam splitter. In the case of the moving mirror the distance from the beam splitter is a linear function of time, and can be written as $l_m(t) = l_f + v_m t$ where v_m is the velocity with which the mirror moves. Radiation reflected from the moving mirror therefore travels a distance of $2(l_f + v_m t)$ between its first and second visit to the beam splitter.

When the beams are recombined they interfere. Constructive interference occurs when the path difference is an integral number of wavelengths of the radiation, i.e. when:

$$n\lambda = 2v_m t \tag{VI-4}$$

and likewise destructive interference when

$$\left(n + \tfrac{1}{2}\right)\lambda = 2v_m t \tag{VI-5}$$

Initially imagine monochromatic radiation. The generalized expression for the intensity of the recombined beam (of which Eqs (VI-4) and (VI-5) are derived as special cases) is:

$$I = I_o \cos\left(\frac{4\pi v_m}{\lambda} t\right) = I_o \cos\left(\frac{4\pi v_m \nu}{c} t\right) \tag{VI-6}$$

This is shown diagrammatically in Fig. (VI-2) (upper panel). From this it will be observed that a radiation of a single wavelength (frequency) corresponds to a time varying cosine output from the interferometer. In the jargon used in the study of such systems "A delta function in the frequency domain corresponds to a cosine in the time domain."

Suppose we now complicate the issue by using incident radiation comprising five different wavelengths. Each wavelength will give rise to a pattern similar to that obtained from a single wavelength as described above, but with a different time between the peaks and the troughs. (Fig. VI-2), middle panel). The output from the interferometer is the sum of these five signals, which is indicated in the right hand graph, which is known as an interferogram.

Now 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 contained in the time domain will be different, and the interferogram will be modified accordingly. This is indicated in the lower panel of Fig. VI-2).

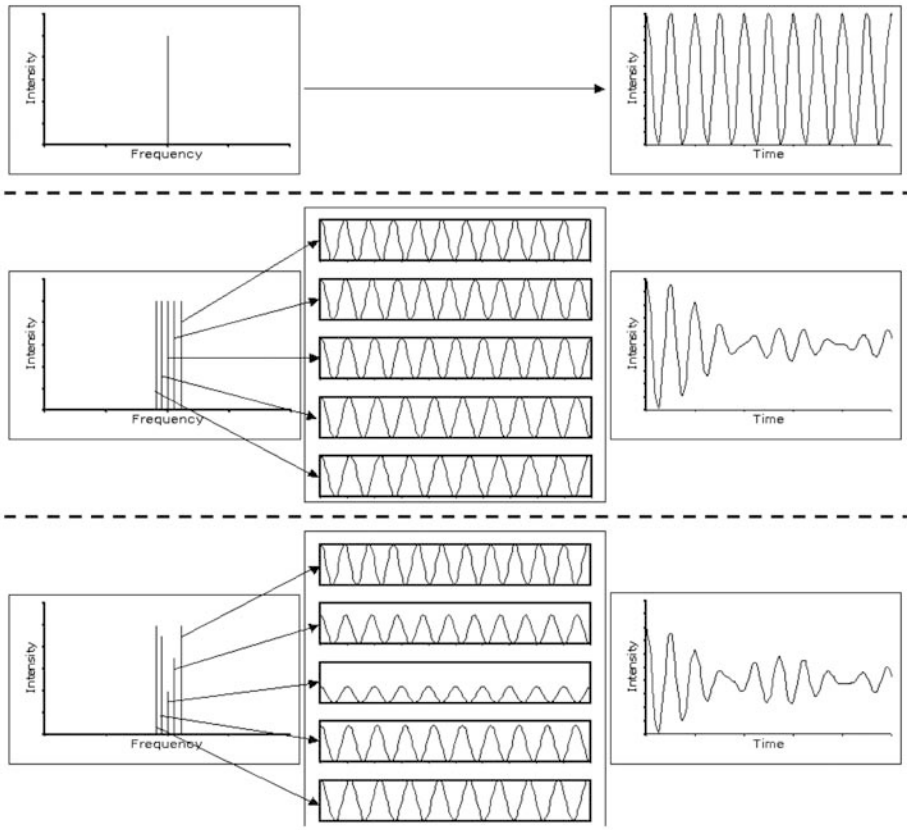


FIG. VI-2. A simplified description of interferometer operation. In the upper panel a single frequency of incident radiation (left) produces an intensity output, which varies cosinusoidally (right). In the middle panel five discrete components are present in the incident radiation (left). Each gives rise to a co-sinusoidal component in the output, but with a different period (middle). These are summed to give the total output or interferogram (right). Finally, the effect of an absorbing material placed in the exit beam is indicated. The amplitudes of the five components of transmitted radiation are perturbed (middle), which is reflected in the resultant interferogram (right).

In a real instrument where the frequencies of the radiation from the source are a continuum the total intensity of the light passing through the sample is given by the sum of expressions like those given in Eq. (VI-6), and:

$$I(t) = \sum_{\nu} I_o(\nu) \cos\left(\frac{4\pi v_m \nu}{c} t\right) \qquad \text{(VI-7)}$$

The FTIR detector is usually a pyroelectric material, such as triglycine sulphate (TGS), which has the property of producing an electrical signal if its temperature changes. The heating effect of the infrared radiation used is fairly uniform across the energy range employed, and so these detectors have a flat response over the whole of the spectral range of interest. The output from the detector is routed to a pre-amplifier, and converted into a time varying voltage which is an accurate representation of the total transmitted intensity described by Eq. (VI-7).

In this expression (Eq. (VI-7)), the coefficients $I_o(\nu)$ represent the infrared spectrum of the sample, and these are extracted by a mathematical procedure called Fourier transformation. The first step is to digitise the signal from the detector. For the transformation process to be optimized it is important that the intervals in the digitisation correspond precisely to increments in the moving mirror motion. This is accomplished by adding a source of laser radiation to the optical system. The laser radiation traverses the interferometer in exactly the same way as the infra-red and generates an interference pattern in exactly the same way (see Fig. VI-2, upper panel). The digitization can be triggered every time the cosine function describing this output passes through zero; for sufficient digital resolution of the infrared signal this should happen several times during the average period of the infrared radiation. This means that the frequency at which the laser operates should be much higher than that of the infrared, and visible laser radiation is used. A He/Ne device operating at 15800 cm^{-1} (632.8 nm, 474 THz), in the red part of the spectrum is suitable. For an interferometer with a mirror moving at 5 $mm.s^{-1}$ this triggers a conversion every 63.3 μs, but crucially the conversion occurs whenever the mirror has advanced precisely another 158 nm. This so-called fringe detection method accounts for the extremely high stability of FTIR spectrometers.

