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No. 7

Stable Isotope Technique to Assess Intake of Human Milk in Breastfed Infants

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STABLE ISOTOPE TECHNIQUE TO ASSESS INTAKE OF HUMAN MILK IN BREASTFED INFANTS

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STABLE ISOTOPE TECHNIQUE TO ASSESS INTAKE OF HUMAN MILK IN BREASTFED INFANTS

INTERNATIONAL ATOMIC ENERGY AGENCY
VIENNA, 2010

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FOREWORD

Exclusive breastfeeding for six months, followed by the introduction of appropriate complementary foods and continued breastfeeding, as recommended by the World Health Organization, are cornerstones in infant nutrition. However, only limited information is available on the quantities of human milk consumed and the time of introduction of other foods into infants' diets. The lack of information is, at least partly, due to the difficulties involved in assessing intake of human milk. The conventional technique is to weigh the infant before and after each feed, which is called 'test weighing'. However, this technique is time consuming and may disturb the normal feeding pattern.

These practical problems can be overcome by using a stable (i.e. non-radioactive) isotope technique, the deuterium oxide 'dose-to-mother' technique, as the normal feeding pattern is not influenced and the total volume of human milk consumed by the baby over a period of 14 days is assessed. Furthermore, the method is non-invasive as the dose of deuterium oxide is consumed orally by the mother and saliva samples are collected for analysis.

This method, which is elegant in its simplicity, was developed by the late A. Coward in the United Kingdom in the early 1980s. This publication, the first of its kind, is largely based on his pioneering work. The IAEA has fostered the more widespread use of this technique in Member States by supporting national and regional nutrition projects through its technical cooperation programme, in particular in Africa, and through coordinated research projects addressing priority areas in infant nutrition, for example, within the recent project on zinc nutrition during early life.

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 methodology to assess the intake of human milk in breastfed infants by the stable isotope technique, based on analysis of deuterium by Fourier transform infrared spectrometry.

The IAEA is grateful to the major contributors to this publication (L. Bluck and C. Slater, United Kingdom) for sharing their technical expertise and extensive experience in the stable isotope technique in nutrition. Photographs are based on recent training events in Africa to illustrate the use of the deuterium oxide dose-to-mother technique. However, it should be noted that the application of this methodology offers the opportunity to address priority areas in infant nutrition globally. The IAEA officer responsible for this publication was L. Davidsson of the Division of Human Health.

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1. INTRODUCTION

1.1. BACKGROUND

Appropriate feeding practices play an important role in ensuring optimum growth, development and health in the first years of life. The World Health Organization (WHO) recommends exclusive breastfeeding for six months, followed by the introduction of appropriate complementary foods, while breastfeeding continues until two years of age or beyond [1–3]. Exclusive breastfeeding means that the infant receives human milk without any additional food or drink, not even water. In many countries, although breastfeeding is widespread, only a small proportion of infants are exclusively breastfed and only limited information is available on the quantities of human milk consumed and the time of introduction of other foods into infants' diets. This lack of information is due, at least partly, to the difficulties involved in measuring the intake of human milk.

The conventional technique to measure the intake of milk is to weigh infants before and after each feed. This is known as 'test weighing'. The technique is time consuming and the procedure can disturb the normal feeding pattern [4]. In many settings, infants are nursed frequently, on demand, including during the night, which results in practical limitations to the use of test weighing. The practical problems associated with test weighing can be overcome by using the stable isotope technique. The amount of human milk consumed by the baby over a period of 14 days can be assessed using the deuterium oxide 'dose-to-mother' technique (Fig. 1), which involves giving the mother a drink of deuterium labelled water and following the disappearance of the deuterium from the mother and its appearance in the baby (Fig. 2). The technique also allows the baby's intake of water from sources other than human milk and the mother's body composition to be estimated [5–7]. For example, the deuterium oxide dose-to-mother technique can be used to: evaluate the efficacy of counselling and education programmes on infant feeding practices [8, 9]; evaluate the association between the intake of human milk by breastfed infants and maternal body composition [10]; evaluate community nutrition programmes for lactating women [11]; evaluate the effect of the introduction of complementary foods on human milk intake by breastfed babies [12]; and quantify nutrient flux or transfer of toxic elements from mother to baby [13, 14].

Deuterium is a stable (non-radioactive) isotope of hydrogen with the symbol ^2H. It is given orally as deuterium oxide (^2H_2O) and after mixing with body water is eliminated from the body in urine, saliva, sweat and human milk. Deuterium oxide is metabolized in the body in the same way as water, and is

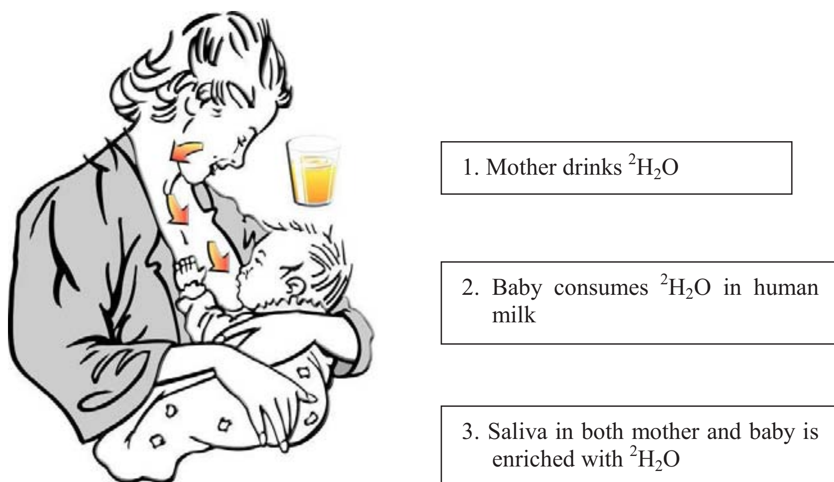

FIG. 1. Dose-to-mother technique for assessing human milk intake.

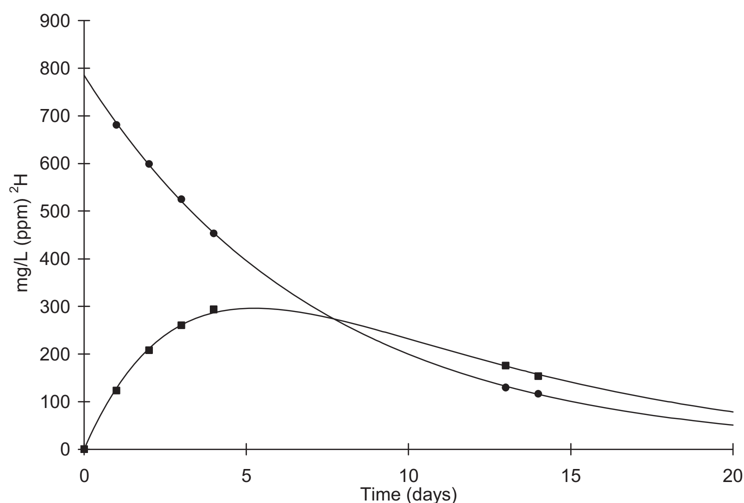

FIG. 2. Disappearance of deuterium from the body water of the mother (●) and appearance in her baby (■).

dispersed through the body water within a matter of hours. Body water can be sampled in the form of saliva, urine, plasma or human milk and the enrichment of deuterium can be measured by isotope ratio mass spectrometry (IRMS) or Fourier transform infrared spectrometry (FTIR). FTIR is not suitable for analysis of urine

or human milk samples. The technique is not as sensitive as IRMS and, therefore, a larger dose of deuterium oxide is required. However, FTIR instrumentation is easier to use and maintain than that of IRMS, is less expensive to buy and the cost of analysis is lower; FTIR is, therefore, particularly suitable in settings where resources are limited.

1.2. OBJECTIVE

This publication is a practical guide to the assessment of human milk intake by breastfed babies using the deuterium oxide dose-to-mother technique. It is intended for nutritionists, analytical chemists and other health professionals, who may have no previous experience of using stable isotope techniques.

1.3. SCOPE

This manual describes a quantitative method of assessing human milk intake and the intake of fluids from sources other than human milk by breastfed babies. It includes a description of the analysis of deuterium enrichment in saliva samples by FTIR.

1.4. STRUCTURE

Following this introduction, Section 2 gives some background information on the deuterium oxide dose-to-mother technique of assessing human milk intake by breastfed babies. Section 3 gives detailed instructions for the procedure including anthropometry of the mother and her baby; preparation of deuterium oxide doses; consumption of the dose by the mother; and collection of saliva samples from the mother and from the baby. Section 4 discusses the analysis of deuterium enrichment by FTIR. Section 5 describes the precautions that should be taken to ensure good quality data, while Section 6 lists the equipment needed, both in the laboratory and in the field. Finally, four appendices describe: the mathematical model used for calculation of human milk intake and the baby's intake of water from sources other than human milk; general information on the safety of deuterium oxide; isotopic fractionation; and FTIR.

2. THE DOSE-TO-MOTHER TECHNIQUE FOR ASSESSING HUMAN MILK INTAKE

2.1. TWO COMPARTMENT STEADY STATE MODEL OF WATER FLOW IN A MOTHER–BABY PAIR

2.1.1. Introduction

The deuterium oxide dose-to-mother technique was first described by A. Coward and co-workers in 1982 [5]. Assessment of human milk intake and intake of water from sources other than human milk is based on a two compartment model (Fig. 3). For more detailed information, see Appendix I.

In the two compartment model, the mother's total body water (TBW) is the first compartment and the baby's TBW is the second compartment. These two compartments are connected by the flow of human milk from the mother to the baby. In the steady state, water input is equal to water output, and the amount of water in the compartment does not change. Over the timescale of the experiment, this is a good approximation for the mother, but the baby's TBW changes as the baby grows.

2.1.2. Curve fitting and calculations

Intake of human milk and water from sources other than human milk can be assessed by fitting the deuterium enrichment data to a model for water turnover in the mother and the baby. Human milk intake by the baby is calculated from the

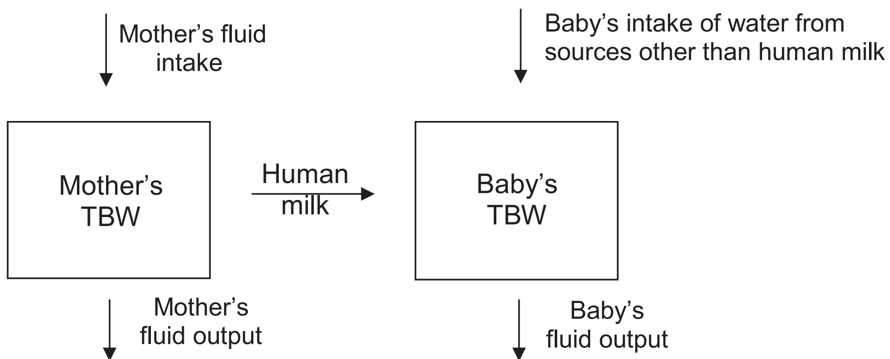

FIG. 3. Two compartment steady state model of water flow in a mother–baby pair.

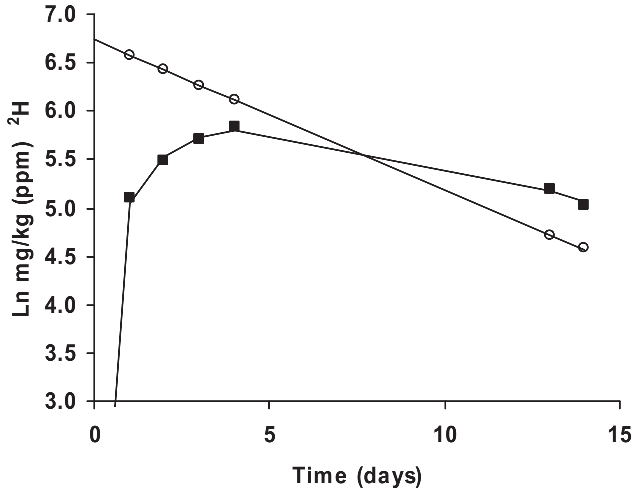

FIG. 4. Deuterium enrichment in the body water of a mother and her baby. The symbols (○ mother, ■ baby) represent the measured deuterium enrichment.

flow of water from the mother to the baby. The baby's total water intake includes water from the oxidation of human milk proteins, lipids and carbohydrates, and water from sources other than human milk. Allowance must be made for the baby's growth during the two week study duration. For more information, see Appendix I.

Figure 4 shows the natural logarithm (ln) of deuterium enrichment in the body water of a mother and her baby plotted against the time since the dose of deuterium oxide was consumed by the mother.

Curves are fitted to this data using the 'Solver' function in Microsoft Excel. Solver uses non-linear regression to determine the value of the constants that gave the line of best fit through the data. The elimination of deuterium from the mother (○) is linear on this plot. The mother's TBW and, therefore, her body composition can be estimated by back extrapolation to the y intercept (see Section 2.1.4).

There is an error associated with the estimate of the baby's intake of water from sources other than human milk because of the assumptions used in the model. This error (25 ± 62 g/d) results in a small apparent intake of water from sources other than human milk in babies who are reported to be completely exclusively breastfed [9].

