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Assessment of Zinc Metabolism in Humans Using Stable Zinc Isotope Techniques

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ASSESSMENT OF ZINC METABOLISM IN HUMANS USING STABLE ZINC ISOTOPE TECHNIQUES

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FOREWORD

Zinc is essential for growth, sexual maturation, reproduction, healing, immunity and myriad functions that may be limited when diets are low in animal products or high in factors such as phytate that inhibit zinc absorption from plant based diets. Although inadequate zinc nutrition cannot be sensitively detected in individuals by measuring zinc concentration in blood, stable isotopes of zinc can be safely and successfully used to better understand zinc metabolism in humans to improve zinc absorption from diets and meet nutritional requirements.

The IAEA assists Member States in their efforts to develop evidence based interventions to combat nutrient deficiencies and improve nutrition and health. The IAEA contributes to the development and transfer of technical expertise in the use of stable isotope techniques to address micronutrient deficiencies in Member States through support to national and regional nutrition projects through the technical cooperation programme and coordinated research projects addressing priority areas in nutrition. Using stable isotope techniques, new data have been generated concerning absorption of zinc from indigenous diets and foods biofortified with zinc through selective agricultural breeding or fortified with zinc compounds. These data provide information needed for designing effective food fortification programmes for infants and children. This publication is part of the IAEA's continuing efforts to contribute to capacity building in this field by providing information on the theoretical background and practical application of state of the art methodologies for assessing human zinc metabolism to better understand absorption, dietary bioavailability and nutritional requirements.

The publication was developed with input from international experts and is intended for nutritionists, analytical chemists and other professionals interested in the application of stable isotope techniques to evaluate human zinc nutrition and metabolism.

The primary contributors to this publication were N.M. Lowe (United Kingdom) and L.R. Woodhouse (United States of America). In addition, several procedural descriptions were shared by the research group of N.F. Krebs, L.V. Miller and J.L. Westcott (United States of America). The generous sharing by all contributors of their technical expertise and experience is gratefully acknowledged. The IAEA officers responsible for this publication were J.R. Hunt, L. Davidsson and C. Slater of the Division of Human Health.

EDITORIAL NOTE

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CONTENTS

1. INTRODUCTION

1.1. BACKGROUND

Zinc deficiency in humans can result when diets are limited in animal products and are plentiful in unrefined grains, legumes, nuts and seeds containing phytic acid, a potent inhibitor of zinc absorption. The affects of severe zinc deficiency, such as impaired growth, sexual maturation, reproduction and healing, have been reversed with zinc supplementation. However, moderate or mild zinc deficiency cannot be sensitively detected in individuals biochemically using markers such as blood zinc concentrations. Much of the information about zinc absorption from different diets, as well as zinc excretion and requirements in humans, has relied on the safe use of zinc stable isotopes to understand human zinc metabolism.

1.2. OBJECTIVE

The aim of this publication is to provide background information on zinc metabolism in humans that has been obtained through the use of zinc isotopes. Detailed descriptions are provided on how to use stable isotope techniques to assess human zinc absorption, endogenous faecal zinc (EFZ) excretion and zinc kinetics. This publication also aims to enable increased use of stable zinc isotope approaches worldwide to provide for improved nutrition and to evaluate proposed nutritional interventions for all population groups, particularly those at risk of zinc deficiency. This publication was developed by an international group of experts and is intended for nutritionists, analytical chemists and other professionals interested in the application of stable isotope techniques to evaluate human zinc nutrition and metabolism. It includes advice on planning a study, administering isotopes, sample preparation and analysis, and calculation of the physiological end points. It is designed as a detailed practical overview for new users with general scientific training (e.g. nutritionists and other health professionals) who will work in collaboration with an established zinc stable isotope laboratory to develop food and nutrition strategies to improve zinc nutrition in populations at risk of zinc deficiency.