The digitized signal can then be sent to a Fourier Transform (FT) processor, which can either be a dedicated device or can be a piece of software running on a computer. Modern desktop workstations are fast and efficient enough to perform the transform in addition to storing data and performing other spectral manipulations, thereby generating a fully reconstructed infrared spectrum over the whole of the mid-infrared range in approximately one minute.

VI.1.6. Scanning parameters in FTIR

Resolution

Spectral resolution is a measure of the ability of the spectrometer to distinguish two closely spaced peaks. For an FTIR instrument it can be shown that the resolution is inversely related to the distance that the moving mirror travels. In practice the mirror travel is determined as a trade-off between achieving best resolution with a long mirror travel without compromising scan time. A reasonable velocity for the moving mirror is about 5 $mm.s^{-1}$, and a scan time of 2 s allows the mirror to move 1 cm, giving a spectral resolution of 1 cm^{-1}. If the mirror velocity remains the same, but the travel is restricted to 0.25 cm, then the resolution decreases to 4 cm^{-1}.

Apodization

The mathematical basis of the Fourier transformation actually requires that the sum indicated by Eq. (VI-7) be replaced by an integral between infinite limits. This can be achieved only by a system operating at infinite resolution, i.e. with a moving mirror with infinite travel. The effect of restricting the mirror movement is to introduce distortions to the spectrum recovered by the Fourier transformation, broadening the peaks and introducing side-lobes. This distortion can be reduced mathematically by convoluting the output from the detector with an envelope, which is zero at the beginning and end of the scan. This process is known as apodization, and the shape of the envelope used is known as the apodisation function. There are a number of apodization functions in common use; the choice is made on the anticipated spectral line widths. In general, a Box Car function is used for narrow lines (such as when rotational fine structure of vibrational states is being studied), and either triangular or Happ apodization employed when the broad bands exhibited by solid or liquid samples are investigated.

Advantages of FTIR

The advantages of FTIR instruments over the dispersive instruments are:

— **Multiplexing**. Because the whole of the FTIR spectrum is measured simultaneously in FTIR compared with sequential wavelength scanning used by dispersive instruments the spectrum is obtained much more quickly.

— **Ease of spectral summing**. The fringe detection method used in FTIR instruments guarantees stability of frequency measurement, and therefore multiple spectral scans can be added with confidence. Combination of the ease of summing with multiplexing means that many spectra can be acquired and summed on an FTIR instrument in a shorter time than a single spectral scan can be obtained on a dispersive instrument
— **Fewer optical losses.** An FTIR instrument does not use a narrow source slit to select near monochromatic radiation from the source as a dispersive instrument does. Furthermore there are fewer mirrors in an FTIR, and consequently a smaller percentage of radiation losses. The higher amount of energy available for measuring the spectrum increases the signal to noise ratio (quality) of the spectrum obtained.
— **Long term stability.** A dispersive instrument has no internal analogue of the laser calibration of the wavelength scale inherent in FTIR. This means that if spectra collected over a considerable time period are to be compared there must be frequent external wavelength calibrations of the dispersive instrument. These are not required in FTIR.

VI.1.7. Representation of infrared spectra

In the case of the original dispersive instruments, which scanned the spectrum by mechanically rotating a prism or grating, it is fairly simple to arrange for a scan with a constant rate of change of wavelength. Since these scans were usually output to a y–t pen recorder it became customary to plot infrared spectra with wavelength as the x axis, and this was decreasing so that high energy features appeared to the right of the graph. FTIR instrumentation operates in such a way that the natural measure of radiation energy is frequency, and for this reason FTIR output is often plotted with this parameter as the x-axis. In this case the x axis is drawn extending from low to high values, to retain the convention that high energy features occur to the right. However care must be taken when comparing spectra drawn according to the two conventions as low energy features look narrower when plotted on a linear frequency scale than on one linear in wavelength, while high energy features appear broader.

If the infrared spectrometer is run without a sample in the beam the spectrum produced is not perfectly flat as might be imagined. First, this is because the output of the radiation source is not perfectly uniform across the entire spectral region. Second, while the detector is optimised for a flat response across the incident energy range the detection efficiency is always slightly dependent on the precise frequency of radiation being measured.

Finally, the optical components themselves do not transmit radiation perfectly, and the degree of radiation absorbed in its passage through the spectrometer depends upon the frequency. Furthermore some gases exhibit infrared absorption lines, and their presence in the optical path gives rise to residual spectral features. To minimize this, the majority of the optical components of the spectrometer are sealed in an evacuated chamber. However this is not generally feasible for the sample compartment, and the presence of atmospheric gases in this region (particularly CO_2) gives rise to a background spectrum.

To eliminate these effects the infra-red spectrum of a sample is usually 'ratioed' to that of the background. In the case of a dispersive instrument the background spectrum is usually acquired at the same time as the sample spectrum by splitting the beam into two, passing one through the sample and the other through a blank, and finally utilizing a method of beam switching so that radiation from the two paths is focussed alternately on the detector. This is known as double beam operation.

In the case of FTIR the exceptional frequency stability of the instruments and the inherent digitization of the output allow the background spectrum to be acquired first and all subsequent spectra corrected for it mathematically. In either case the spectrum of the sample is represented by:

$$T(\lambda) = \frac{I_s(\lambda)}{I_b(\lambda)} \text{ (dispersive instrument) or } T(\nu) = \frac{I_s(\nu)}{I_b(\nu)} \text{ (FTR)} \qquad \text{(VI-8)}$$

where I_s is the detector output for the sample and I_s that for the background. The quantity T is called the *transmittance*, and related to the absorbance introduced earlier, Eq. (VI-3) by:

$$A = -Log\{T\} \qquad \text{(VI-9)}$$

Spectra can be displayed on either the transmittance or absorbance scales. There are, therefore, four combinations of x and y axes which can be chosen to represent a spectrum: transmittance versus wavelength, transmittance versus frequency, absorbance versus wavelength, and absorbance versus frequency. For comparison a simple infra-red spectrum displayed on all four possible combinations is illustrated in Fig. (VI-3).