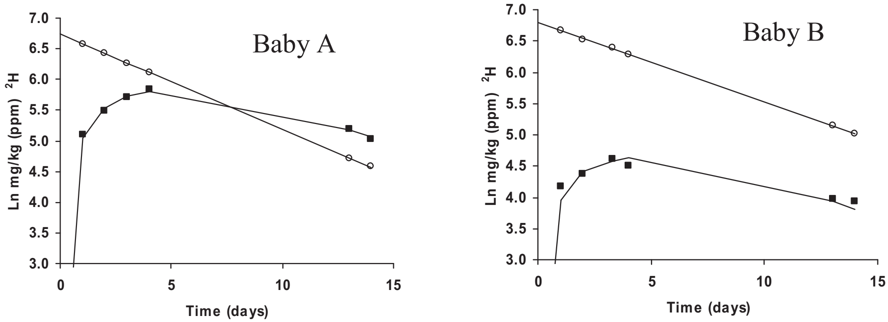

FIG. 5. ^{2}H enrichment in body water (saliva) collected from mother (○) and baby (■).

2.1.3. Example of data output

Figure 5 illustrates results from two different mother–baby pairs:

- The enrichment of ^{2}H in the baby's saliva was noticeably higher in the baby on the left (Baby A) than the baby on the right (Baby B);
- Baby A consumed 950 g of human milk per day and less than 25 g from sources other than human milk;
- Baby B consumed 230 g of human milk per day and 773 g of water per day from sources other than human milk.

2.1.4. Estimating maternal body composition

The mother's body composition is estimated from her TBW, which is measured by deuterium dilution. The calculations assume that the body is composed of fat and fat free mass (FFM). Fat mass (FM) is the difference between body weight and FFM. FFM can be estimated from TBW as follows.

The volume of TBW is slightly less than the volume of distribution of the deuterium dose because some of the deuterium is sequestered in non-aqueous substances (mainly proteins) by a process known as non-aqueous exchange.

V_D is the volume of distribution of deuterium (also known as the pool space). When the natural logarithm of deuterium enrichment in the mother's body water is plotted against time, the distribution is a straight line. V_D is calculated from the y intercept of the linear regression line through the data (Fig. 6).

The value of the y intercept is given the notation, $E_{m(0)}$ (enrichment of ^{2}H in the mother's body water at time zero):

$$V_D \text{ (kg)} = \text{deuterium oxide dose (mg)}/(E_{m(0)})$$

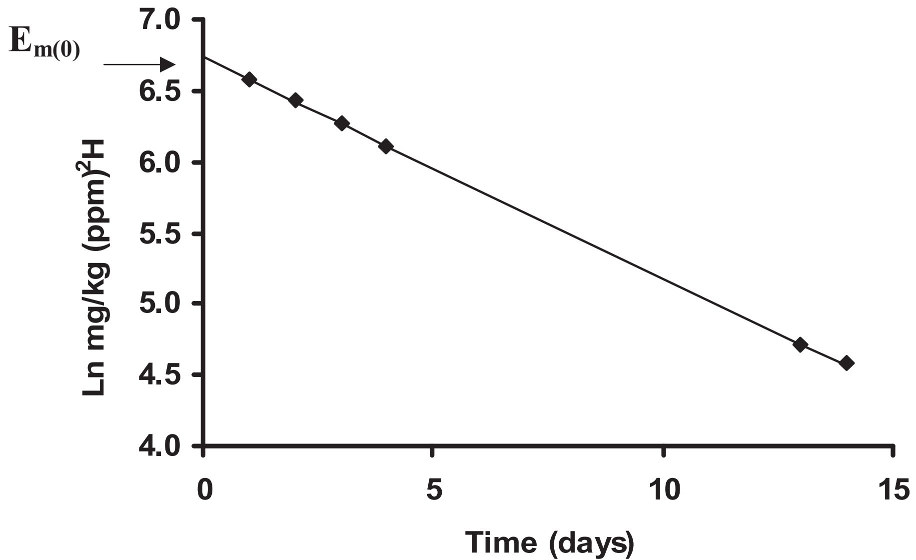

FIG. 6. Elimination of deuterium from the mother.

V_D must be corrected for non-aqueous isotopic exchange. Non-aqueous isotopic exchange for deuterium is assumed to be 4.1% of the pool space. TBW is, therefore, calculated by dividing V_D by 1.041:

TBW (kg) = V_D/1.041

The hydration of FFM in the body is remarkably constant between species, but is higher in infants than in adults. Here, we are only concerned with the mother. FFM in adults is assumed to be 73.2% water. This is known as the hydration of FFM. Thus:

FFM (kg) = TBW (kg)/0.732

FM is calculated as the difference between FFM and body weight:

FM (kg) = body weight (kg) – FFM (kg)
FM% = FM (kg)/body weight (kg) × 100

2.2. PLANNING THE STUDY

Careful planning is essential for a successful outcome in any study. The most important part of any study is determining the purpose of the study. It is necessary to concentrate on one main issue and to ask what the hypothesis being tested is:

- How many mother–baby pairs are required to address the issue? A sample size calculation should be performed (Section 2.3). A biostatistician should be consulted.
- How will the data be handled? What statistical tests will be performed? Expert advice should be sought at the planning stage, not after the data have been collected.
- What is the procedure for obtaining ethical approval?

2.2.1. Ethics

All studies involving human participants must be reviewed and approved by the local ethics committee. The ethics committee is usually composed of medical doctors, scientists and lay people, including religious and community leaders, and an advocate or lawyer. It could be based at the Ministry of Health, Ministry of Science or the local university. The committee should be contacted at an early stage to determine the process for seeking ethical approval and to obtain copies of the required documentation.

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

An example of the kind of information required by the ethics committee is shown below, but the details will vary, depending on local circumstances:

- The purpose of the proposed study must be clearly stated;
- A summary of the study design and methodology including details of the proposed sample size, giving indications of the calculations used to determine the required sample size;
- An outline of the ethical considerations involved in the proposal;
- Details of how consent is to be obtained, including an information sheet written in simple non-technical language;
- Who will have access to the data and what measures will be adopted to maintain the confidentiality of the participants;

— Who the investigators are (including assistants) that will conduct the study, and what their qualifications and experience are;
— Location(s) where the project will be carried out;
— Proposed start date;
— Proposed completion date.

Appendix II contains some information about the safety of deuterium oxide that may be useful in the preparation of applications for ethical approval.

2.2.2. Pilot study

If the technique has not been used before before, it is advisable to do a pilot study before starting.

A pilot study is important to:

— Practise and test the procedures, including sampling, analysis of samples and data handling;
— Train all people involved;
— Develop routine and teamwork;
— Develop strategies to overcome practical difficulties.

A pilot study is usually conducted with a relatively small number of mother–baby pairs.

2.3. SAMPLE SIZE CALCULATION

In any study, it must be ensured that an appropriate number of participants are included to be able to obtain a reliable answer to the question being asked. Sample size calculations are an important component of any study design, and are required by ethical review committees and funding bodies. A power calculation can be used to determine the sample size necessary to obtain a reliable answer.

Example

To calculate the required sample size for a study of human milk intake in breastfed babies, it is necessary to know the standard deviation of daily human milk intake in breastfed babies and to define the difference between study groups that will be regarded as significant. For example, a difference of 100 g of human milk intake per day might be regarded as defining the difference between predominantly breastfed and partially breastfed infants [7].

If no other information is available, a standard deviation (σ) of 130 g/d for human milk intake and a difference in human milk intake between groups (δ) of 100 g should be assumed. For a power of 80% and a significance level of 0.05, with two study groups, the required sample size (n) can be calculated using the following equation:

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

where 7.85 is the multiplication factor f (α, power) for a power of 80% and $\alpha = 0.05$ obtained from statistical tables. Therefore:

$$n = 2 \times 7.85 \times \left(\frac{130}{100}\right)^2 = 27$$

At least 27 mother–baby pairs in each group are required to achieve statistically significant results. If more power or a higher level of significance is required, then more participants will be required for the study. It is also sensible to add a factor to allow for drop-outs based on local experience. At least 30 mother–baby pairs in each group would be required in the above example, assuming a 10% attrition rate, but this could be much higher depending on the nature of the study.

3. PROCEDURE FOR ASSESSING HUMAN MILK INTAKE

The procedure for assessing human milk intake is summarized in Fig. 7. The following sections give detailed instructions on the procedure for assessing human milk intake.

3.1. DOSE PREPARATION AND STORAGE

All of the equipment used for preparing doses must be completely dry to avoid contamination by water. The standardized dose of deuterium oxide for assessing human milk intake, with analysis of deuterium using FTIR, is 30 g (99.8 at.% ^{2}H) for all mothers, regardless of their body weight.

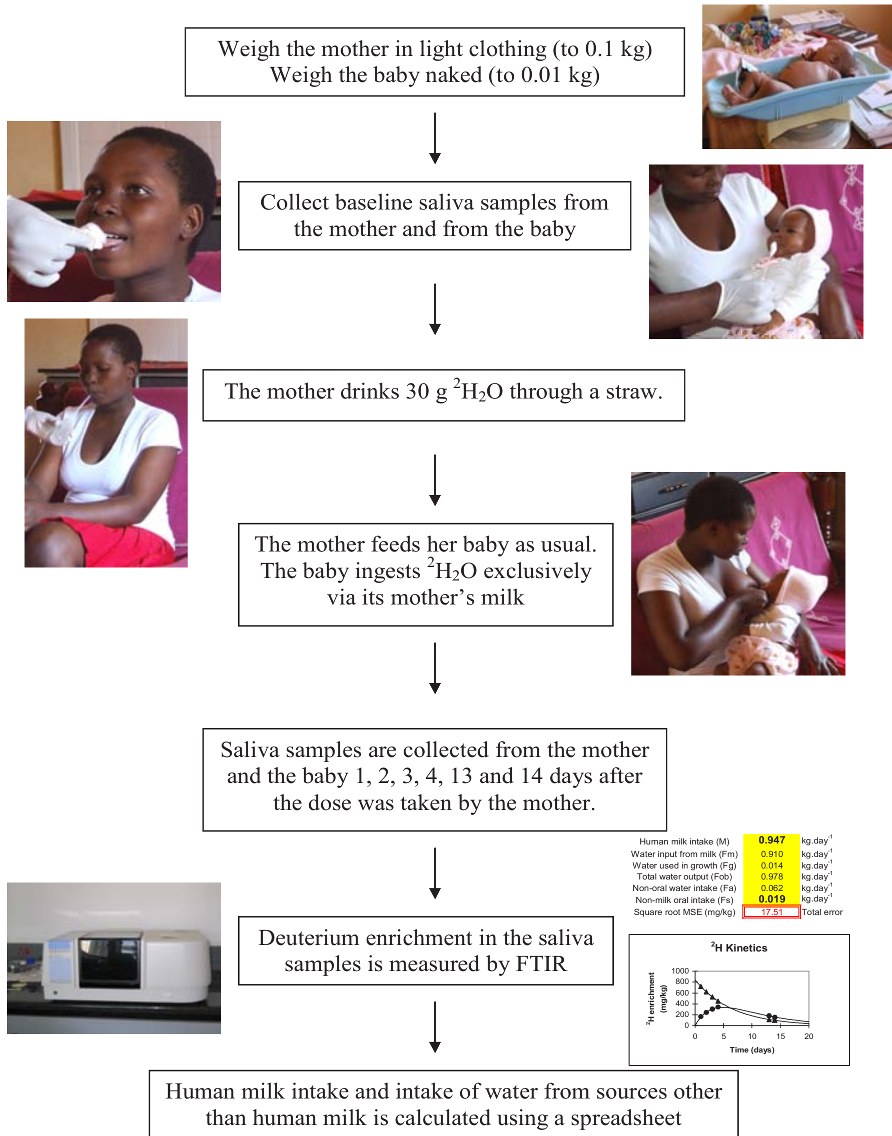

FIG. 7. Summary of the procedure for assessing human milk intake by the deuterium oxide dose-to-mother technique.

Dose bottles must be screw capped and leak proof (e.g. 60 mL wide mouth, leak proof, autoclavable, polypropylene bottles) to avoid losses during storage and contamination by moisture from the atmosphere. It is not necessary to autoclave the bottles. The doses can be made in batches and stored in a refrigerator until required. Bottles should be labelled with a dose number and the date the dose was prepared. Doses should be prepared in a food preparation area, not a chemistry laboratory.

Doses must be accurately weighed to at least 0.01 g. The batch number of the stock solution of deuterium oxide used to make the doses, the date the doses were prepared, the dose number, the weight of the bottle, the weight of the bottle plus the dose, and the weight of the dose should be recorded in a laboratory notebook. This information can be transferred to a spreadsheet later.

The procedure for preparing doses is illustrated in Fig. 8 and is as follows:

FIG. 8. Dose preparation. Doses should be prepared in a food preparation area, not in a laboratory.

— The labelled bottle plus lid should be weighed and the weight recorded.
— A measuring cylinder should be used to add 30 mL of ^2H_2O to the bottle and the lid should be replaced.
— The bottle plus ^2H_2O should be weighed.
— The exact weight of deuterium oxide in each dose should be recorded.
— The weight of ^2H_2O will not be exactly 30 g, as the density of deuterium oxide is greater than the density of water (density of ^2H_2O = 1.105 g/mL and density of H_2O = 1.000 g/mL at 25ºC). This does not matter as long as the exact weight is recorded and used in subsequent calculations.
— The same measuring cylinder can be used for all doses prepared in a single session, since it is only used for highly enriched deuterium oxide. It should not be washed between doses. This would contaminate it with water, leading to an error in the amount of ^2H_2O given to the mother.