1.3. SCOPE

This publication focuses on methods to quantify zinc absorption, the amount of readily exchangeable endogenous zinc and zinc excretion in humans using zinc stable isotopes. These methods can provide information useful for the assessment of nutritional requirements for zinc and for improving the availability of zinc from diets. General scientific background is provided to relate these methods to others that are less extensively addressed, such as early methods employing zinc radioisotopes or methods of intrinsically labelling foods with zinc isotopes. The procedures described include those commonly handled by different collaborating members of a multidisciplinary team such as specialists in nutrition, clinical chemistry, trace element chemistry, mass spectrometry, medicine and statistics. This publication can help prepare the reader to participate in or lead investigations using these methods, while recognizing the need to collaborate with other experts. Guidance provided here, describing good practices, represents expert opinion but does not constitute recommendations made on the basis of a consensus of Member States.

1.4. STRUCTURE

After this introduction, Section 2 provides background information on the role of zinc as an essential micronutrient. Section 3 introduces the use of zinc isotopes to study human zinc metabolism, and Sections 4 and 5 provide advice on planning, preparing and administering doses of zinc stable isotopes to humans. Section 6 describes the mass spectrometry instrumentation available for analysis of zinc stable isotopes, and Section 7 provides advice on collecting and preparing samples for zinc isotope ratio analysis. Section 8 describes the calculation of enrichment from isotope ratios, and how these data can be applied to determine zinc absorption, exchangeable pool size and endogenous zinc excretion in faeces. Sections 9 and 10 provide information on study protocols and safety of administering zinc stable isotopes, and some additional considerations when planning a study. This is followed by appendices containing an equipment and supplies list, an example of how to prepare an IV isotope dose, guidance on administration of the IV isotope dose, protocols for purification of zinc from faeces and from urine, plasma or serum to measure isotope ratios, and calculations of sample enrichment from stable isotope ratios, using three zinc isotope sources. Finally, the references are followed by a glossary and a list of abbreviations of terms.

2. HUMAN ZINC NUTRITION

Zinc is an essential nutrient required for a wide array of physiological functions in all tissues and cells. Zinc is a transition element with a single valence state as a divalent cation (Zn^{+2}). This zinc ion has an affinity for negatively charged hydroxyl and thiol groups, and readily forms complexes with amino acids, peptides, proteins and nucleotides. Primarily an intracellular ion, zinc's multiple functions include catalytic, structural and regulatory roles. It is present in six classes of enzymes and in over 2500 specific zinc finger proteins involved in transcriptional and translational genetic control [1]. Although zinc's roles in major biochemical pathways, gene regulation and cellular division have been extensively investigated and described [1], they have not been clearly linked to the adverse consequences of severe zinc deficiency, including depressed growth and genital development, immune and cognitive dysfunctions, diarrhoea and reproductive teratogenesis.

Zinc is considered a trace element, along with elements such as copper, manganese, iron, nickel and selenium, because it is present in low concentrations in body tissues. An adult man contains about 2.2 g of zinc, with approximately 63% of this located within muscle tissue, 20% within bone, 3% in the liver and only 0.1% in the plasma [2]. Zinc depletion in experimental animals resulted in a decline in zinc concentrations in bone, liver, testes and plasma, without reducing concentrations in skeletal muscle, skin and the heart [2].

The European Food Safety Authority estimated average requirements for zinc as approximately 7.5 mg and 6.2 mg daily for men and women, respectively, who consume relatively refined (low phytate) diets [3]. Recommended dietary intakes were set higher to allow for individual variation and to meet the needs for most (97.5%) of the population and to further allow for variation in dietary phytate (an inhibitor of zinc absorption) resulting in recommended ranges of 9.4–16.3 mg daily for men and 7.5–12.7 mg daily for women [3]. Zinc recommendations in Canada and the United States of America were estimated as 11 mg daily for adult men and 8 mg for adult women, assuming diets relatively low in phytate [4].