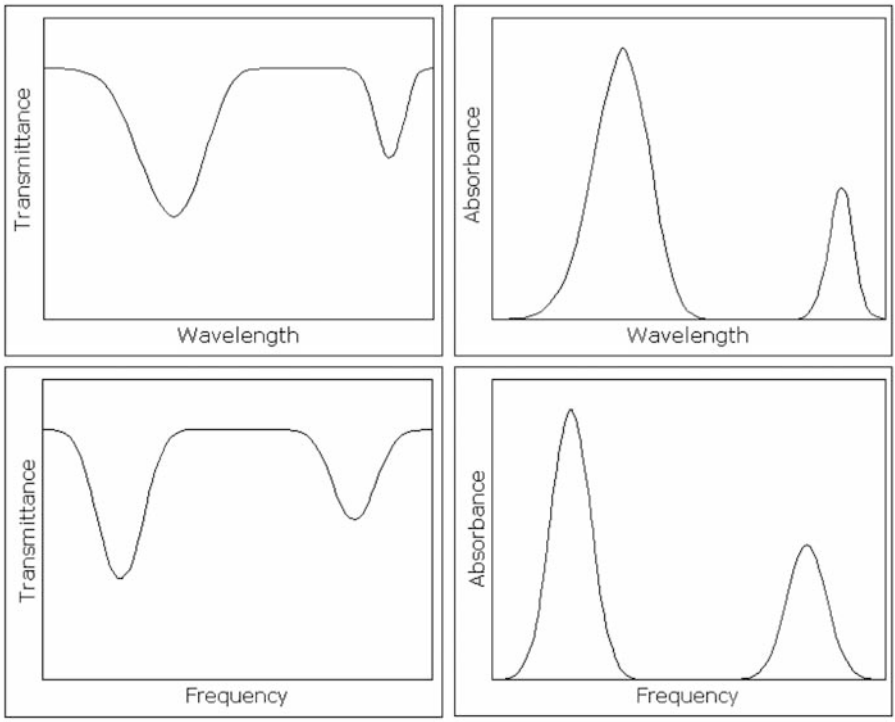


FIG. VI-3. The four ways commonly used to present infrared spectra. These four plots are of the same spectrum to illustrate that care must be taken to pay proper regard to the x and y axis scales when making comparisons of spectra.

VI.1.8. Quantitative analysis of deuterium in water

The infrared spectrum of H_2O, HDO, and ^2H_2O was discussed in the body of this report where it was demonstrated that the band at 2504 cm^{-1} was a suitable candidate for determining the deuterium concentration of an aqueous sample. For the application of TBW determination, where it is necessary to limit the dose administered to a minimum, measurements will be made in dilute samples (less than 1000 ppm deuterium). Liquids usually exhibit broad infra-bands in their infrared spectrum, and this is the case with water, where the bands are sufficiently broad for them to be incompletely resolved.

This is illustrated in Fig. (VI-4). This spectrum immediately indicates the major difficulties that have to be overcome in order to use the peak at 2504 cm^{-1} for quantitative analysis at low levels. The band appears as a shoulder on the

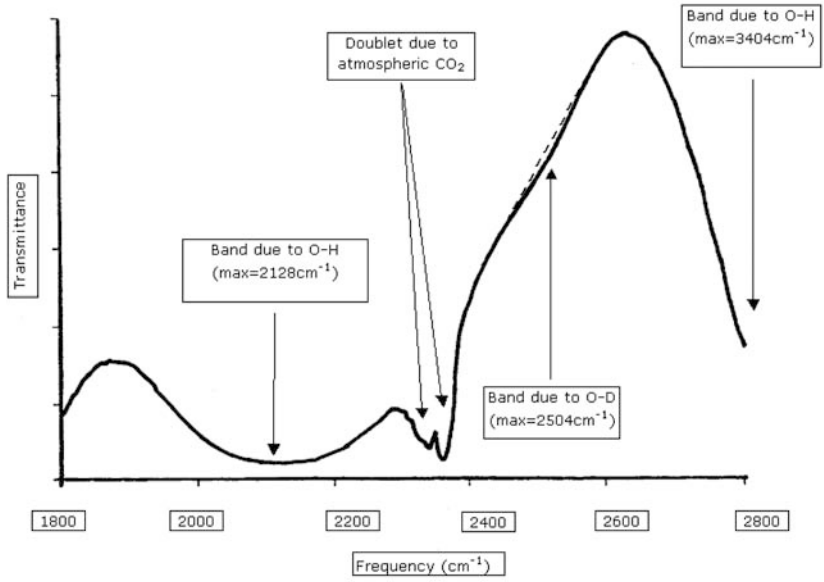


FIG. VI-4. The infrared spectrum of a 1000 ppm solution of ^2H_2O in H_2O. The band due to the O–D bond appears as a shoulder on the intense band due to O–H.

intense band due to the O-H bond, which at this level (1000 ppm) is three orders of magnitude more abundant, which makes precise estimation of the baseline (indicated in the figure by the dotted line) very difficult, particularly since there is almost unavoidable interference to low energy due to atmospheric CO_2.

These problems can be resolved by a combination of a change in the way that spectra are acquired, together with a more sophisticated method of accounting for the baseline under the band. The latter requires that special software be written, which interfaces directly with the machine output. Full details of the algorithm will be given later.

The principal change to the way that the spectrum is acquired is in the sample used for the background. Instead of environmental air or a blank cell being used for the background the cell is filled with water at natural abundance. The output of the spectrometer in the region of the peak at 2504 cm^{-1} now shows the band due to the O–D bond much more clearly, see Fig. (VI-5). The five spectra in this figure were obtained sequentially using FTIR, and reloading the sample each time. Atmospheric CO_2 obscures the low energy side of the D–O