Note that if this is the first time the technique has been used, it is necessary to keep a few millilitres of the 99.8 at.% ^2H stock solution to make a calibration standard for analysis with the saliva samples (see Section 4.3).

To ensure good hygiene and avoid cross-contamination, doses should not be stored in the same place as saliva samples. Samples will have an enrichment of ^2H of up to 1000 mg/kg (parts per million (ppm)), whereas the doses are enriched to at least 99.8 at.% excess, i.e. 998 000 mg/kg. The dose, therefore, contains approximately 1000 times as much deuterium as the biological specimens. In addition, doses should not be stored with saliva samples to avoid microbial cross-contamination. When transporting doses to and from the field, separate boxes for doses and saliva samples should be used.

3.2. MEASURING WEIGHT AND HEIGHT OR LENGTH

An accurate measure of the mother's body weight is required for estimating her body composition, which is based on the measurement of TBW. Mothers should be asked to empty their bladder (and if possible bowels) before being weighed, and should be weighed in minimal clothing. Standardizing conditions in this way is particularly important in longitudinal studies. The mother's weight should be measured at the baseline and again on Day 14 to make sure that it has not changed substantially. The mother's height is not required for the assessment of human milk intake, but most nutrition studies include basic anthropometry measurements of both the mother and baby. The body mass index (BMI) of the mother is calculated from her weight (in kg) and height (in cm):

$$\text{BMI (kg/m}^2\text{)} = \text{weight (kg)}/(\text{height (cm)}/100)^2$$

FIG. 9. Weight measurement. Weight is measured in light clothing without shoes.

3.2.1. Measuring the mother's weight

The mother's weight must be measured to the nearest 0.1 kg using electronic scales or any balance with adequate precision (Fig. 9).

The mother's weight is measured as described below:

- — The balance must be placed on a level surface. This should be checked using a spirit level;
- — Mothers should wear minimal clothing and no shoes. If mothers do not wish to wear minimal clothing during the weighing procedure, their clothes could be weighed separately afterwards, and the weight of their clothes subtracted to obtain an accurate measure of body weight;
- — The mother's weight should be recorded on the mother's information sheet to 0.1 kg;
- — The accuracy of the scales should be checked daily using a calibration weight of known mass.

FIG. 10. Measuring height: hair down, eyes forward.

3.2.2. Measuring the mother’s height

The mother’s height must be measured to the nearest 0.1 cm using a stadiometer.

The mother’s height is measured as described below:

- The stadiometer must be placed on a level surface, which should be checked using a spirit level. The accuracy of the stadiometer should be checked periodically using measuring rods of known length.
- Height should be measured without shoes.
- The mother should stand upright with her heels to the wall or touching the vertical post on the stadiometer. Her knees should be straight.
- The mother should be asked to look straight ahead. It should be ensured that her eyes are at the same level as her ears (Fig. 10).
- The beam should be lowered until it touches the top of her head. Elaborate hair arrangements must be undone. The mother’s height (in cm to the nearest 0.1 cm) should be recorded on the mother’s information sheet. The measurement should be repeated. Both measurements should be recorded and the mean calculated.

3.2.3. Measuring the baby’s length and weight

An accurate measurement of the baby’s weight (W) is required to estimate the volume of distribution of the deuterium in the baby (V_b) [15]:

$$V_b = 0.84W^{0.82}$$

The baby’s weight should be measured at the baseline and on Day 14.

WHO has developed detailed instructions on how to measure a child's weight and length/height. The materials can be downloaded from the following web site:

http://www.who.int/childgrowth/training/en/index.html

3.2.3.1. Measuring the baby's weight

The baby must be weighed without clothes using scales accurate to 0.01 kg (Fig. 11).

The baby's weight should be measured as described below:

— A cloth should be left in the weighing pan to prevent chilling the child;
— The scales should be adjusted to zero with the cloth in the pan;
— The naked child should be gently placed on the cloth in the weighing pan;
— It is necessary to wait for the child to settle and the weight to stabilize;
— The weight should be measured (to the nearest 10 g, 0.01 kg) and recorded immediately.

It is important to take proper care of the scales to ensure that measurements are accurate. The scales should be kept clean and stored at normal indoor temperature, protected from humidity.

Standardization (levelling) of the scales should be performed weekly, for example, every Monday or whenever the scales are moved.

FIG. 11. Measuring the baby's weight.

Checking the scales

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

To check tared weighing, a 10 kg weight should be weighed, the scales tared and then a 3 kg weight should be added. The scales should indicate the 3 kg weight.

If the weights are not accurate, the scales should be calibrated if possible. If it is not possible to recalibrate, the scales need to be replaced.

3.2.3.2. Measuring the baby's length

The baby's length is measured using a measuring board (sometimes called an infantometer). Two people are needed to measure the child's length (Fig. 12).

One person should:

- — Assist in positioning the child face up on the measuring board, supporting the head and placing it against the headboard;
- — Position the crown of the head against the headboard, compressing the hair. Check that the child is lying straight along the centre line of the board and does not change position. Shoulders should touch the board, and the spine should not be arched;
- — It is usual for this person to stand or kneel behind the headboard.

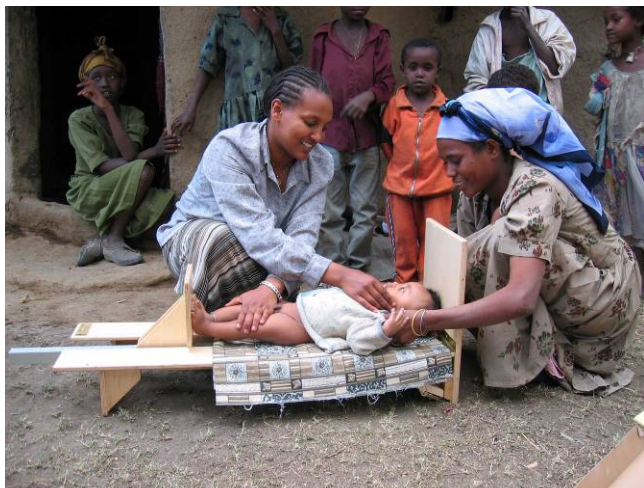

FIG. 12. Measuring the baby's length.

The second person should:

- Support the trunk as the child is positioned on the board.
- Lay the child flat along the board.
- Place one hand on the shins above the ankles or on the knees and press down firmly. The footpiece should be placed firmly against the heels with the other hand.
- The length should be measured (to the nearest 0.1 cm) and recorded immediately.

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

3.3. DOSE ADMINISTRATION

Baseline saliva samples must be obtained from both the mother and her baby before the mother consumes the dose. Detailed instructions for saliva sampling are given in Section 3.4. The dose bottle should be inverted several times to mix any condensation on the cap into the bulk of the liquid. This should be done immediately before the dose is consumed. This is because the condensation on the lid will be fractionated relative to the bulk liquid. For more information on fractionation, see Appendix III.

The dose should be administered as described below:

- The bottle should not be opened until it is time for the dose to be consumed;
- The bottle number and the time the dose was taken should be noted on the mother's data sheet;
- Mothers should drink the dose through a straw to avoid spillage (Fig. 13);
- About 50 mL of drinking water should be added to the dose bottle and the participant asked to drink this through the straw. This procedure should be repeated. This will ensure that no deuterium labelled water is left in the bottle.

FIG. 13. The mother drinks the dose through a straw.

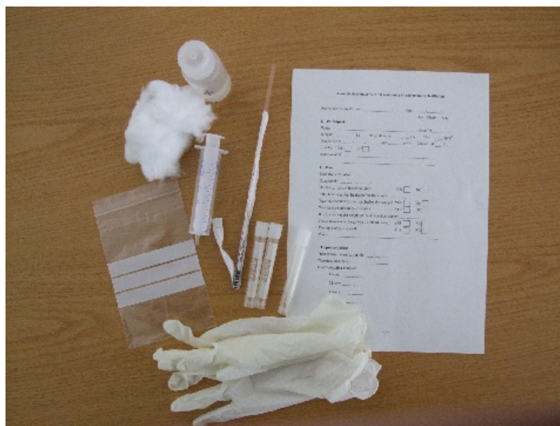

FIG. 14. Equipment required for saliva sampling.

3.4. SALIVA SAMPLING

3.4.1. Preparing for saliva sampling

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

Ensure that the following items are available before starting (Fig. 14):

— Cotton wool and swabs:
  - Cotton wool balls are used to collect saliva samples from the mother;
  - Cotton wool swabs with extra cotton wool are used to collect saliva samples from the baby.

— Sample storage vials:
  - Must be screw capped with a seal to prevent losses, fractionation and cross-contamination during storage, e.g. 4 mL cryovials. It is a good idea to use different coloured caps for baseline and post-dose samples, e.g. blue caps for baseline samples and red caps for post-dose samples.
  - Must be completely dry before use.
  - Must not be reused to prevent cross-contamination between enriched (post-dose) samples and unenriched (baseline) samples.
  - Should be labelled with the participant's identification number, date and time the sample was taken. Names should not be written on sample vials to preserve confidentiality.

— Disposable 20 mL syringes:
  - Must be completely dry before use;
  - Must not be re-used to prevent cross-contamination between enriched (post-dose) samples and unenriched (baseline) samples.

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

— Zip-lock bags:
  - Three small zip-lock bags are needed for each mother–baby pair: one for the baseline samples from both the mother and the baby; one for the mother's post-dose samples; and one for the baby's post-dose samples.
  - One larger zip-lock bag is needed to keep all the samples from the mother–baby pair together.
  - All of the bags must be labelled permanently with the participant's identification number.

— Labels:
  - It should be ensured that the labels are of good quality and cannot come off the containers.
  - A permanent marker should be used to write on the labels, to avoid the writing being smudged or removed, in particular when the samples are thawed.

— Participant data sheets:
  - Printouts of data sheets for each participant need to be available before the first sampling (baseline).
  - Names should not be written on the sheets to preserve confidentiality. The names and corresponding participant identification number must be recorded separately.
  - An example of a participant data sheet is shown in Section 3.5.

3.4.2. Step-by-step instructions for saliva sampling: Mother

The procedure for saliva sampling in the mother is described below and illustrated in Fig. 15:

(1) Clean gloves should be used for each participant.
(2) When collecting samples, it should be ensured that the mothers have not eaten or drunk anything for at least half an hour before collection.
(3) The mother should be given a cotton wool ball to soak up saliva. She should be asked to move it around her mouth for 2 min or until sodden, keeping her mouth closed while doing this. Asking her to think about a favourite food increases salivation.
(4) The plunger should be removed from a new 20 mL disposable syringe.
(5) The mother should be asked to transfer the cotton wool to the front of her mouth and transfer it directly from her mouth into the body of the syringe.
(6) The plunger should be replaced into the body of the syringe.
(7) A sample storage vial should be labelled with the participant's identification number, date and time of collection.
(8) The lid should be removed from the vial, and the syringe plunger used to extract saliva from the cotton wool into the sample storage vial.
(9) If there is not at least 2 mL of saliva, the above steps should be repeated with a new cotton wool ball or swab. If possible, 4 mL should be collected to allow for repeat analysis.

FIG. 15. Saliva sampling in the mother.

(10) The syringe, cotton wool and gloves should be discarded between participants. The sample vials or syringes should not be reused.
(11) The saliva should be sampled at the baseline and then 1, 2, 3, 4, 13 and 14 d after the dose was taken by the mother, preferably at the same time of day on each occasion. The participant's identification number as well as the date and time the sample was taken should be recorded on each vial.
(12) Good records should be kept. All the dates and times of saliva collection should be recorded on a data entry form as well as on the bottles. This information should be copied to a spreadsheet as soon as possible.

3.4.3. Step-by-step instructions for saliva sampling: Baby

The procedure for saliva sampling in the baby is described below and is illustrated in Fig. 16:

(1) Clean gloves should be used for each baby.
(2) When collecting samples, make sure that it is at least 15 min since the baby was last fed, so that there is no residual milk or other foods in the baby's mouth.
(3) In babies, saliva is sampled using a cotton wool swab. An extra piece of cotton wool should be wrapped around the swab. Saliva should be collected by moving the swab around the baby's mouth until the cotton wool is sodden. The time required for this will vary between babies. Patience is required. It may take several attempts to collect the required volume (minimum 2 mL, preferably 4 mL).
(4) The plunger from a new 20 mL disposable syringe should be removed. The cotton wool from the swab should be removed and placed in the barrel of the 20 mL syringe.
(5) The plunger should be replaced into the body of the syringe.
(6) A sample storage vial should be labelled with the participant's identification number, date and time of collection.

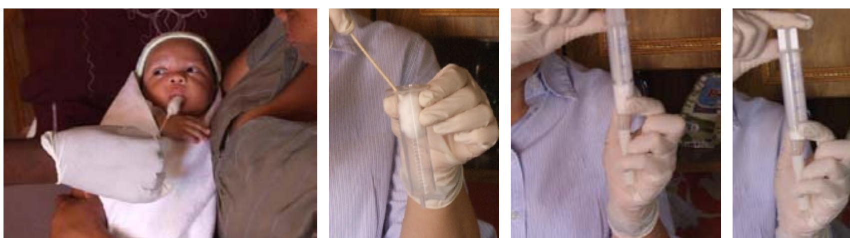

FIG. 16. Saliva sampling in the baby.