The consequences of zinc deficiency can be severe in humans with prolonged total parenteral nutrition with inadequate zinc content, or with a rare genetic condition characterized by zinc malabsorption, acrodermatitis enteropathica. Clinical symptoms of acrodermatitis enteropathica include dermatitis, diarrhoea, alopecia, growth retardation, weight loss, mood changes and birth defects [2]. Although such symptoms emphasize the physiological importance of zinc, severe zinc deficiency is rarely seen. However, chronic mild or moderate zinc insufficiency can occur if the diet is low in animal protein, if there are high intakes of inhibitors of zinc absorption (such as phytate) and

if absorption is suboptimal, such as in elderly populations or in patients with malabsorption, alcohol abuse, or other clinical conditions where zinc deficiency occurs in association with generalized malnutrition [2, 5–8]. Chronic zinc deficiency is especially of concern in developing countries. Countries at high risk of zinc deficiency are those with a stunting prevalence of more than 20% and estimated prevalence of inadequate zinc intake of more than 25%. The national prevalence of zinc deficiency is high in South Asia, most of sub-Saharan Africa and parts of Central and South America, and zinc deficiency is estimated to be responsible for 0.4 million deaths annually [7]. Zinc and protein generally tend to be found in the same foods. However, relative to supply, the human requirement is lower for protein than for zinc; therefore, in conditions of severe acute malnutrition, zinc deficiency is likely (in addition to the deficiency of other micronutrients). Some of the clinical features observed in children suffering from severe protein energy malnutrition are very similar to those of severe zinc deficiency: anorexia, diarrhoea, stunted growth, wasting, skin desquamation and ulceration, hair fragility and dyspigmentation, a reduction in lymphoid tissue and increased susceptibility to infection are all common to both conditions. However, it is difficult to separate the symptoms due to zinc deficiency and those due to deficiencies of other micronutrients, including vitamins A, B_{12}, C, D and folate [2]. Unless attention is paid to their zinc intakes, children recovering from malnutrition are also at risk of becoming zinc deficient due to increased requirements during catch-up growth and recovery.

Perhaps because the physiological roles of zinc are so numerous, there is no clear, sensitive biochemical indicator to assess human zinc deficiency or nutritional status. Together with indicators such as rates of stunting and responsiveness to controlled trials of zinc supplementation, the determination of the amount of dietary zinc that is available for absorption has become an important means to help identify individuals and populations at risk of zinc deficiency [2, 9].

2.1. ASSESSMENT OF ZINC NUTRITIONAL STATUS AND REQUIREMENTS IN HUMANS

A wide range of possible indicators of zinc nutritional status have been investigated, including zinc levels in tissue, the activity of zinc dependent enzymes, zinc concentration in various body fluids, zinc excretion and absorption, and the kinetics of zinc metabolism. Preferably, a good indicator or biomarker would be (a) specific to the nutrient in question, (b) responsive to both increases and decreases in nutrient status, and (c) reliable (i.e. consistently responsive in a predictable manner). A meta-analysis of the data [10] indicated

that, in healthy individuals, plasma zinc concentration responded to changes in dietary zinc intake, both zinc supplementation and acute zinc depletion. In addition, 24 hour urinary zinc excretion and hair zinc concentration responded to zinc supplementation, but data were insufficient to evaluate the effect of zinc depletion, and neither urinary zinc excretion nor hair zinc concentration is regarded as a sensitive indicator of zinc status.

Measurement of blood plasma or serum zinc, together with evaluation of dietary zinc intake and prevalence of stunting, has been recommended to assess the risk of zinc deficiency in populations but not individuals [2, 10]. The reliability of plasma/serum zinc concentration to reflect zinc status is subject to various caveats, including the health status of the individual, diurnal and postprandial factors and the sample collection method. Conditions have been described for optimizing such measurements [2].