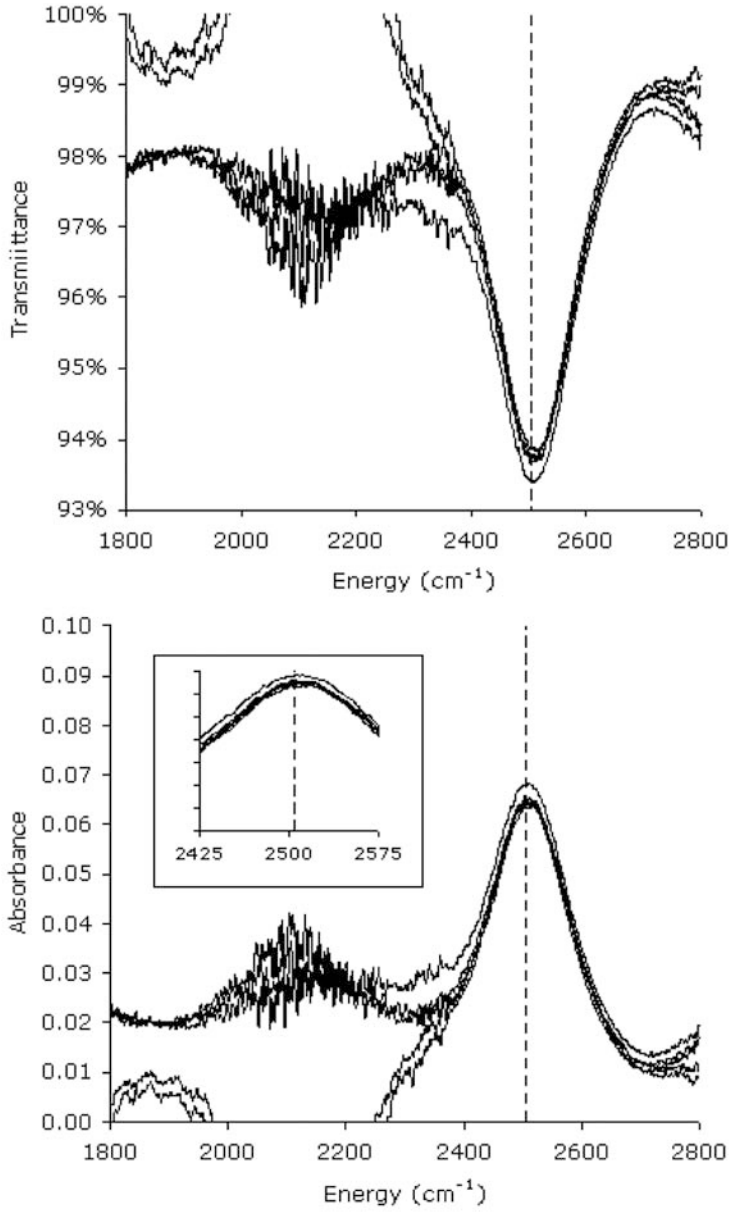


FIG. VI-5. Infrared spectra of deuterium in water (1000 ppm excess) recorded against a naturally abundant sample as background. Upper panel shows transmittance; the lower panel shows absorbance. The graphs show the results obtained from five replicate samples measured against a common background.

band to a highly variable degree (probably due to the operator breathing during sample loading).[1]

For any quantitation to be performed it is necessary to work in terms of absorbance so that the Beer–Lambert Law can be utilized. The same five spectra are shown in this way in Fig. VI-5), where it is clear that even though the deuterium peak is well defined the estimation of the baseline is quite subjective. Furthermore it may be observed from the inset in Fig. VI-5 that the peak maximum is not exactly coincident with the predicted value of 2504 cm^{-1}. This may be due either to the small shift in band position with concentration, or to a sloping baseline distorting the peak envelope. Using FTIR and a specially developed algorithm, which is described in the next section, can solve both the problems of peak displacement and baseline estimation. The algorithm is not commonly used in spectral analysis and is unlikely to be supplied with an instrument. Most FTIR spectrometers have a 'data export' facility, which will save the data as a two column table of absorbance versus energy. Once in this form the data can be manipulated using a spreadsheet, or better by using special software available from MRC-HNR.[1]

Use of peak fitting software

As discussed in the previous section, even if the infrar1

ed spectra are taken with a naturally abundant background subtracted there are severe difficulties in accurately estimating deuterium concentration from the spectra obtained. The algorithm, originally developed for mass spectrometry [107, 108] and later applied to FTIR [109] is used to compare the absorbance spectrum obtained from a sample with that of a known deuterium enrichment (the 'reference'), and will now be derived fully.

It is assumed that the absorption spectrum of the reference can be written as a peak envelope (of unknown form) superimposed on a linear baseline. Mathematically this is written:

$$R(v) = c_{ref}A(v) + a_{ref}v + b_{ref} \qquad \text{(VI-10)}$$

where $R(v)$ describes the observed band shape, $A(v)$is the spectrum of unit concentration of deuterium in the absence of baseline effects, c_{ref} is the

[1] Isotope.exe can be obtained from Medical Research Council Collaborative Centre for Human Nutrition Research, Elsie Widdowson Laboratory, Fulbourn Road, Cambridge CB1 9NL, United Kingdom; telephone (+44 1223) 426356, web site www.mrc-hnr.cam.ac.uk.

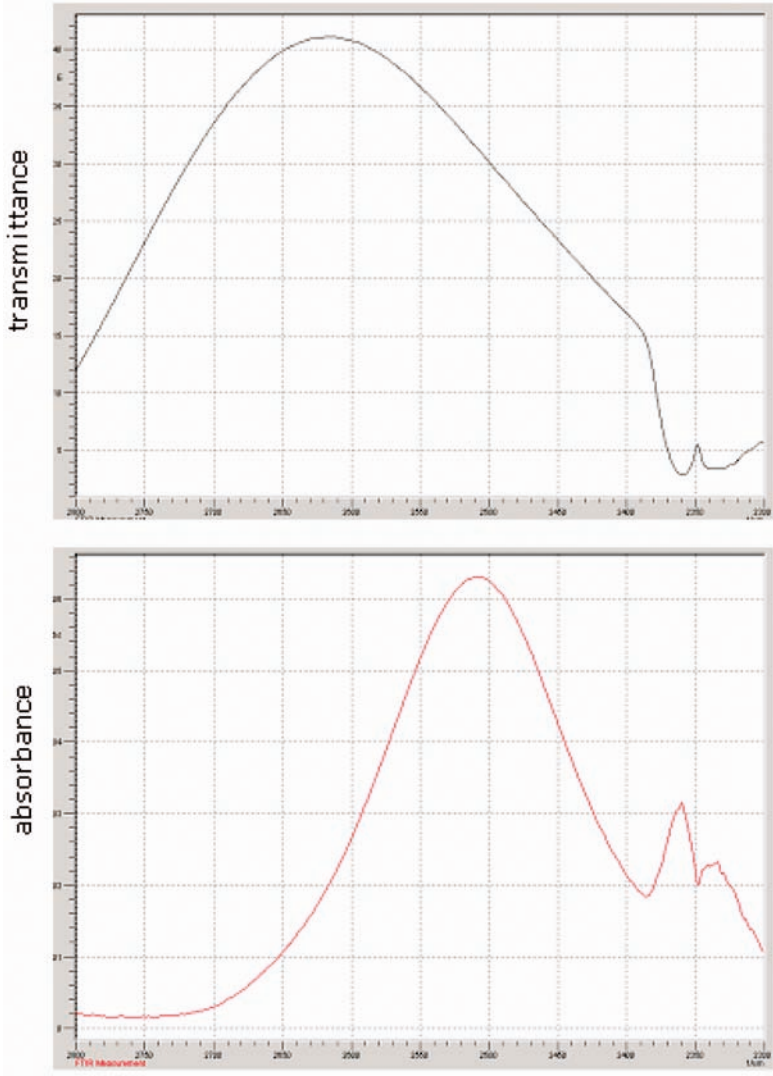


FIG. VI-6. The two stages in obtaining the spectrum of a 1000 mg/L standard. The upper panel shows the spectrum (transmittance) of the basal sample. The lower panel shows the background corrected spectrum (absorbance). In both cases note the doublet due to atmospheric CO_2.

deuterium concentration (mg/kg) in the reference, and a_{ref}, b_{ref} are constants describing the linear baseline. The spectrum of the sample can be written in a similar way, except that it is allowed to be displaced by a small amount, δ, along the frequency axis.