(7) The lid should be removed from the vial and the syringe plunger used to extract saliva from the cotton wool into the sample storage vial.

(8) If there is not at least 2 mL of saliva, the above steps should be repeated with a new cotton wool ball or swab. If possible, 4 mL should be collected to allow for repeat analysis.

(9) The swab, syringe, cotton wool and gloves should be discarded between participants. Sample vials or syringes should not be reused.

(10) The saliva from the baby should be sampled at the baseline and then 1, 2, 3, 4, 13 and 14 d after the dose was taken by the mother, preferably at the same time of day on each occasion. The participant's identification number, the date and time the sample was taken should be recorded on each vial.

(11) Good records should be kept. All the dates and times of saliva collection should be recorded on a data entry form as well as on the bottles. This information should be copied to a spreadsheet as soon as possible.

3.4.4. Storage of saliva samples

There will be a total of seven samples from the mother and seven samples from her baby; therefore, a large study will generate many hundreds of samples. Careful management and labelling of saliva samples is essential. Saliva sample vials from each mother–baby pair should be stored together.

Important points for the storage of saliva samples are listed below:

— It is important to use good quality, screw capped containers for storage of saliva samples;
— Containers must be firmly closed to prevent loss of water by evaporation and cross-contamination between samples;
— Sample vials should be stored in zip-lock bags to prevent cross-contamination between participants, and between pre-dose and post-dose specimens;
— The mother's and the baby's baseline samples should be placed in the same bag;
— A new bag should be used for the mother's post-dose samples and one for the baby's post-dose samples;
— All of these should be placed together in a larger zip-lock bag;
— The participant's identification number should be written on both the sample vials and the zip-lock bags;
— A log of samples should be kept in a spreadsheet;
— To minimize bacterial growth, saliva samples should be stored in a cool box or fridge until they can be transferred to a freezer at –20°C for storage until analysis.

To avoid contamination of samples:

- — Samples and doses should never be stored together;
- — It should always be ensured that the cap of the sample bottles is tightly closed to avoid losses by evaporation and contamination by moisture from the atmosphere.

3.5. DATA INPUTS REQUIRED

The data inputs required are the:

- — Mother's weight, height, study date, dose number, time of dose consumption, date and time of saliva samples at time zero and 1, 2, 3, 4, 13 and 14 d post dose;
- — Baby's weight, length, study date, date and time of saliva samples at time zero and 1, 2, 3, 4, 13 and 14 d post dose.

An example of a data sheet is shown in Fig. 17.

	Mother	Baby
Date of dosing (Day 0)		
Study ID		
Date of birth		
Body weight (kg) Day 0		
Body weight (kg) Day 14		
Height/length (cm)		
Date and Time of baseline saliva sample		
Dose number		Not applicable
Time dose taken		Not applicable
Date and Time Day 1 saliva sample		
Date and Time Day 2 saliva sample		
Date and Time Day 3 saliva sample		
Date and Time Day 4 saliva sample		
Date and Time Day 13 saliva sample		
Date and Time Day 14 saliva sample		

FIG. 17. Example of a participant data sheet.

4. ANALYSIS OF DEUTERIUM ENRICHMENT BY FTIR

The enrichment of deuterium in saliva samples can be measured by FTIR [16]. A typical FTIR instrument is shown in Fig. 18.

4.1. THE FTIR LABORATORY

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

The FTIR instrument should not be moved once it has been installed. If it is necessary to move it, an engineer must be called to check the alignment of the mirrors.

The humidity in the FTIR instrument should be less than 60%. If the FTIR contains a desiccant, it should be changed when the indicator changes colour. This could be once a week in humid climates.

FIG. 18. A typical FTIR instrument.

4.2. CLEANING THE FTIR INSTRUMENT

A water dampened cloth should be used to wipe the exterior of the FTIR instrument to keep it dust free. It is not advisable to wipe the inside of the sample compartment.

If spillage from the cell occurs inside the compartment, it should be cleaned up immediately with an absorbent lint free cloth.

A brief description of the principles of FTIR can be found in Appendix IV. The exact details of the procedure will depend on the make and model of the FTIR instrument, but the main principles and precautions are summarized in the following sections.

4.3. PREPARATION OF THE CALIBRATION STANDARD

A large volume of a calibrating or standard solution of approximately 1000 mg/kg (ppm), or 1 g/L should be prepared (gravimetrically) by weighing 99.8 at.% deuterium oxide (D_2O) and diluting it in normal drinking water from the region. Note that the density of deuterium oxide is 1.105 g/mL.

The following points should be noted:

— It is convenient to make 1 L of the calibration standard in a volumetric flask, and then to transfer it to a borosilicate bottle with a PTFE lined screw cap for storage until required. A second bottle containing 1 L of the water used to make the dilution should also be retained. It is a good idea to store the calibration standards in several smaller, tightly sealed bottles (e.g. 250 mL borosilicate bottles with PTFE lined screw caps). Only one enriched and one natural abundance bottle should be in use at any time, as working standards. The remainder should be kept sealed until required. The calibration standards will last for several years if stored in a cool dark place. The bottles must be well sealed to prevent ingress of water from the atmosphere. They should not be stored in the same place as the D_2O.

— The D_2O should be weighed on an analytical balance accurate to 0.0001 g or preferably 0.000 01 g. Suitable analytical balances and glassware will probably be available in the chemistry department of the local college or university. The standard should be prepared in two stages as described below, but it is important that the weight of deuterium oxide is known to 0.0001 g. Balances must be levelled and calibrated before use.

— Distilled water should not be used to make the calibration standard as it is subject to fractionation. The local drinking water should be used to make the 1000 mg/kg (ppm) standard. A similar volume of the local drinking

water should be kept to serve as a zero standard. If the quality of the local drinking water is poor, the shelf life of the standard will be improved if the water is passed through a sterile 0.22 μm filter.
— All glassware must be clean and dry before use.

The following procedure is recommended:

— Using an analytical balance (accurate to 0.0001 g or preferably 0.000 01 g), a clean, dry 50 mL volumetric flask with its stopper in place, or another similar container, for example a clean, dry glass bottle with a cap, should be weighed.
— A small volume (~20 mL) of drinking water should be added to the flask, the cap replaced and the flask weighed again.
— 1 g of D_2O should be added to the bottle. If an adjustable pipette is being used to transfer 1 g of D_2O, then the volume selected should be 0.9 mL, as the density of deuterium oxide is higher than that of water (1.105 g/mL and 1.000 g/mL, respectively, at 25ºC). The stopper or cap should be replaced to avoid losses by evaporation, and the weight noted. The weight of D_2O in the bottle should be calculated.
— A clean, dry 1 L volumetric flask with its stopper should be weighed. At this stage, a balance weighing to 0.1 g can be used.
— The water from the 50 mL container should be quantitatively transferred into the 1 L volumetric flask using a funnel. Local drinking water should be added to the smaller container and poured into the larger container. This should be repeated at least three times to ensure that all the deuterium oxide is transferred. Care should be taken not to spill any.
— Local drinking water should be added to the 1 L volumetric flask up to the mark. The stopper should be replaced and the flask weighed again.
— After the weight has been noted, the calibration standard should be transferred to a clean, dry glass bottle with a PTFE-lined screw cap.
— A similar volume of the local drinking water should be kept to use as a zero standard or blank to measure the background spectrum.
— The enrichment of the calibration standard should be calculated as follows:
  - If (A) is the weight of D_2O and (B) is the weight of drinking water plus D_2O in the 1 L flask, then the weight of added drinking water is (B – A);
  - For example, weight of D_2O = 1.0015 g (A)
  - Weight of drinking water plus D_2O in the 1 L flask = 1000.1 g (B)
  - Then the weight of added drinking water = 1000.1 g – 1.0015 g
    = 999.0985 g (B – A)
  - Enrichment of D_2O in the calibration standard = A/(B – A) × 10^6 mg/kg
    = 1.0015 g/999.0985 g ×10^6 mg/kg
    = 1002 mg/kg (ppm)

It should be noted that 1 mg/kg = 1 mg/L as the density of H_2O is 1.0 kg/L at 25ºC; therefore, the calibration standard is approximately 1000 mg/L.

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

4.3.1. Shelf life of the calibration standards

The shelf life of the calibration standards will depend on the quality of the local drinking water. The bottles should be stored in a cool dark place out of direct sunlight, but not in the same refrigerator as the highly enriched (99.8 at.%) deuterium oxide. Wrapping bottles in aluminium foil helps to protect the contents from light. Bottles must have good seals and be tightly closed to prevent ingress of atmospheric moisture. Some laboratories recommend storing the bottles upside down. Should there be a leakage, these are less likely to suffer fractionation. Some laboratories recommend storing the calibration standards in several 100 mL or 250 mL bottles, rather than 1 L bottles. This has the advantage of only exposing a small portion of the calibration standard to the atmosphere at any time, but the disadvantage that as the calibrant is used, it will be more prone to fractionation effects.

4.4. PREPARATION OF A STANDARD CURVE

Once the FTIR instrument has been installed, the accuracy of deuterium analysis over the range of enrichments likely to be encountered should be checked using gravimetrically prepared standards (Fig. 19). Smaller volumes (e.g. 100 mL) of these standards can be prepared by diluting D_2O with local drinking water in a volumetric flask, as described above. The enrichment should range from 0 (natural abundance in drinking water) to 2000 mg/kg, i.e. an enrichment above that likely to be encountered in saliva samples.

Standards should be made (in 100 mL of local drinking water) according to the following table (Table 1). The D_2O can be pipetted into the volumetric flask (column 2), but it must be accurately weighed (column 3). The weight of the drinking water added to make up the volume should also be noted (column 4). The actual enrichment (mg/kg) can be calculated from the weights as described previously. An example of a standard curve is shown in Fig. 20.

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

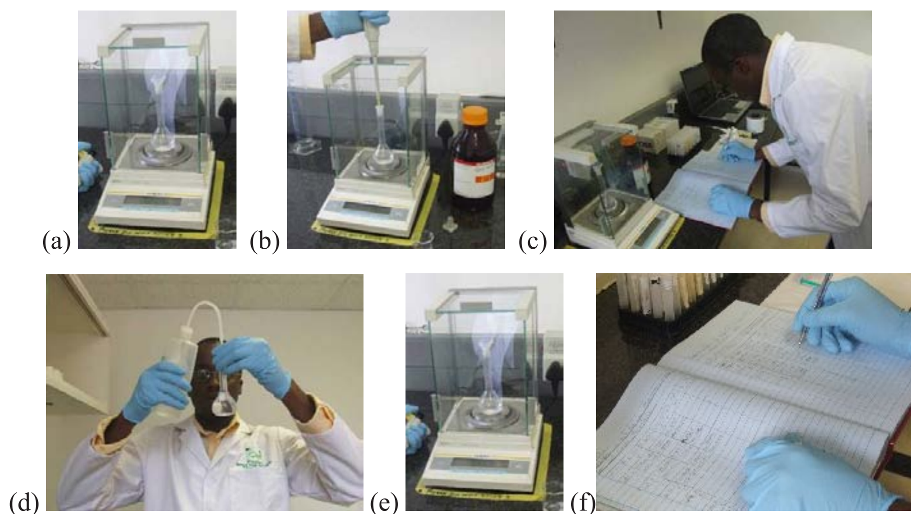

FIG. 19. Preparation of calibration standards. (a) The volumetric flask with its lid should be weighed; (b) D_2O should be pipetted into a weighed amount of water; (c) it should be weighed again and the weight noted; (d) it should be filled to the mark; (e) it should be weighed again; (f) the weight should be noted. The enrichment of D_2O in the standard should be calculated.

TABLE 1. PREPARATION OF FTIR STANDARDS

Target enrichment (mg/kg D_2O)	µL D_2O	Weight D_2O (g) to four decimal places	Weight of drinking water added (g)
0	0		
100	10		
200	20		
400	40		
600	60		
800	80		
1000	90		
1500	140		
2000	180		

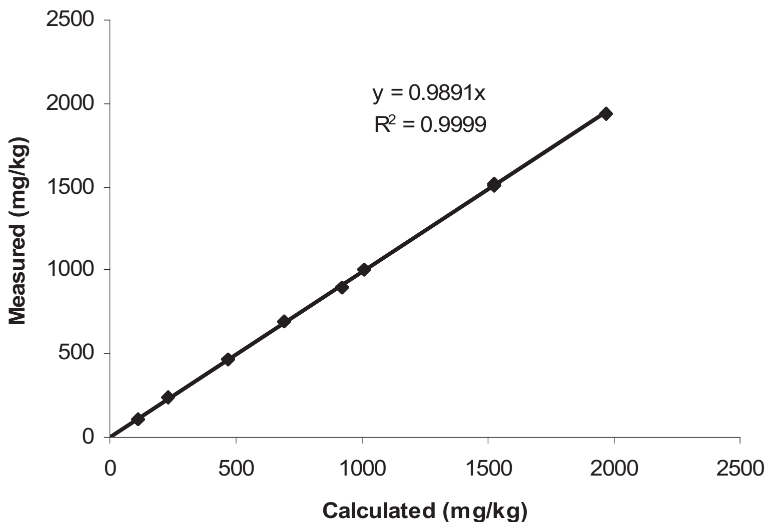

FIG. 20. Deuterium calibration curve measured by FTIR.