A sensitive and reliable functional or biochemical indicator has not been developed for use in detecting moderately impaired zinc nutrition in individuals. In the absence of a biomarker of individual zinc status, nutritional assessment may be limited to comparing estimated zinc intakes to estimated requirements. The determination of human zinc requirements is based principally on a factorial analysis of zinc excretion and the amounts of zinc that must be absorbed to replace losses and maintain a healthy balance, plus allow for growth as needed [2–4]. Determining the amount of dietary zinc required should take into account the incomplete bioavailability of zinc for absorption from different human diets and from fortification or supplementation sources. Much of the knowledge of quantitative zinc absorption and excretion in humans has been gained from studies using zinc isotopes.

2.2. ZINC ABSORPTION

Zinc absorption mainly takes place in the distal duodenum and proximal jejunum of the small intestine [2]. The mechanism by which zinc present in the lumen of the small intestine is transported across the basolateral membrane and into the portal circulation involves both saturable (carrier mediated) and unsaturable (diffusion mediated) processes. Saturable processes involve zinc transporter proteins such as the ZnT and ZIP families of proteins that mediate transfer of zinc into and out of cells, respectively [11]. It is suggested that specific proteins of these families control the cellular uptake and efflux of zinc, and their expression is responsive to changes in dietary zinc intake. However, the coordination and regulation of transport mechanisms have not yet been fully determined.

The percentage of zinc entering the gastrointestinal tract that is absorbed — commonly referred to as the fractional zinc absorption (FZA) — varies depending on:

(a) The amount of zinc ingested;
(b) The presence of inhibitors or enhancers;
(c) The physiological state of the individual (for example pregnancy, lactation or certain morbidities).

Within a typical range of dietary zinc intakes, small increases in the amount of zinc consumed substantially reduce the fraction of zinc absorbed [12–14]. As the amount of ingested zinc increases, FZA is reduced, and the total amount (mg) of zinc absorbed increases in a non-linear manner consistent with a saturable response [15, 16]. Unlike the nutrient iron, which is better absorbed in response to low body iron status, it is not clear that zinc absorption adjusts to compensate for low zinc status. Although continuous consumption of low zinc diets (<11 mg/d) for 4–8 weeks increased FZA in adults [16], at such low intakes, increases in fractional absorption do not meaningfully increase the absolute amount of zinc absorbed.

The most influential inhibitor of dietary zinc absorption is phytic acid, which is also known as 6-phosphoinositol or myo-inositol hexaphosphate, commonly referred to as the salt form, phytate. It binds or chelates positively charged ions, such as zinc, iron and calcium, precipitating them and thereby decreasing their absorption in the gastrointestinal tract. Phytate is present in cereal grains, beans, legumes, nuts and seeds. Its inhibitory effect on zinc absorption was established in animals in 1960 [17] and evidence for an effect in humans was presented in 1975 by Pecoud et al. [18] who demonstrated that the administration of 102 mg of phytate as sodium phytate hydrate (equivalent to the amount contained in 100 g brown bread) taken with 50 mg of zinc (as zinc sulfate) significantly reduced the plasma response. In adults, the amounts of zinc and phytate ingested are the two principal predictors of zinc absorption from human diets [15, 16]; however, similar modelling has suggested that dietary phytate may be less influential on zinc absorption in children [19].

Competitive interactions between zinc and other positively charged ions, including calcium, iron, copper and tin, may reduce zinc absorption by competing with zinc for common transport mechanisms, or may possibly increase zinc absorption by competitively complexing with inhibitors such as phytic acid. Metal ions with a similar size, electron configuration and coordination number were thought to compete for sites on transport proteins. However, evidence from in vitro studies suggests that the interaction may be more influenced by physiochemical interactions in the gut lumen than physiological competition

for carriers into and across the enterocyte [20]. In humans, iron and zinc may competitively interact, reducing zinc absorption in the presence of inorganic iron. However, the amount of iron required to reduce zinc absorption is greater than that typically found in food [21]. Iron supplements may result in inhibition of zinc absorption in vulnerable population groups [22, 23].