Therefore:

$$S(\nu) = c_{\text{samp}} A(\nu + \delta) + a_{\text{samp}} (\nu + \delta) + b_{\text{samp}} \tag{VI-11}$$

The constants in this equation are defined in the same way as in Eq. VI-10), except that the subscripts have been changed to identify them as relating to the spectrum of the sample. Expand the term $A(\nu + \delta)$ as a Taylor series truncated after the third term to obtain:

$$S(\nu) = c_{samp} \left\{ A(\nu) + \delta \frac{dA(\nu)}{d\nu} + \frac{\delta^2}{2} \frac{d^2 A(\nu)}{d\nu^2} \right\} + a_{samp} (\nu + \delta) + b_{samp} \tag{VI-12}$$

From Eq. (VI-10)

$$A(\nu) = \frac{R(\nu) - a_{ref}\nu - b_{ref}}{c_{ref}} \tag{VI-13}$$

$$\frac{dA(\nu)}{d\nu} = \frac{\frac{dR(\nu)}{d\nu} - a_{ref}}{c_{ref}}$$

$$\frac{d^2 A(\nu)}{d\nu^2} = \frac{\frac{d^2 R(\nu)}{d\nu^2}}{c_{ref}}$$

If we now make the approximations

$$\frac{dR(\nu)}{d\nu} \approx \frac{R(\nu + \varepsilon) - R(\nu - \varepsilon)}{2\varepsilon} \tag{VI-14}$$

$$\frac{d^2 R(\nu)}{d\nu^2} \approx \frac{R(\nu + \varepsilon) - 2R(\nu) + R(\nu - \varepsilon)}{\varepsilon^2}$$

Equation (VI-13) becomes

$$A(\nu) = \frac{R(\nu) - a_{ref}\nu - b_{ref}}{c_{ref}} \tag{VI-15}$$

$$\frac{dA(\nu)}{d\nu} = \frac{R(\nu+\varepsilon) - R(\nu-\varepsilon)}{2\varepsilon c_{ref}} - \frac{a_{ref}}{c_{ref}}$$

$$\frac{d^2A(\nu)}{d\nu^2} = \frac{R(\nu+\varepsilon) - 2R(\nu) + R(\nu-\varepsilon)}{\varepsilon^2 c_{ref}}$$

These may be substituted into Eq. (VI-12) to obtain

$$S(\nu) = \frac{c_{samp}}{c_{ref}}\left\{\frac{\delta}{2\varepsilon}\left(\frac{\delta}{\varepsilon}+1\right)R(\nu+\varepsilon) + \left(1-\frac{\delta^2}{\varepsilon^2}\right)R(\nu) + \frac{\delta}{2\varepsilon}\left(\frac{\delta}{\varepsilon}-1\right)R(\nu-\varepsilon)\right\}$$
$$+\left(a_{samp} - \frac{c_{samp}}{c_{ref}}a_{ref}\right)\nu + (\nu+\delta) + \left(\delta a_{samp} + b_{samp}\right) - \frac{c_{samp}}{c_{ref}}\left(\delta a_{sref} + b_{sref}\right) \tag{VI-16}$$

The utility of this equation is demonstrated by noting that it is of the form

$$S(\nu) = \chi_1 R(\nu+\varepsilon) + \chi_2 R(\nu) + \chi_3 R(\nu-\varepsilon) + \chi_4\nu + \chi_5 \tag{VI-17}$$

where χ is a constant and

$$\chi_1 + \chi_2 + \chi_3 = \frac{c_{samp}}{c_{ref}} \tag{VI-18}$$

In other words, using just the overall band profiles of sample and reference spectra, *without any recourse to the estimation of likely baselines* equation, Eq. (VI-17) indicates that the ratio of the concentrations of deuterium in sample and reference can be obtained directly.

In practice, the output from the FTIR is in the form of a series of absorbance data obtained at incremental steps over the range of infrared frequencies being measured. If this extends from $\nu_{\min}$ to $\nu_{\max}$ then the forgoing theory suggests that a series of equations can be written:

$$
\begin{aligned}
&S(\nu_{\min}+\varepsilon) = \chi_1 R(\nu_{\min}+2\varepsilon) + \chi_2 R(\nu_{\min}+\varepsilon) + \chi_3 R(\nu_{\min}) + \chi_4 \left(\nu_{\min}+\varepsilon\right) + \chi_5 \\
&S(\nu_{\min}+2\varepsilon) = \chi_1 R(\nu_{\min}+3\varepsilon) + \chi_2 R(\nu_{\min}+2\varepsilon) + \chi_3 R(\nu_{\min}+\varepsilon) + \chi_4 \left(\nu_{\min}+2\varepsilon\right) + \chi_5 \\
&S(\nu_{\min}+3\varepsilon) = \chi_1 R(\nu_{\min}+4\varepsilon) + \chi_2 R(\nu_{\min}+3\varepsilon) + \chi_3 R(\nu_{\min}+2\varepsilon) + \chi_4 \left(\nu_{\min}+3\varepsilon\right) + \chi_5 \\
&\vdots \\
&S(\nu_{\min}+n\varepsilon) = \chi_1 R(\nu_{\min}+(n+1)\varepsilon) + \chi_2 R(\nu_{\min}+n\varepsilon) + \chi_3 R(\nu_{\min}+(n-1)\varepsilon) + \chi_4 \left(\nu_{\min}+n\varepsilon\right) + \chi_5 \\
&\vdots \\
&S(\nu_{\max}-\varepsilon) = \chi_1 R(\nu_{\max}) + \chi_2 R(\nu_{\max n}-\varepsilon) + \chi_3 R(\nu_{\max n}-2\varepsilon) + \chi_4 \left(\nu_{\max n}-\varepsilon\right) + \chi_5
\end{aligned}
\qquad \text{(VI-19)}
$$

where $S(\nu)$ is the digitized absorption spectrum of the sample, $R(\nu)$ that of the reference, ε the increment of digitization, and χ_1, χ_2, χ_3, χ_4, and χ_5 are constants.