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

4.5. OPERATION OF THE FTIR INSTRUMENTATION

The FTIR instrument should be switched on 30–40 min before use to allow the electronics to stabilize. It should be ensured that both the interface and the mirror are working properly.

In addition, make sure that the following are set:

— Measurement mode: Absorbance
— Apodization: Triangular
— No. of scans: 32
— Resolution: 2.0
— Range (cm^{-1}): Minimum 2300 Maximum 2900

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

The peak due to deuterium should have a maximum at about 2504 cm^{-1} (Fig. 21).

When the absorption spectrum of the local drinking water is ratioed to that of the reference, the instrument is calibrated directly in terms of mg/kg (ppm) excess of deuterium.

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

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

4.5.1. Typical FTIR spectra

Atmospheric CO_2 causes a sharp doublet on the shoulder of the deuterium oxide signal. These peaks can be either positive (Fig. 21) or negative (Fig. 22). Negative peaks are obtained if there is a lower CO_2 concentration present in the sample compartment when the enriched sample is scanned than when the background was scanned.

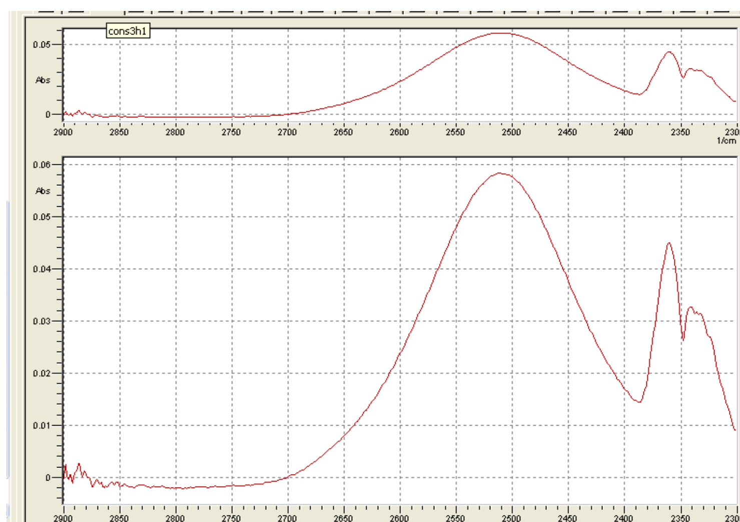

FIG. 21. Typical FTIR spectrum from an enriched water sample after background correction.

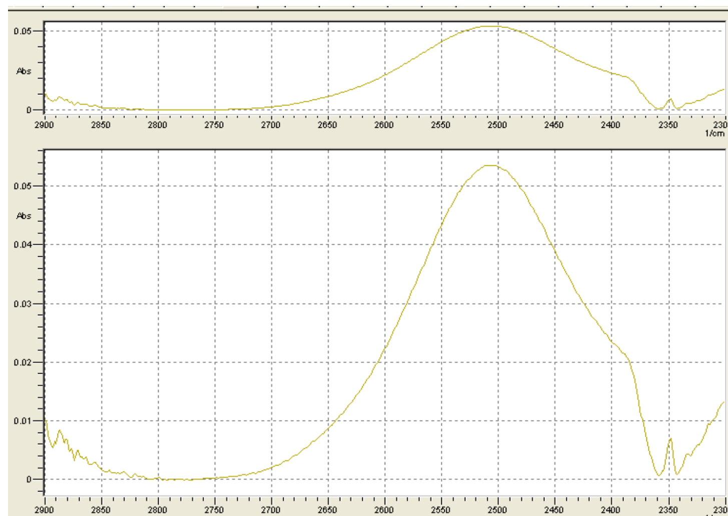

FIG. 22. Typical FTIR spectrum after background correction showing a negative CO_2 peak.

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

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

— The FTIR instrument should be located in a well ventilated or air-conditioned room;
— The cells should be carefully filled as described in Section 4.7 to avoid air bubbles;
— Samples containing bubbles should not be analysed. They should be flushed out by adding more sample.

4.6. THE FTIR CELL

Calcium fluoride cells with a cell thickness (path length) of 10^{-4} m (100 μm) are recommended for analysis of deuterium in saliva samples. These cells cannot be used for the analysis of urine samples, because they are damaged by the ammonium and phosphate content of urine. Figure 23 shows a schematic diagram of the FTIR cell (Omni-cell, Specac part number 1800). The procedure for filling the cell is described in Section 4.7. Sodium chloride cells, which are

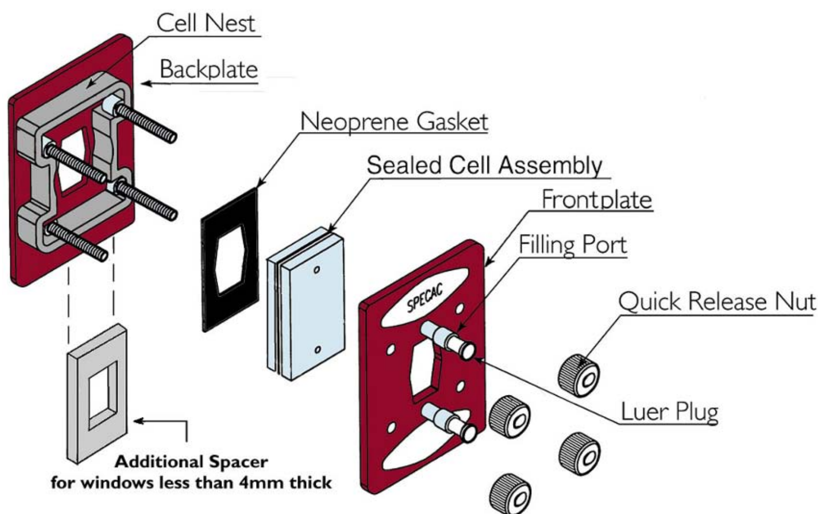

FIG. 23. Schematic diagram of the sealed cell assembly (© Specac Ltd, United Kingdom; reproduced with permission.)

often supplied with the FTIR instrument, are not suitable for analysis of samples containing water.

4.6.1. Care of the cells

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

4.7. FILLING THE FTIR CELL

The cells are filled using a 1 mL disposable syringe (Fig. 24).

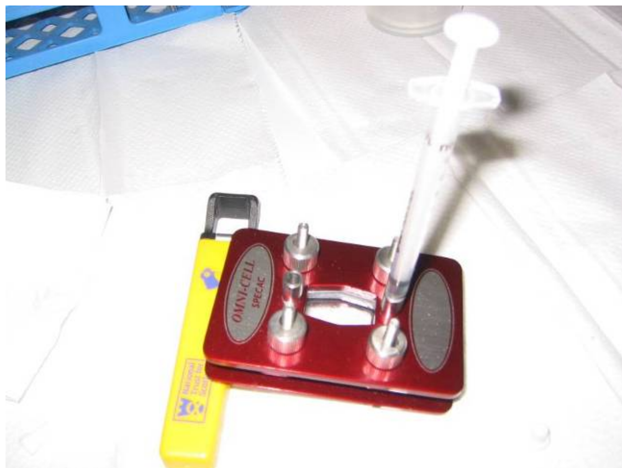

FIG. 24. FTIR cell with a 1 mL syringe in the filling port. The cell is raised at one end by propping on any suitable object, e.g. a pencil.

4.7.1. Introduction

The following precautions should be taken:

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

4.7.2. Recommended procedure for filling the FTIR cell

The recommended procedure for filling the FTIR cell is as follows (Fig. 25):

(1) A 1 mL syringe should be filled with the sample (standard or saliva).
(2) Folded absorbent paper should be firmly pressed over the exit port to absorb excess sample and to prevent ingress of air.

FIG. 25. Procedure for filling the cell. The cell should be filled using a 1 mL syringe, bubbles should be checked for by holding the cell up to a light, and the cell should be placed in the sample chamber of the FTIR instrument.

(3) The cell should be filled by gently pushing the syringe plunger or using firm taps on the plunger with the index finger.
(4) Excess/splashes should be removed from the outside of the cell window using absorbent paper.
(5) Bubbles should be checked for by holding the cell up to a light.
(6) If there are visible bubbles in the cell, more sample should be added as described above until all of the bubbles have been pushed out.
(7) The absorbance from 2300–2900 cm^{-1} should be measured.
(8) The sample should be removed using the same syringe that was used for filling. The syringe should be discarded.
(9) A new syringe should be used for each sample to avoid cross-contamination.
(10) For the next sample, the procedure should be repeated from Step 1.
(11) When all the samples have been analysed, the cell should be rinsed with drinking quality water before storing.

5. CRITICAL STEPS FOR GOOD QUALITY DATA

5.1. DOSE PREPARATION

Doses should be weighed accurately to at least 0.01 g. This should be done by trained staff in an analytical laboratory.

5.2. IN THE FIELD

The following points are important in the field:

- Well trained field workers can help with anthropometry and collection of saliva samples, but it is important that they appreciate the importance of care in making measurements of height and weight, and accurate record keeping. Training of field workers is, therefore, crucial.
- Mothers should be weighed in minimal clothing (to 0.1 kg). Babies should be weighed to 0.01 kg.
- It should be ensured that mothers do not eat or drink for 30 min before saliva sampling.
- Make sure that babies have not been fed for 15 min before saliva sampling.
- Before opening the dose bottle, it should be inverted a few times to mix in condensation on the lid.
- The bottle should not be opened until it is time for the dose to be consumed.
- Make sure that 100% of the dose is consumed by adding water to the bottle and asking the mother to drink this as well, twice.
- Make sure that no cross-contamination takes place between dose bottles and sample vials.
- Sample vials should be labelled with the participant's identification number, time and date.
- All of the data should be recorded on the information sheet.
- The data should be transferred to a spreadsheet, for example Microsoft Excel, as soon as possible.
- Paper records should be kept as a backup.

5.3. IN THE LABORATORY

The following points are important in the laboratory:

- The FTIR instrument should not be moved after it has been installed. If it is necessary to move it, an engineer must be called to check the alignment of the mirrors.
- The humidity in the FTIR instrument should be less than 60%. If the FTIR contains a desiccant, it should be changed when the indicator changes colour. This could be once a week in humid climates.
- Make sure that saliva samples are properly thawed before analysis.
- The specimen vials containing saliva should be inverted to mix in condensation on the lid.
- The saliva samples should be centrifuged at 1000g for 10 min before analysis.

6. EQUIPMENT LIST

6.1. IN THE LABORATORY

Deuterium oxide (99.9 or 99.8 at.% ^2H).
Dose bottles (screw cap, leak proof, e.g. 60 mL wide mouth, polypropylene leak proof, autoclavable bottles).
Labels for dose bottles.
Permanent ink pens for writing on labels.
Glass measuring cylinder to transfer 30 mL ^2H_2O to dose bottles.
Glass or plastic funnel.
Electronic balance weighing to 0.01 g for weighing doses.
Electronic balance weighing to 0.0001 g for making calibration standard.
Refrigerator for storing doses.
Freezer (-20ºC) for storing saliva samples.
Voltage stabilizers for all electronic equipment (electronic balances/FTIR).
Centrifuge with buckets to take sample vials, ideally refrigerated.
Fourier Transform Infra Red Spectrometer (FTIR).
Calcium fluoride cells for FTIR.
1 mL disposable plastic syringes with Luer tip for filling FTIR cell.
Paper tissues/absorbent paper.
Lens paper to clean window of FTIR cell.
Volumetric flasks (1 L, 100 mL and 50 mL) for making calibration standards.
Automatic pipettes plus tips (1mL, 200 µL, 20 µL) for making calibration standards.
Wash bottle for filling volumetric flasks.
Two borosilicate glass reagent bottles (1 L) with PTFE lined screw caps for storing calibration standard and sample of local drinking water used to make calibration standard.
100 or 250 mL borosilicate glass bottles with PTFE lined screw caps for the aliquots of the calibration standard and local drinking water used as ‘working standards’ on a daily basis.

6.2. IN THE FIELD

Doses (prepared in the laboratory).
Drinking water.
Drinking straws.
Balance weighing to 0.1 kg for weighing mothers.

- Balance weighing to 0.01 kg for weighing babies.
- Stadiometer for measuring the height of the mother.
- Measuring board or infantometer for measuring the length of the baby.
- Saliva sampling vials with screw cap (e.g. 4 mL internal thread self standing cryovials).
- Labels for sample vials.
- Permanent ink pens for writing on labels.
- Cotton wool balls for saliva sampling in the mothers.
- Cotton wool swabs for saliva sampling in the babies.
- 20 mL plastic syringes.
- Disposable gloves
- Plastic zip-lock bags for storing saliva samples. Place the baseline samples of the mother and baby in the same bag. Use a new bag for the mother's post-dose samples and one for the baby's post-dose samples. Place all of these together in a larger zip-lock bag. Three medium and one large zip-lock bags per mother/baby pair.
- Watch (to note time of saliva sampling).
- Refrigerator for storing doses if working in the field for several days without returning to base.
- Cool box with ice pack (for storing samples in the field until they can be frozen).

Appendix I

TWO COMPARTMENT STEADY STATE MODEL OF WATER FLOW IN A MOTHER–BABY PAIR

I.1. INTRODUCTION

The deuterium oxide dose-to-mother technique was first described by A. Coward and co-workers in 1982 [5]. This appendix is based on Ref. [5] and the more recent work of Haisma, under Coward's supervision [7]. The notation used here follows the convention set out in those documents.