A number of dietary factors may have a positive influence on zinc absorption, including amino acids, such as histidine and methionine, and other low molecular weight ions, such as ethylenediaminetetraacetic acid (EDTA) and organic acids (e.g. citrate) [24]. The amount of protein in a human meal has been shown to be positively related to zinc absorption from that meal [12]. This may be due to amino acids released from the protein that keep the zinc in solution rather than the effect of a specific type of protein, such as animal protein, as was previously suggested [24]. Although both dietary protein and calcium, but not iron, may modestly enhance zinc absorption from human diets, absorption is primarily influenced by the amounts of zinc and phytate [25].

The physiological state of the individual can also affect zinc homeostasis. Zinc absorption has been shown to increase during pregnancy and lactation [26–28]. Certain morbidities, such as diarrhoea and tropical enteropathy, that affect the integrity of the gastrointestinal tract, can also affect zinc absorption and endogenous faecal zinc excretion, a major homeostatic regulator of zinc homeostasis [29].

2.3. ZINC EXCRETION AND HOMEOSTASIS

Zinc is lost from the body via faecal and urinary excretion and through skin sloughing (integumental losses), sweat and semen. Faecal zinc is made up of unabsorbed dietary zinc, as well as endogenous zinc from intestinal cells that are sloughed off, and from pancreatic and, to a lesser extent, bile secretions. This endogenous faecal zinc excretion is positively correlated with the amount of zinc absorbed and plays a major role in zinc homeostasis [2, 4, 30]. Urinary zinc accounts for approximately 15–25% of total zinc losses, but is not a sensitive indicator of zinc intake within the range of typical dietary zinc consumption [2, 10]. In adult humans, zinc losses from other routes besides faeces and urine, including skin desquamation and sweat, account for approximately 17–19% of total zinc losses [2].

Unlike other micronutrients, such as iron, there is no storage form of zinc that can be readily mobilized when required. Therefore, a regular dietary supply is needed to meet physiological needs. The human body has a highly efficient homeostatic mechanism that enables the conservation of zinc when intakes are low. The primary site of homeostatic regulation is the gastrointestinal tract via

control of endogenous faecal zinc excretion (Fig. 1) [2, 4, 30], whereas the zinc status of the individual does not appear to play a major role in the regulation of the amount of zinc absorbed [14, 16]. Homeostasis can be achieved over a wide range of dietary zinc intakes, but there may be a limit to the adaptations that can compensate for chronically marginal intakes, particularly in infants and children, as well as during pregnancy and lactation when there are increased demands for growth and development.

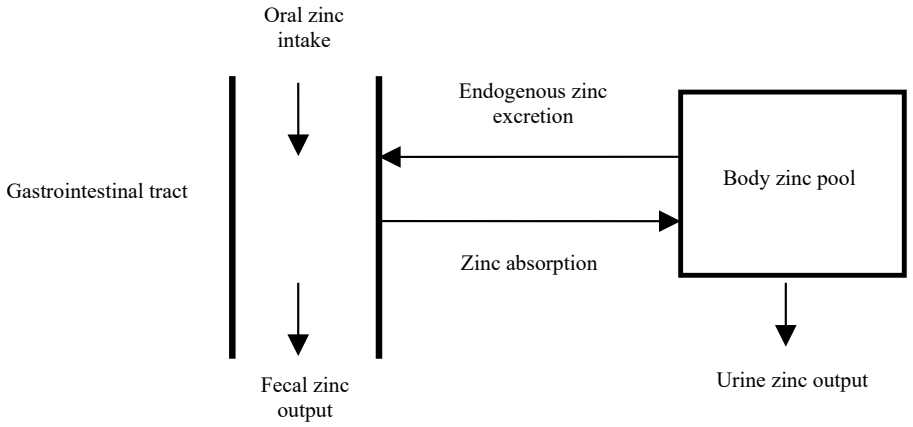


FIG. 1. Diagram of the sites of zinc absorption and excretion.