This is a set of simultaneous equations in five unknowns, which will in general be heavily overdetermined. The best way of obtaining a general solution to these equations is to use matrix algebra. The equations can be written in matrix form as:

$$
\underline{S} = \underline{\underline{R}}\,\underline{\chi} \qquad \text{(VI-20)}
$$

with the general solution:

$$
\underline{\chi} = \left(\underline{\underline{R}}^{+}\,\underline{\underline{R}}\right)^{-1}\underline{\underline{R}}^{+}\,\underline{S} \qquad \text{(VI-21)}
$$

Infrared liquid cells

From the Beer–Lambert Law, the absorbance of the beam is linearly related to the thickness of the sample (more correctly termed 'path length'). For optimal performance the peak absorbance should be around 0.05. Consider now a sample of 1 g in 1 L of water, which represents the upper limit of enrichment likely to be obtained in practice. This sample is 1000 ppm (weight), and has an excess deuterium concentration of 2 ×1/20 = 0.1 M. The reported extinction coefficient (ε) for the peak at 2504 cm^{-1} is 7150 $M^{-1}.m^{-1}$ [104], and hence the optimal path length is found from:

$$
l_{optimal} = \frac{A_{optmal}}{\varepsilon c} = \frac{0.05}{0.1\times 7150} = 70\ \mu\text{m} \qquad \text{(VI-22)}
$$

Liquid cells are readily obtainable with path lengths of 25, 50, 100, 200, 500, or 1000 μm, with the recommendation that 100 μm be used for the application of TBW measurement.

It is essential that the cell material be transparent to the infrared radiation used. Commonly used cell materials are summarized in Table below. Each material has a usable energy range and the material from which laboratory glassware is typically made (borosilicate glass) has a lower limit of usability that is higher than the energy of the spectral band we need to use.

The alkali halides, which are the most common materials for infrared work, are unsuitable for this application since they are soluble in water. The alkaline earth fluorides are useful when the sampled body water is saliva or plasma [109, 110]. If urine is sampled, CaF_2 and BaF_2 are unsuitable as they are damaged by the ammonium and phosphate content, leading to clouding. Although the lower cut-off quoted for quartz is above the energy required, it has been used (with limited success) for urine. It is possible that LiF may be suitable for this analysis, but currently this has not been investigated.

The cells themselves comprise two windows separated by a spacer. They may be supplied sealed by amalgamation or in demountable form where the spacer acts as a self-sealing gasket. In either case the cells are mounted into a cell nest, which has, a back plate suited to the sample holder of the FTIR, held in position with a front plate with two integral Luer filling ports.

The advantage of the demountable cell is the ease of cleaning. However, there is no guarantee that the cell path length does not change marginally on disassembly and reassembly. Therefore this must be avoided between measuring standard and samples. If a sample shuttle is fitted to the FTIR to

TABLE VI.1. COMMONLY USED CELL MATERIALS

Material[a]		Usable energy range		Comments
		Low (cm^{-1})	High (cm^{-1})	
×	Borosilicate glass	4000	30 000	Laboratory glassware
√	CaF_2	1100	77 000	Unsuitable for urine
√	BaF_2	740	67 000	Unsuitable for urine
√	LiF	1700	95 000	
×	NaCl	625	40 000	Dissolves in water
×	KBr	400	40 000	Dissolves in water
(√)	SiO_2 (quartz)	2700	65 000	Has been used for urine

[a] Commonly used infrared window materials.

allow baseline correction to be obtained without opening the sampling compartment between baseline and sample data being acquired, then it is important that a matched pair of sealed cells be used. These are a pair of cells that have been selected to have optical properties that correspond with a small degree of tolerance.

Appendix VII

THE HNR SOFTWARE 'ISOTOPE.EXE'

Installing the software (see footnote 1, page 110)

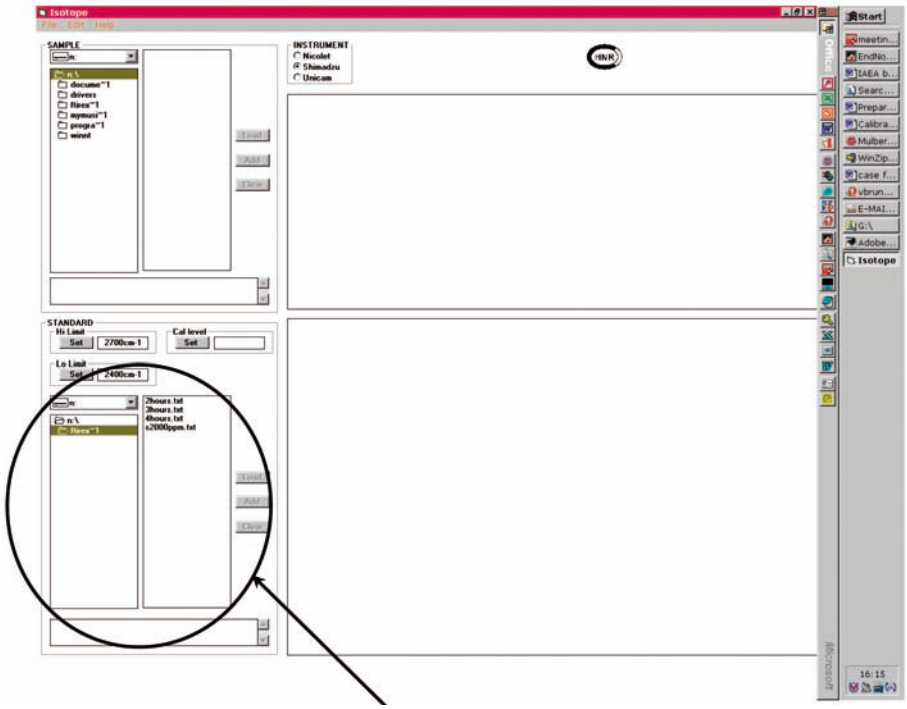
The software comprises two files, 'isotope.exe' and 'vbrun300.dll'. The first of these is the executable (programme) file that has been specially written by HNR, the second is the visual basic runtime library. Both of these files need to be copied to your computer.