Calculation of human milk intake and intake of water from sources other than human milk is based on a two compartment steady state model [17]. This is illustrated in Fig. 26.

In the two compartment model, the mother's body water (V_m) is the first compartment and the baby's body water (V_b) is the second compartment. These two compartments are connected by the flow of milk from the mother to the baby (F_{bm}). In a steady state model, the total water input is equal to the total water output. In Fig. 26, F signifies a flow of water. Conventionally, in compartmental

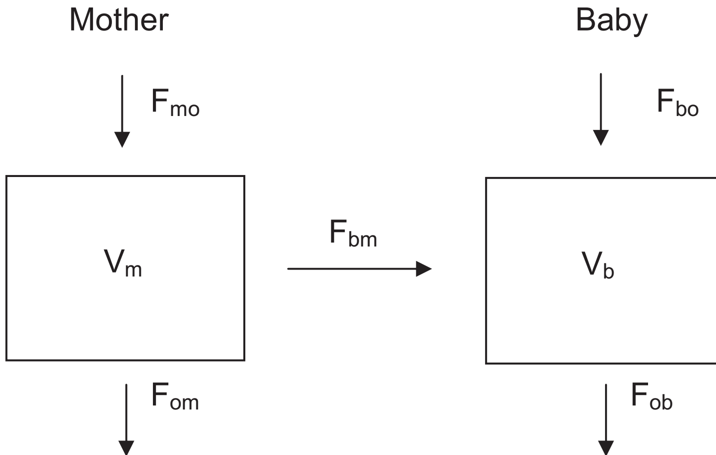

FIG. 26. Two compartment steady state model of water flow in a mother–baby pair. F = flow; m = mother; b = baby; o = outside; V = volume TBW; V_m = mother's TBW volume; V_b = baby's TBW volume; F_{mo} = from outside to mother; F_{bo} = from outside to baby (non-breast fluid intake); F_{bm} = from mother to baby (breast milk intake); F_{om} = from mother to outside; F_{ob} = from baby to outside.

models, the first letter after the F indicates where the flow goes to, and the second letter indicates where the flow is from, so that F_{bm} is the flow from the mother to the baby, i.e. human milk intake by the baby. Flows are also shown from the outside (o) to the mother (F_{mo}), i.e. the water she drinks, and from the mother to the outside, i.e. the water she loses from her body in urine, faeces, sweat and breath. Similarly, F_{bo} is the flow from the outside to the baby, i.e. water intake by the baby from sources other than human milk. The final flow is from the baby to the outside, F_{ob}, i.e. the water lost from the baby's body in urine, faeces, sweat, saliva and breath.

I.2. ASSUMPTIONS OF THE MODEL

The two compartment steady state model is based on a number of assumptions. Where these assumptions do not hold true, an adjustment is included in the calculations.

The assumptions are:

- The body water pool in both the mother and the baby is a single compartment in each individual.
- The deuterium dose equilibrates rapidly and uniformly throughout the body water pool of the mother and her baby.
- The size of the body water pool in the mother is constant. The baby's body water pool is assumed to change linearly with time due to growth.
- All water regardless of the route of exit is labelled with deuterium in proportion to deuterium in the body water pool.
- Deuterium leaves the system only as water.
- Water intake by the baby is only by ingestion.

The deuterium dose is in the form of deuterium labelled water, also referred to as deuterium oxide (^2H_2O or D_2O).

I.2.1. Validity of the assumptions

- Assumption 1: The body water pool in both the mother and the baby is a single compartment in each. This is true.
- Assumption 2: The deuterium dose equilibrates rapidly and uniformly throughout the body water pool of the mother and her baby. This is true. The deuterium dose is fully equilibrated in the mother's body within a few hours after she has consumed the dose.

— Assumption 3: The size of the body water pool in the mother is constant, but the baby's body water pool changes linearly with time. In weight stable healthy adults, water input is equal to water output over a two week period. If the mother's weight changes, it could be due to changes in body fat, which does not affect the body water pool size, or changes in FFM, which will affect the body water pool size. The baby will grow during the two week assessment period; therefore, the baby's TBW will increase. An adjustment must be made for the change in size of the baby's TBW pool. The mother's TBW at the baseline is measured by isotope dilution, but when the deuterium oxide dose is given to the mother, it is not possible to measure the baby's TBW unless a second stable isotope (e.g. ^{18}O) is given to the baby. This would complicate the procedure and make it much more expensive. In addition, ^{18}O requires mass spectrometry for analysis. The baby's TBW is predicted from its body weight (W) using the formula of Wells [15]:

$$TBW = 0.84\ W^{0.82}$$

— Assumption 4: All water, regardless of the route of exit, is labelled with deuterium in proportion to deuterium in the body water pool. This is not true. An adjustment has to be made for isotopic fractionation in water lost from the baby's body as water vapour in breath and by transdermal evaporation.
— Assumption 5: Deuterium leaves the system only as water. This is not strictly speaking true. A small amount of deuterium exchanges with hydrogen atoms (mainly in proteins) in both the mother's and the baby's body. This process is known as non-aqueous exchange. The error introduced by predicting rather than measuring TBW in the baby is greater than non-aqueous exchange; therefore, for the purposes of assessing human milk intake, non-aqueous exchange is ignored.
— Assumption 6: Water intake by the baby is only by ingestion. Atmospheric water can be absorbed through the skin and the lungs of the baby, with alveolar exchange being the largest component. A correction is necessary to take account of this non-oral water intake, which is estimated to be 6.3% of total water intake.

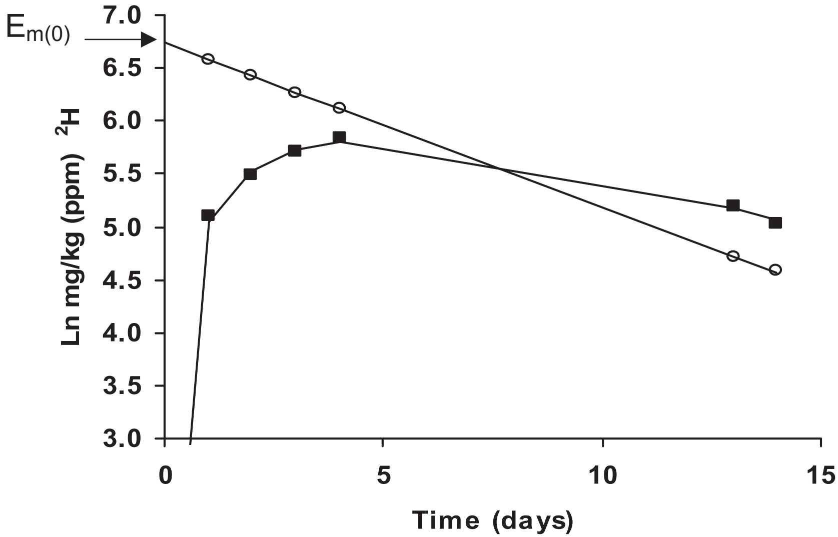

FIG. 27. Deuterium enrichment in the body water of a mother (○) and her baby (■).

I.3. CALCULATION OF HUMAN MILK INTAKE (M) AND INTAKE OF WATER FROM SOURCES OTHER THAN HUMAN MILK (F_S) IN THE BABY

Intake of human milk and water from sources other than human milk can be calculated by fitting the deuterium enrichment data to a model for water turnover in the mother and in the baby. An example is illustrated in Fig. 27.

In the steady state, water turnover in the mother is given by a single exponential equation:

$$\frac{E_{m(t)}}{E_{m(0)}} = e^{-k_{mm}t}$$

where

$E_{m(t)}$ is the deuterium enrichment in the mother's body water at time t, in mg/kg or ppm;

t is the time since the dose was taken, i.e. time post-dose in days;

$E_{m(0)}$ is the deuterium enrichment in the mother's body water at time zero mg/kg (ppm), i.e. the y intercept of the isotope elimination curve (log/linear plot of enrichment of ^2H in body water versus time) (see Fig. 27);

k_{mm} is the fractional water turnover in the mother (kg/d), i.e. the gradient of the isotope elimination curve (see Fig. 27).

Data from the baby are fitted to the following multi-exponential model:

$$E_{b(t)} = E_{m(0)}\left(\frac{F_{bm}}{V_b}\right)\left(\frac{e^{-k_{mm}t} - e^{-(F_{bb}/V_b)t}}{(F_{bb}/V_b) - k_{mm}}\right)$$

where

$E_{b(t)}$ is the deuterium enrichment in the baby's body water at time, t, in mg/kg (ppm);

t is the time since the dose was taken by the mother, i.e. time post-dose in days;

$E_{m(0)}$ is the deuterium enrichment in the mother's body water at time zero mg/kg (ppm), i.e. the y intercept of the mother's isotope elimination curve (log/linear plot of enrichment of ^2H in the mother's body water versus time) (see Fig. 27);

F_{bm} is the transfer of water from the mother to the baby via human milk (kg/d);

V_b is the baby's total ^2H distribution space (kg). V_b is assumed to change linearly with initial and final values determined from the baby's weight (W, kg). $V_b = 0.84\ W^{0.82}$ [15];

k_{mm} is the fractional water turnover in the mother (kg/d), i.e. the gradient of the mother's isotope elimination curve (see Fig. 27);

F_{bb} is the total water loss in the baby (kg/d).

Curve fitting can be performed using the 'Solver' function in Microsoft Excel. To use the 'Solver', it is necessary to go to 'Tools' and then click 'Solver'. This will produce a box that allows a target cell to be set whose value in this case has to be minimized. Note that if 'Solver' does not appear in the 'Tools' menu, it should be added to it. This is achieved by using 'Tools, Add-Ins' and checking the box marked 'Solver'. 'Solver' uses non-linear regression to determine, by iteration, the value of the constants that gave the line of best fit through the data, i.e. to minimize the sum of the squares of the differences between observed and fitted values for mother and baby data combined. This procedure requires initial estimates for the unknown parameters ($C_{m(0)}$, F_{bm}, k_{mm} and F_{bb}) and, subsequently, refines them to converge on best fit values. Maternal body water volume (V_m) can

be calculated from the dose given, and $C_{m(0)}$ and maternal water intake can be estimated as:

$$F_{mo} = V_m \times k_{mm}$$

I.4. CALCULATION OF M: HUMAN MILK INTAKE BY THE BABY

Human milk intake by the baby is calculated from the flow of water from the mother to the baby, assuming that human milk is 87.1% water [18].

$$M = F_{bm}/0.871 \text{ kg/d}$$

Measured human milk intake is often expressed as g/d.

I.5. CALCULATION OF F_S: THE BABY'S INTAKE OF WATER FROM SOURCES OTHER THAN HUMAN MILK

The baby's total intake of water includes water from the oxidation of milk solids (protein, fat and carbohydrate) and water from sources other than human milk. The total water input derived from human milk is F_m. Calculation of F_s assumes that water input equals water output. Allowance must be made for the baby's growth (F_g) and for an increase in TBW during the two weeks of saliva sampling and the fact that water lost in the baby's breath and by transdermal evaporation (F_{ob}) is subject to isotopic fractionation (see Appendix III for more information on isotopic fractionation), and for absorption of atmospheric water by the skin, mainly in the lungs (F_a). Water input = ($F_m + F_a + F_s$).

Water input ($F_m + F_a + F_s$) equals water output plus water from growth ($F_{ob} + F_g$); therefore:

$$F_s = F_{ob} + F_g - F_m - F_a$$

I.5.1. Calculation of total water input to the baby derived from human milk (F_m)

The flow of water from the mother to the baby (F_{bm}) represents free water in milk and does not include water from the oxidation of milk solids (protein, fat and carbohydrate):

— Human milk is assumed to contain 87.1% water, 1.3% protein, 4.1% fat and 7.2% carbohydrate [18];
— The yield of water from 1 g of protein is 0.41 g, from 1 g of fat 1.07 g and from 1 g of carbohydrate 0.55 g.

Therefore, oxidation of milk solids gives about 9 g of water per 100 g of human milk.

Total water input to the baby derived from human milk (F_m) is given by:

$$F_m = F_{bm} + 0.09M$$

I.5.2. Adjustment for the baby's growth (F_g)

Growth of the baby during the experimental period will result in a small change in the baby's deuterium distribution space, which is related to its TBW, and in this context is known as V_b. V_b is assumed to change linearly with initial and final values determined from the baby's weight (W, kg). $V_b = 0.84\ W^{0.82}$ [15].

Water gained during the experimental period, F_g, is given by:

$$F_g = (V_{b,\ day14} - V_{b,\ day0})/14$$

I.5.3. Adjustment for isotopic fractionation (F_{ob})

Deuterium is lost from body water via breath and insensible routes via the skin (transdermal evaporation) more slowly than hydrogen, for the reasons described above; therefore, F_{bb} must be corrected for isotopic fractionation.

Total water output from the baby, i.e. flow from the baby to the outside (F_{ob}), which includes water lost as urine, sweat, in faeces and in breath, includes a correction for isotopic fractionation. The isotopic fractionation factor for deuterium between water vapour and water liquid is 0.946 at 37ºC. It is assumed that 85% of the baby's water output is not fractionated and that the remaining 15% is fractionated by a factor of 0.946. Thus, the correction factor is $0.85 + (0.946 \times 0.15) = 0.9919$.