3. USING ZINC STABLE ISOTOPES TO STUDY HUMAN ZINC METABOLISM

Zinc stable isotopes have been used in studies of human nutrition for more than 40 years and have played a crucial role in advancing our understanding of zinc requirements and metabolism. The present report addresses three key areas of zinc in human nutrition that can be investigated using stable isotope methodology:

(a) Zinc absorption and bioavailability;
(b) Zinc kinetics and exchangeable pools;
(c) Endogenous faecal zinc excretion and homeostasis.

After an orientation to zinc stable isotopes, this section describes these three stable isotope methods. The subsequent sections will go into greater detail about implementing these methods, ending with example outlines of protocols and related data analyses.

3.1. STABLE ZINC ISOTOPES

Isotopes are the atoms of an element that have the same number of protons (30 in the case of zinc) but a different number of neutrons, resulting in differences in total mass. In the case of radioactive isotopes (radioisotopes) the nucleus is chemically unstable, and decays at a known rate (half-life) into atoms of the same or other elements, producing stable end products, together with radioactive emissions that are often the basis for detection. In contrast, stable isotopes are the atoms of an element that do not decay and do not produce or emit ionizing radiation, providing the advantage that when working with stable isotopes, there are no health risks associated with exposure to radioactivity. This advantage enables the safe administration of stable zinc isotopes in the study of pregnant women or children (see Section 10.5 for more on the safety of administering zinc stable isotopes). Stable isotopes can be separated and quantified by mass spectrometry according to mass to charge ratio.

3.1.1. Isotopes as tracers to study biological processes

Isotopes can be useful as tracers (also referred to as labels, such as when an isotopic zinc tracer has been incorporated into foods for testing zinc absorption). A tracer is an identifiable substance that can be introduced into a biological system and followed through the course of a process, providing information on the process or on the redistribution of the substance. The use of tracers in studies such as those described in this publication involves the following assumptions [31, 32]:

(a) The tracer behaves in the same way as the tracee, or natural substance it represents;
(b) The mass of the tracer administered does not perturb the kinetics of the system;
(c) An intravenous (IV) tracer behaves the same as an absorbed orally administered tracer;
(d) The tracee is in steady state (i.e. the amount of tracee in the system is unchanged in the timeframe of the experiment).

As suggested by these assumptions, an isotopic tracer needs to be metabolized in the same way as the substance that it represents and the amount of tracer used should not alter the biological process or distribution. In contrast with radioisotope tracers of zinc, which can be detected in such small amounts that they are essentially 'mass free', stable isotopes of zinc have greater limits of detection requiring more isotope to be administered for accurate measurement. Whereas radiotracers are detected directly, stable isotope tracers are quantified only indirectly via induced changes in isotope ratios. Although more isotope needs to be administered with stable isotope studies than with radioisotope studies, the stable isotope is still regarded, and referred to in this publication, as a tracer. Experimentally, the goal is to employ sufficient stable isotope for measurement precision without introducing quantities that substantially alter the usual biological concentrations of the substance being examined.

3.1.2. Stable isotopes of zinc, abundance and sample enrichment

Five stable isotopes of zinc occur in nature, in the following proportions [33]:

(a) ^{64}Zn 49.17%;
(b) ^{66}Zn 27.73%;
(c) ^{67}Zn 4.04%;
(d) ^{68}Zn 18.45%;
(e) ^{70}Zn 0.61%.

The proportion of a stable isotope that occurs naturally is referred to as its natural abundance. Zinc sources can be prepared with higher proportions of a single isotope, and are considered enriched for that isotope when that natural abundance is exceeded. Highly enriched sources of isotopes that have a low natural abundance can be used as tracers in studies related to zinc nutrition and metabolism. As a result, biological samples, such as plasma, urine and faeces, become slightly enriched with this isotope above the baseline or natural abundance level, and detecting these changes enables quantitative measurements of zinc metabolism.