(1) Using Windows Explorer, open a new folder for isotope work.
(2) Copy both 'isotope.exe' and 'vbrun.exe' to this new folder.
(3) If desired, create a shortcut to 'isotope.exe', and drag it onto the desktop.

Using the software

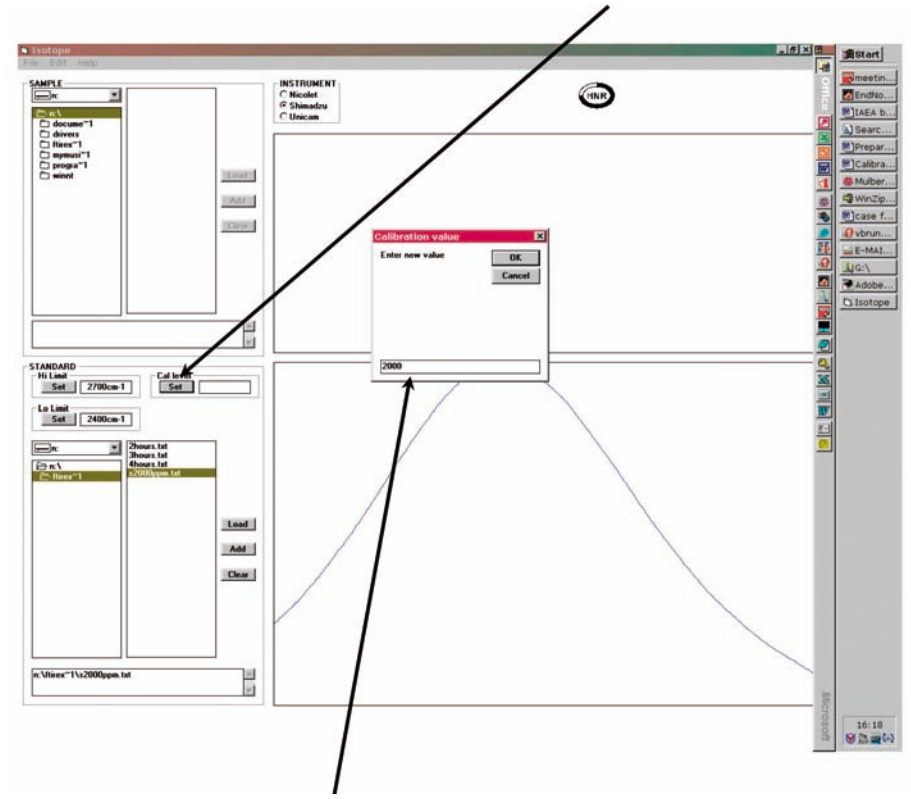
When the software is launched the screen shown on the next page will appear. (Note the files and folders in the directory structure shown for standard and sample, as well as some of the other screen detail will vary depending on the contents of the computers hard disk drive(s) and the desktop configuration.

Because exported data from FTIR instruments differ the software has been configured to run with instruments available from three manufacturers. Others may be added to the repertoire as and when they become used for deuterium analysis. The formats of the files exported are given in Section 7.4.7. The appropriate instrument type is selected using the radiobuttons at the top of the screen. If 'Unicam' is selected then only files with the extension (filetype) '.tbl' are available for processing. If 'Shimadzu is selected then the expected file extension is '.txt', and if 'Nicolet' then files of type '.csv' are required.



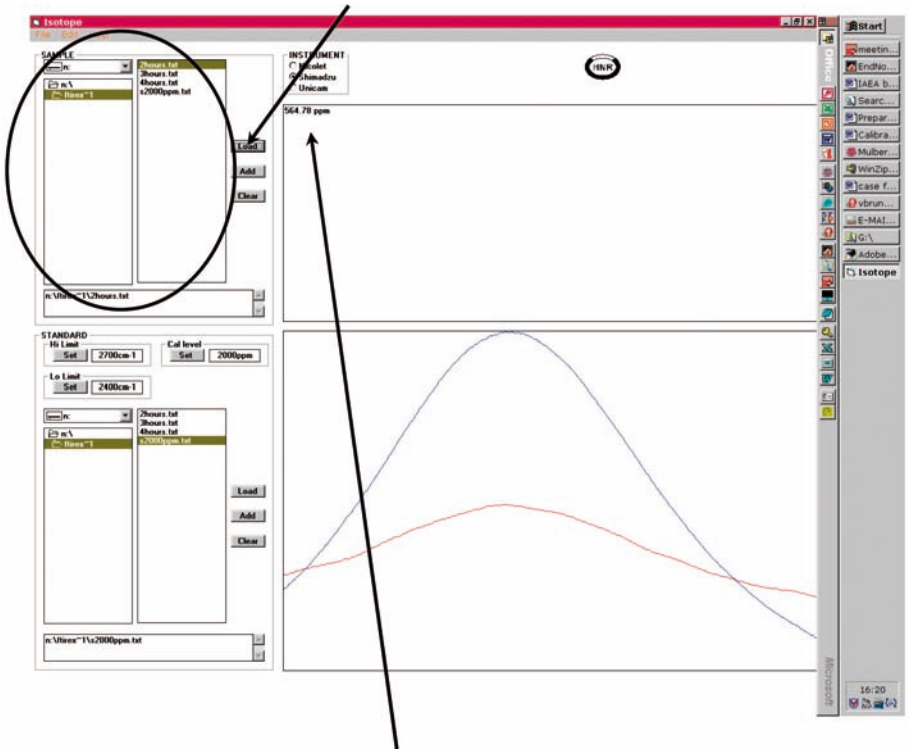
In the section for the standard select the appropriate file that has been exported from the FTIR control software, and load it.