F_{ob} is given by:

$$F_{ob} = F_{bb}/0.9919$$

I.5.4. Adjustment for water absorbed by the skin (F_a)

For non-oral water intake in the infant (F_a), a correction factor is necessary for environmental water influx to the baby, which is composed of atmospheric water absorbed through the skin and the lungs. Alveolar exchange is the largest component. Non-oral water intake is estimated as 6.3% of total water intake [19]. As total water intake is equal to total water output, F_a is given by:

$$F_a = 0.063(F_{ob} + F_g)$$

I.6. CALCULATION OF ORAL WATER INTAKE FROM SOURCES OTHER THAN HUMAN MILK (F_S)

$$F_s = F_{ob} + F_g - F_m - F_a$$

There is an error associated with the estimate of the baby's intake of water from sources other than human milk, because of the assumptions made in this calculation. This error (25 ± 62 mL/d) results in a small apparent intake of water from sources other than human milk in babies who are truly exclusively breastfed [9].

Appendix II

GENERAL INFORMATION ON THE SAFETY OF DEUTERIUM OXIDE

II.1. ISOTOPES OF HYDROGEN

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

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

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

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

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

Deuterium oxide is water (^2H_2O) in which 99.8 or 99.9% of the hydrogen atoms are in the form of deuterium. This is referred to as 99.8 (or 99.9) atom% ^2H_2O or as D_2O. Deuterium oxide can be used to measure the size of the body water pool (total body water) by isotope dilution, and the flow of water from one pool to another (e.g. from the mother's body water to the baby's body water in human milk).

II.2. DEUTERIUM OXIDE SAFETY

Stable isotopes have been used in human metabolic studies for over half a century. Stable isotopes of hydrogen emit no potentially harmful radiation; the mass of deuterium is two (^2H) and the mass of hydrogen is one (^1H). The mass difference (as a proportion of the atomic mass of hydrogen) between deuterium and hydrogen is a factor of two, and is greater than for any other stable isotopes of the same element. This mass difference may cause significant 'isotope effects' at very high concentrations (>15%) of deuterium oxide in tissues. Isotope effects are caused by the fact that the presence of deuterium in a molecule shortens covalent bonds, making them stronger and more resistant to breakage. Molecules containing deuterium, therefore, display slightly different reaction rates than those containing only hydrogen. The difference in rate constants between a reaction involving a molecule containing only hydrogen and that involving a molecule containing deuterium is known as the 'kinetic isotope effect', and can occur during enzymatically catalysed reactions in the body. Animal studies have shown that tissues containing more than 15% of deuterium labelled water exhibit a multitude of effects including impaired protein and nucleic acid synthesis, altered conformation and stability of biopolymers, altered rates of enzymatic reactions, impaired cell division and morphological changes [20]. The overall effect of deuterium labelling appears to be a depression of tissue metabolism due to lower reaction rates of deuterium labelled compounds in vivo. While some toxic effects of deuterium labelling are reversible, very high concentrations may prove lethal. Levels of deuterium labelling of 15% must be maintained by continual dosage before adverse effects become evident [20]. In mammals, concentrations of deuterium below 15% have not been associated with harmful effects. However, lesser effects, such as transitory episodes of vertigo, have been reported in human adults consuming an amount of deuterium oxide sufficient to enrich body water to 0.35–0.65% [20]. It has been suggested that a threshold for noticeable transitory side effects exists when body water is enriched above 0.2%.

The threshold of deuterium toxicity has been defined as 15% and is far in excess of concentrations conceivable for use in human studies [20]. The amount of deuterium consumed in studies of human milk output and body composition enriches body water to a maximum in the region of 0.1% in the mother and less than half of this in her baby. At this level, no adverse side effects have been reported.

Appendix III

ISOTOPIC FRACTIONATION

Deuterium oxide (^2H_2O) is not identical to water with respect to its physical properties.

When deuterium oxide mixes with body water, three isotopic forms are found (Fig. 28). For example, in a water sample containing 1000 mg/kg (ppm) deuterium oxide, the probability of any particular H being ^2H is 0.001, and the probability of it being ^1H is 0.999.

For any molecule of water, the probability of both H being ^1H ($^1H–O–^1H$) is:

$$P(^1H–O–^1H) = 0.999 \times 0.999 = 0.998\,001 \text{ or } 99.800\,1\%$$

The probability of both H being ^2H ($^2H–O–^2H$) is:

$$P(^2H–O–^2H) = 0.001 \times 0.001 = 0.000\,001 \text{ or } 0.000\,1\%$$

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

$$P(^1H^2HO) = 2 \times 0.999 \times 0.001 = 0.001\,998 \text{ or } 0.1998\%$$

The factor of 2 arises because there are two possible arrangements, $^1H–O–^2H$ and $^2H–O–^1H$, which are equivalent.

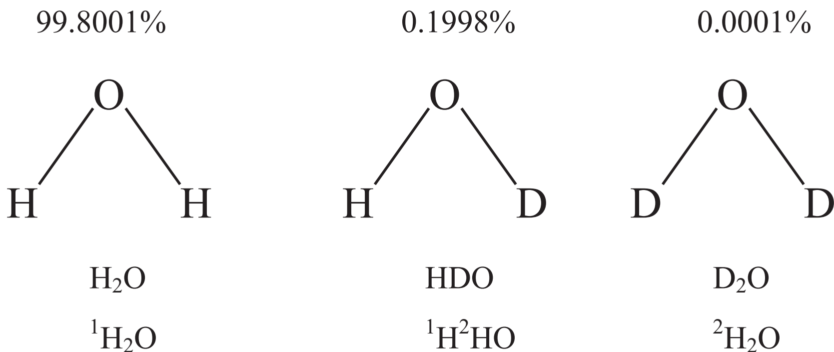

FIG. 28. Abundance of different molecules in a water sample containing 1000 mg per kg water.

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

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

There is very little isotopic fractionation of water within the body. Plasma, urine, human milk and sweat show little fractionation. However, water leaving the body as water vapour in breath and transdermal evaporation contains less deuterium than body water. Transdermal evaporation is insensible water loss from the skin through routes other than the sweat glands. The effect of increased insensible water losses, which contain less deuterium than body water, is to concentrate the deuterium oxide left behind, which could lead to an underestimation of TBW in the mother and, therefore, an overestimation of her body fat, and an underestimation of non-milk water intake by the baby. For this reason, participants should not take part in excessive physical activity during the 14 day sampling period, but continue their normal daily living activities.

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

The following example (Fig. 29) shows the effect of fractionation if 100 μL of condensation is clinging to the lid of a sample vial containing 4 mL of saliva, which originally contained 1000 mg/kg ^2H.

The effect of fractionation is more pronounced when the volume of saliva is small. For example, if a vial containing 1 mL of saliva is left open to the atmosphere and 100 μL evaporates, there will be only 900 μL (0.9 mL) left behind, and this will contain 1006 mg/kg ^2H (Fig. 30).

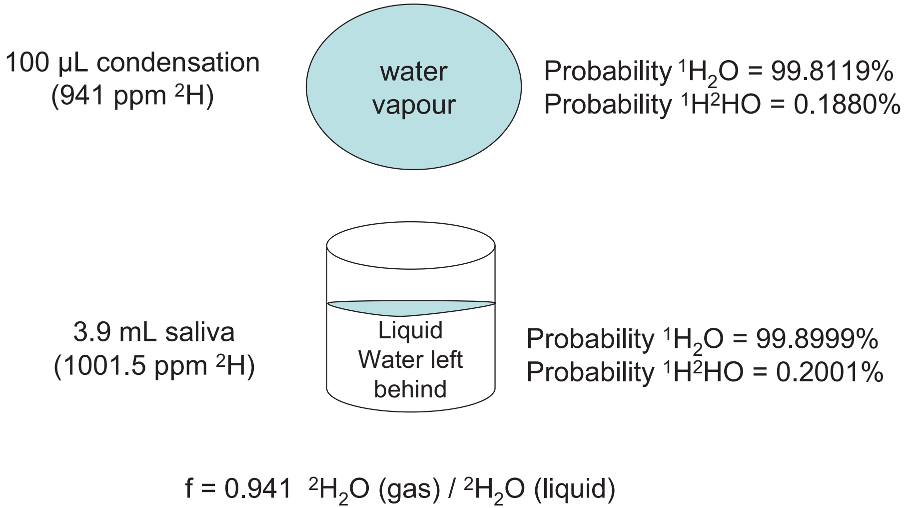

FIG. 29. Effect of isotopic fractionation in 4 mL of a saliva sample originally containing 1000 mg/kg (ppm) ^2H_2O.

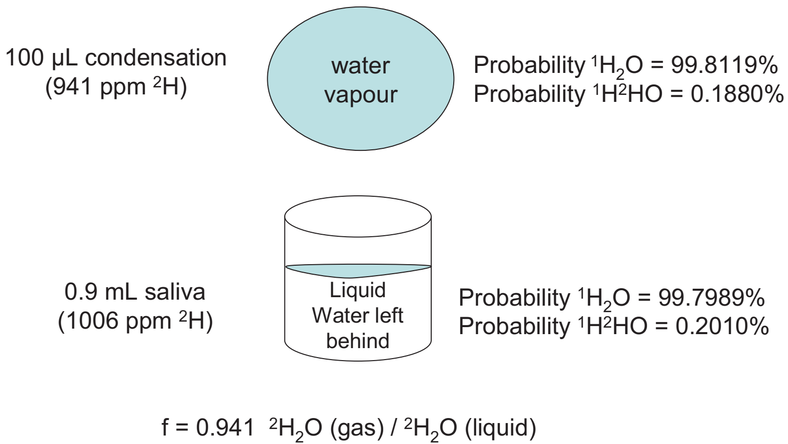

FIG. 30. Effect of isotopic fractionation in a 1 mL saliva sample originally containing 1000 mg/kg (ppm) ^2H_2O.

Appendix IV

FOURIER TRANSFORM INFRARED SPECTROMETRY

The enrichment of deuterium in saliva samples can be measured by FTIR. Practical aspects of using FTIR, including preparation of standards and filling cells, are described in Section 4. This appendix gives an introduction to the principles of FTIR.

IV.1. PRINCIPLES OF FTIR

Absorbance in the middle of the infrared region of the electromagnetic spectrum is due to molecular vibrations. Water exhibits three vibrational modes, which can be regarded as modes of vibration of the O–H bond (Fig. 31).

The energy of vibration depends on the mass of the atoms between which the bond is made. The effect of substitution of deuterium for hydrogen is a shift to a lower energy (Fig. 32).

Peak positions are commonly expressed in terms of wave number (cm^{-1}), frequency (THz) or wavelength (μm) (Fig. 33). The peak due to deuterium oxide is at 2504 cm^{-1} (75.07 THz or 3.994 μm).

It should be noted that as energy increases, frequency and wave number increase, but wavelength decreases.

An FTIR instrument is comprised of a source of infrared radiation, a beam splitter, two mirrors (one fixed and one moving) and a detector (Fig. 34). The beam splitter and mirrors make up the interferometer. One of the mirrors is fixed,

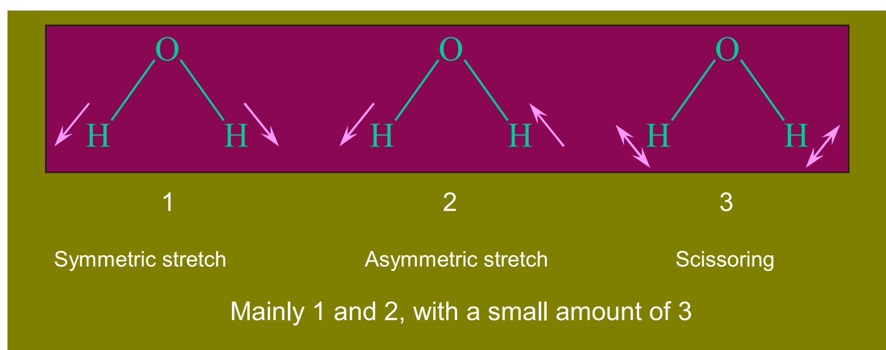

FIG. 31. Modes of vibration of the O–H bonds in water.

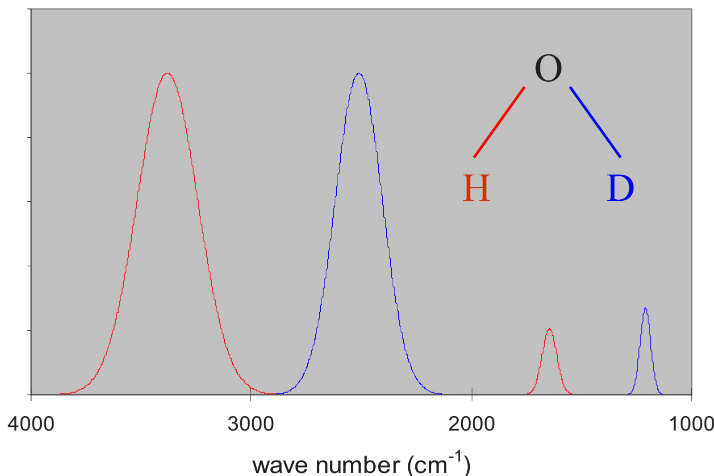

FIG. 32. Schematic diagram of the infrared spectrum due to O–H and O–D bonds.