3.2. ISOTOPE METHODS TO DETERMINE ZINC ABSORPTION

The measurement of zinc absorption is fundamental to the ability to set nutrient recommendations for a given population. The absorption of zinc from typical diets can vary widely depending upon the composition of the diet, especially the amount of zinc ingested and the presence of inhibitors or enhancers

of zinc absorption. Applications include studies of bioavailability from traditional diets in developing countries, understanding the adaptations during pregnancy and lactation and the effects of ageing.

3.2.1. Older methods to measure zinc absorption

A number of methods have been used to measure zinc absorption. Traditionally, zinc absorption was determined by measuring the difference between dietary zinc intake and faecal output, an elemental mass balance approach that is commonly referred to as apparent zinc absorption. Accurately quantifying zinc balance or apparent absorption is challenging. Large errors can result from incomplete faecal collections, difficulty in matching faecal samples with the specific times that the diet was consumed, and the subtraction of non-zero analytical blank measurements from relatively low concentrations of zinc in faecal aliquots. In addition, the traditional balance technique does not distinguish between two types of faecal zinc: (a) zinc from endogenous gastrointestinal secretions, and (b) zinc from the diet that was not absorbed (Fig. 1). In theory, and in contrast to apparent absorption, true absorption represents the dietary intake minus the faecal output of unabsorbed dietary zinc, as follows:

(a) Apparent zinc absorption

$$\text{Apparent Zn absorption} = \text{dietary Zn} - \text{faecal Zn} \quad (1)$$

(b) True zinc absorption corrected for endogenous faecal zinc excretion

$$\text{True Zn absorption} = \text{dietary Zn} - (\text{faecal Zn} - \text{EFZ}) \quad (2)$$

Single doses of isotopes, administered either intravenously or orally, have been used to measure absorption, but have the disadvantage of requiring quantitative assessment of elemental zinc and zinc isotope in faeces.

Single, orally administered isotopes have been used to measure zinc absorption by expressing the amount of isotope excreted in the faeces as a proportion of the amount ingested, as described in Ref. [34]. This faecal isotope monitoring, or isotope apparent absorption method, requires a complete collection of faeces for 8–12 days [35], and because these time periods may still be inadequate for isotope clearance by some participants [36], faecal dyes or markers may be useful to correct for incomplete faecal collections (see Section 3.4.2). Although this isotope based method may be more accurate than the elemental mass balance method without isotopes [35, 36], it does not account for the absorption and re-excretion of isotopes during the collection.

A proposed correction that estimates endogenous re-excretion based on the rate of faecal isotope excretion after most of the unabsorbed isotope is likely to have been excreted [37] may not consistently improve the accuracy of this method [35].

Single, intravenously administered stable isotopes have been used to adjust elemental apparent absorption determinations for endogenous faecal zinc excretion based on relative isotopic enrichment in faecal and plasma samples [38] or in faecal and urine samples [39]. Such a correction involves the quantitative collection and both elemental and isotopic zinc analyses of faecal excreta. Although this single IV tracer method for measuring absorption has been verified against subsequently accepted dual isotope absorption methods in one report [40], the difficulty in accurately quantifying total faecal excretion may explain a lack of verification in another report [35]. This method, discussed further in Section 3.4 on measuring endogenous faecal zinc excretion, may be especially useful for testing assumptions that an oral isotope tracer added to the diet is absorbed similarly to the natural (intrinsic) zinc in the diet. However, because the method is more laborious and subject to the errors associated with faecal collections, the dual isotope method described below is preferred for most absorption measurements.