Now set the calibration level. Click the “Cal level set” button:



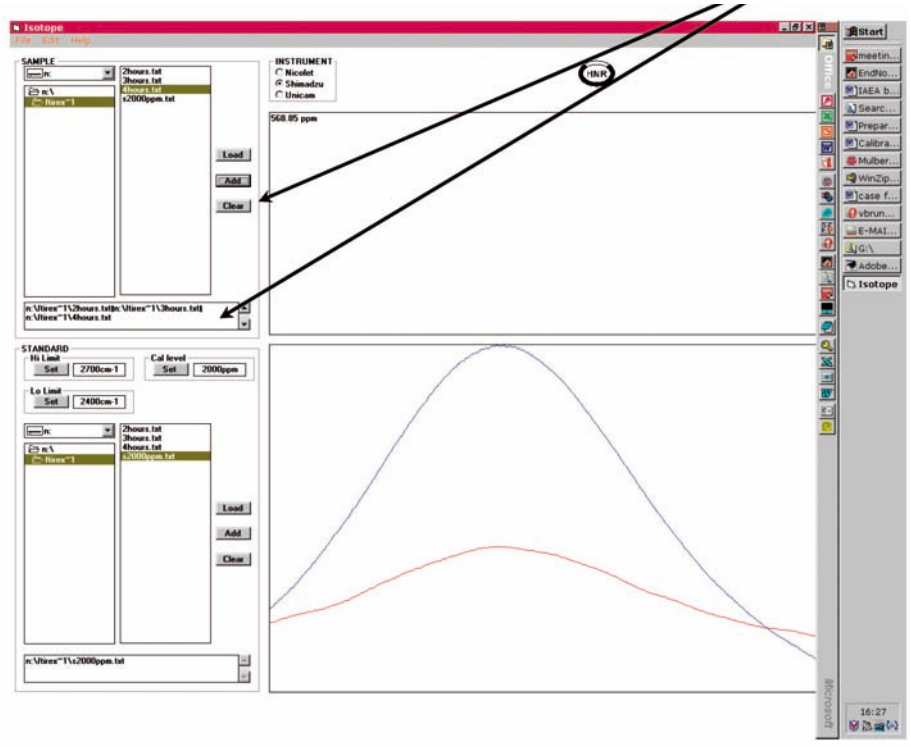
Enter the true excess ppm by weight (mg/kg) value — in this example this was calculated to be 2000 — in the dialogue box and then click “OK”.

Now load an exported sample file in a manner analogous to the standard file.

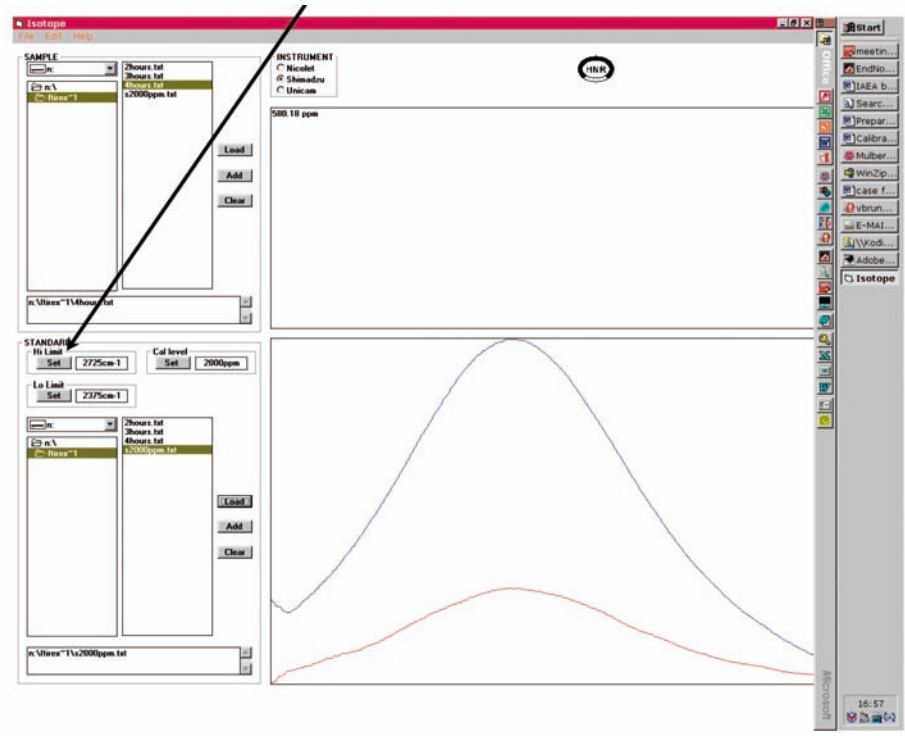


The isotopic composition of the sample is displayed in ppm by weight (mg/kg)

If required, up to four sample or standard spectra can be summed using the add function (note the file names in the box below)



It is possible to set different limits for the spectrum comparison between sample and standard.



(Note that if these values are changed the spectrum display will be blank, and the files for the sample and standard will have to be reloaded).

Appendix VIII

LABORATORY ITEMS

IRMS

- **Dose preparation:** Stable isotope stocks; separate refrigerator for dose stocks and reference waters; clean and dry 5 L glass bottle with PTFE lined top; high range (~10 kg) and medium range (~500 g) analytical balances; dose bottles; glass funnels; measuring cylinder; and labels.
- **Working standard preparation:** Stable isotope stocks; refrigerator for dose stocks and reference waters; 1 L and 100 mL clean and dry glass storage bottles; pipettes; funnels; beakers; measuring cylinder; and labels.
- **Preparation of samples and standards for analysis:** Freezer for sample storage, separate from doses and standards; disposable gloves; laboratory coat; pipettes; catalyst; Exetainers; tube racks; gassing station, unless an IRMS autosampler is being used for this; reference waters; centrifuge (should samples contain sediment); and labels.
- **Analytical equipment:** An IRMS suitable for analysis of ^{2}H abundance in body fluids, housed within a temperature controlled laboratory with secure electricity supply.
- **Data reduction:** A separate workstation for processing and archiving data and generating reports.

FTIR

- **Dose preparation:** Stable isotope stocks: separate refrigerator for dose stocks and reference waters; dose bottles; high range (~10 kg) and medium range (~500 g) analytical balances; dose bottles; glass funnels; measuring cylinders; labels.
- **Work station preparation:** Stable isotope stocks; refrigerator for dose stocks and reference waters; 1 L and 100 mL clean and dry glass storage bottles; pipettes; funnels; beakers; measuring cylinder; and labels.
- **Preparation of samples and standards for analysis:** Freezer for sample storage, separate from doses and standards; disposable gloves; laboratory coat; 1 mL disposable syringes; absorbent paper; reference waters; centrifuge (should samples contain sediment); and labels.
- **Analytical equipment:** An FTIR suitable for analysis of ^{2}H enrichment in saliva; and a desiccant.
- **Data reduction:** A separate work station for processing and archiving data and for generating reports.

Appendix IX

SUPPLIERS OF INTERNATIONAL STANDARDS

- IAEA http://www.iaea.org
- National Institute for Standards and Technology (USA) http://www.nist.gov

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This publication provides information on the theoretical background as well as the practical application of state of the art methodologies to monitor changes in body composition and total energy expenditure, and reflects recent advances in analytical techniques. It was developed by an international group of experts as an integral part of the IAEA's efforts to contribute to the transfer of technology and knowledge in this field among nutritionists, analytical chemists and other professionals.

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