FIG. 33. Comparison of wavelength, absorbance and frequency.

while the other is mounted on an assembly, which is designed to move back and forth at constant velocity (the moving mirror). Radiation from the source is directed towards the beam splitter. This is a semi-transparent/semi-reflective material, which reflects half the incident radiation towards the fixed mirror and transmits half towards the moving mirror. After reflection from the mirrors, the two beams are recombined at the beam splitter and their sum passed through the sample and focussed on the detector. When the beams are recombined, they interfere. As the mirror moves, the interference pattern changes from constructive to destructive and back cyclically (Fig. 35).

This happens for all the different wavelengths of light simultaneously. These are summed to give the total output or interferogram (Fig. 36).

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

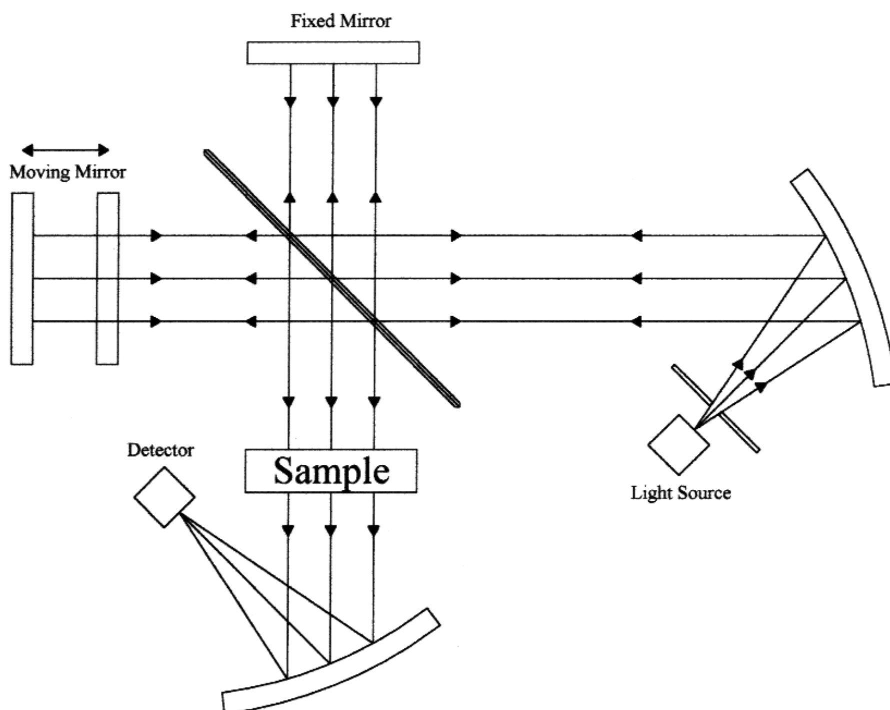

FIG. 34. Schematic diagram of an FTIR instrument.

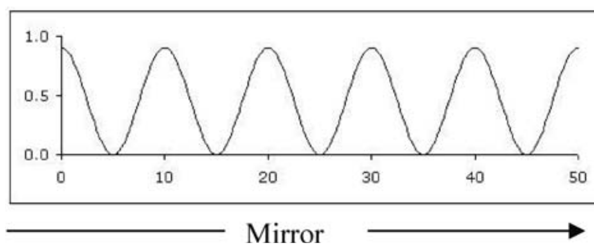

FIG. 35. Cosine wave formed from the recombined beam from a single wavelength.

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 output from the detector is converted into a time varying voltage, which is an accurate representation of the total intensity of light passing through the sample. This is transformed back to the usual kind of spectrum mathematically by the process of Fourier transformation.

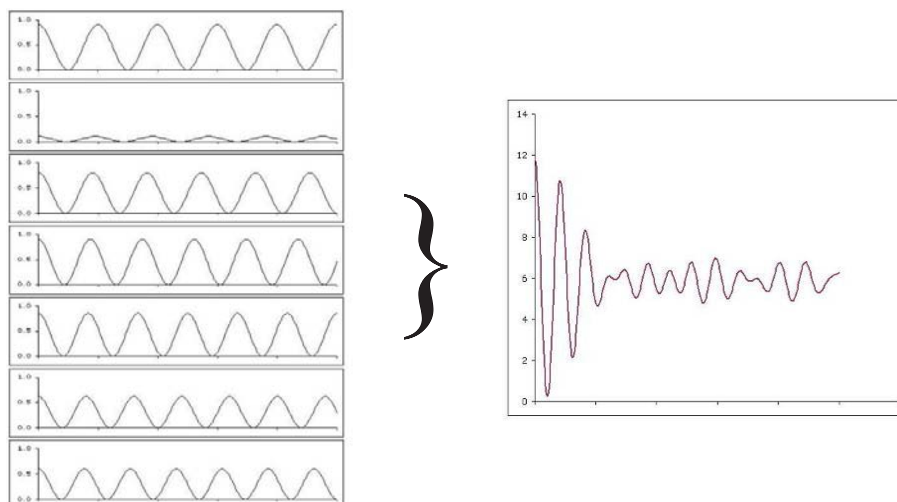

FIG. 36. Representation of interferogram formed from seven different wavelengths.

As the fraction of deuterium is very small (Fig. 37), approximately 1000 mg/kg (ppm), and the dynamic range of the detectors is not great enough to allow accurate measurement of the intensities of the peaks due to O–H and O–D in the same sample, only the intensity of the O–D peak is used, and the concentration of deuterium is estimated using the Beer-Lambert law, which states that “for a parallel beam of monochromatic radiation passing through a homogeneous solution, the amount of radiation absorbed (A) is proportional to the product of the concentration (*c*) and path length (*l*).”

$$A\ \alpha\ c\ l$$

$$A = \varepsilon\ c\ l$$

$$c = A/\varepsilon\ l$$

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

At the low levels of deuterium encountered, the O–D signal appears as a small peak superimposed on the tail of the much larger peak due to the O–H bond (Fig. 38). In addition, atmospheric CO_2 causes a sharp doublet on the shoulder of the O–D signal. This makes the estimation of the baseline under the O–D peak difficult when using air as a background reference. This difficulty can be avoided

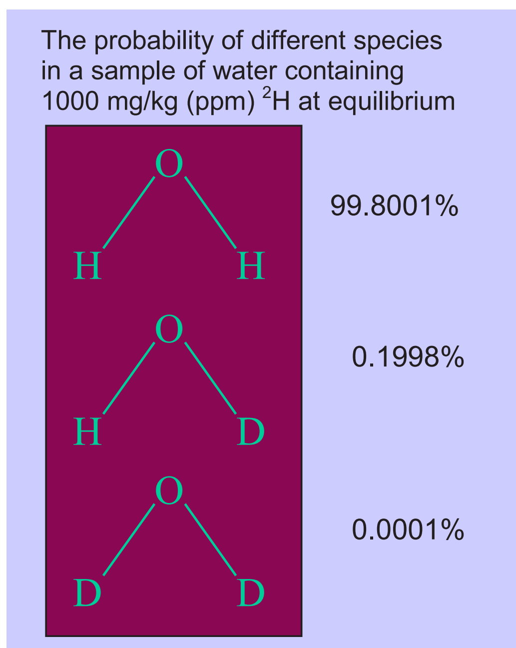

FIG. 37. Deuterium content of a sample of water containing 1000 mg/kg (ppm) ^{2}H.

by using a sample of local drinking water as a background reference, removing much of the O–H background, and revealing the O–-D peak at 2504 cm^{-1}. The absorbance from the enriched samples is automatically corrected for the background by the instrument software (fig D.9). When analysing saliva samples, the baseline sample is used for the background correction. The absorbance from the enriched samples is automatically corrected for the background by the instrument software (Fig. 39). When analysing saliva samples, the baseline sample is used for the background correction.

A mathematical method for comparing the spectrum of the calibrant and sample, which automatically fits baselines to the peaks, has been described in the literature [16]. This method is now available as a program[1] which runs under Microsoft Windows on the same data system as supplied with the instrument, and

---

[1] The software comprises two files 'isotope.exe' and 'vbrun300.dll'. The first of these is the executable (program) 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. They can be obtained from the Medical Research Council Collaborative Centre for Human Nutrition Research (MRC-HNR), Elsie Widdowson Laboratory, Fulbourn Road, Cambridge CB1 9NL, United Kingdom. Telephone No. +44 1223 426357. (http://www.mrc-hnr.cam.ac.uk)

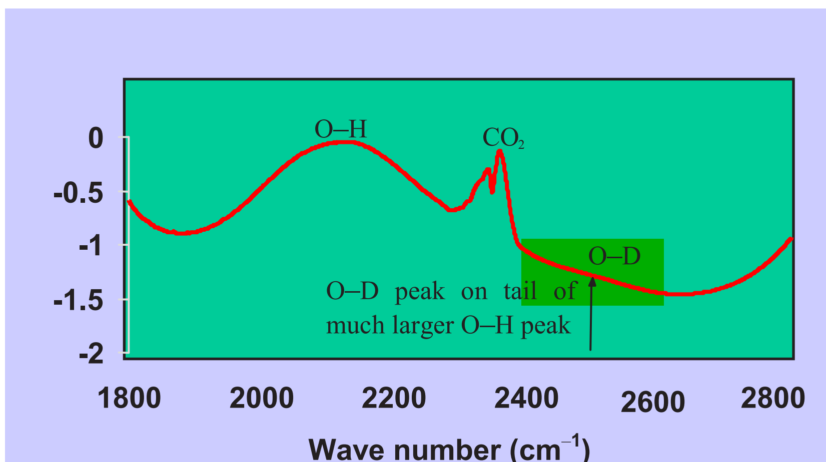

FIG. 38. FTIR absorbance spectrum from water containing 1000 mg/kg (ppm) D_2O.

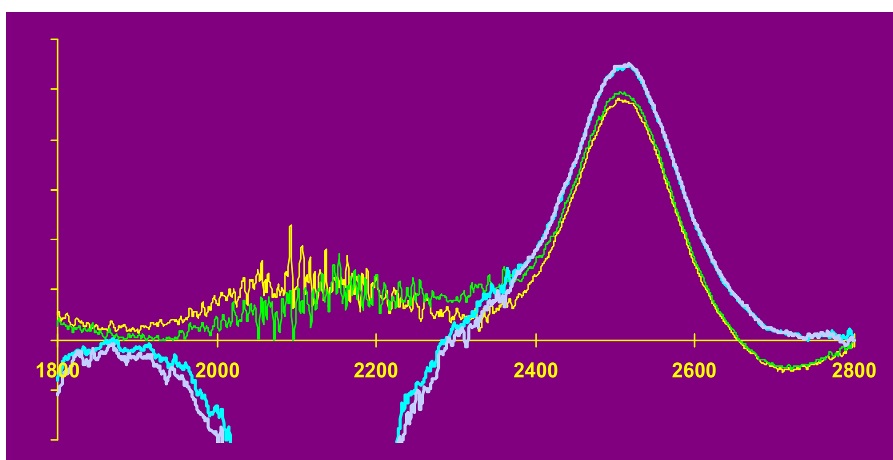

FIG. 39. FTIR spectrum after correction for the background.

operates directly on text files generated by the FTIR vendor. Since most manufacturers export files in slightly different formats from each other, the HNR software must be preconfigured to match the instrument used. Versions are available for use with instruments made by Thermo, Unicam and Shimadzu.

IV.2. UNITS

In FTIR, enrichment is usually expressed as the concentration of deuterium in ppm by weight (mg/kg) above the amount naturally present. The enrichment entered into the 'isotope.exe' software should be in mg/kg.

It should be noted that the units of enrichment in IRMS are at.% excess ^{2}H, also sometimes reported as ppm excess ^{2}H. These ppm are a molar ratio, ppm (mol/mol), and not a weight ratio (mg/kg). The two kinds of ppm are not the same, and are not interchangeable. The use of ppm is discouraged to avoid this confusion.

GLOSSARY

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

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

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

deuterium. The stable isotope of hydrogen with the symbol ^{2}H.

deuterium oxide. Water ($^{2}H_2O$) in which 99.8% of the hydrogen atoms are in the form of deuterium.

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

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

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

equilibration. The hydrogen atoms in water molecules in the body are not permanently attached to the oxygen atoms, but are constantly exchanging. They are in a state of constant flux. When a person drinks a dose of deuterium oxide, it is not a simple matter of the deuterium oxide ($^{2}H_2O$) mixing with water in the body. The deuterium atoms in $^{2}H_2O$ exchange with

hydrogen atoms in water molecules, so that after a few hours, the probability of finding a molecule of $^{2}H_2O$ is very low. Most water molecules are still in the form of $^{1}H_2O$, but a few are in the form of $^{1}H^{2}HO$ after exchange of ^{1}H with ^{2}H. This is the process of equilibration.

fat free mass. The term used in body composition studies to refer to the part of the body that is not fat. FFM includes water, protein, bone minerals and non-bone minerals. FFM contains 73.2% water in healthy adults [21], but the hydration of FFM is higher in children, the latter stages of pregnancy and certain clinical conditions.

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

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

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

isotope. An element with the same number of protons and a different number of neutrons.

Hydrogen has one proton in the nucleus	^{1}H (stable isotope)
If a neutron is added to its nucleus, **deuterium** is formed	^{2}H (stable isotope)
If two neutrons are added to the nucleus, **tritium** is formed	^{3}H (radioactive isotope)

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

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

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

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

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

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

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

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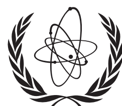

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