Techniques based on faecal collections are labour intensive for both the participant and the investigator, with accuracy dependent on participant compliance and regular gastrointestinal transit as well as a collection period sufficient to collect the unabsorbed isotope without substantial collection of isotope that has been absorbed and re-excreted. Although total faecal collections are possible in the field, a complete, uncontaminated collection is much easier to implement in a metabolic unit with research staff supervising the biological collections. Faecal isotope monitoring may be useful to measure zinc absorption in children when IV dosing is difficult [41, 42].

3.2.2. The dual isotope zinc absorption method

It is now most common to determine FZA by administering two stable isotope sources orally and intravenously, and measuring the resulting enrichment of plasma or urine samples. This dual isotope method, introduced by Friel et al. in 1992 [43] is based on calcium absorption techniques and has been widely used in field situations in developing countries and with very young children. The dual isotope method is based on the principle that after nearly simultaneous administration, both the IV isotope and the absorbed portion of the oral isotope are similarly incorporated into plasma and then endogenously excreted at similar rates. Thus, it is assumed that the IV isotope is 100% incorporated endogenously, bypassing the absorption process, and that retention of the IV dose can serve

as a reference for calculating absorption of the oral dose, without the need for faecal collections. Eliminating faecal collections greatly simplifies the method, generally improving the accuracy of the results [35]. The isotopic enrichment of both plasma and urine samples reaches a plateau about three days after oral or IV administration, and the two isotopes follow a similar rate of loss for at least seven days after isotope administration [43]. Fractional absorption is calculated from the resulting isotope enrichment in urine or plasma, which can be determined in spot urine or plasma samples collected during the 3–7 days after isotope administration [35, 43–46].

FZA is calculated using the following equation that expresses the retention of the oral isotopic tracer in relation to its dose, as corrected for the retention of the IV isotopic tracer in relation to its dose:

$$\text{FZA} = \frac{\text{oral isotopic enrichment in urine} \,/\, \text{dose of oral isotopic tracer}}{\text{IV isotopic enrichment in urine} \,/\, \text{dose of IV isotopic tracer}} \qquad (3)$$

where isotopic enrichment is in mg tracer per mg total zinc and the tracer doses are in mg.

Although isotopic methods of measuring zinc absorption initially assess fractional absorption, the key nutritional measurement of interest is the amount of zinc absorbed. To determine the amount of zinc absorbed, the FZA is multiplied by the total amount of zinc that was labelled for ingestion (naturally occurring plus the added isotope label); this can be a single labelled solution, a labelled meal, or multiple meals in which the zinc label was added in proportion to the natural zinc content of the meals. The absolute amount of zinc absorbed (mg) is:

$$\text{Zn absorbed} = \text{FZA} \times \text{Zn ingested} \qquad (4)$$

This calculation is based on the assumption that the zinc intrinsic to the food is fully interchangeable and similarly absorbed as the zinc in the label (further discussed in Section 5.3 on intrinsic and extrinsic labelling of foods). Zinc absorbed from single meals (under conditions of similar zinc content) can be useful for evaluating zinc bioavailability from different foods. However, measurement of zinc absorbed from one or more whole days of a diet has the advantage of enabling comparison of the results to nutritional adequacy and physiological requirements, which are currently based on daily zinc excretion.

3.3. ISOTOPE METHODS TO DETERMINE ZINC KINETICS AND EXCHANGEABLE POOLS

In addition to studies of zinc absorption and excretion, stable isotopes can be used to study the movement and distribution of zinc in the body. Measurement of isotope enrichment in accessible samples, such as blood and excreta (urine and faeces) provides data which, along with known physiology, can be mathematically modelled using compartmental analysis computer programs to yield information about the flux rates, turnover rates and sizes of metabolic pools of zinc in healthy individuals [47–49].

These techniques have been applied in the study of zinc metabolism and homeostasis in various physiological states, including the postprandial effect [50, 51], the effect of exercise [52], the physiological response to acute severe and marginal zinc depletion and repletion [53, 54], small for gestational age infants [55], lactation [26], nutrient interactions [23] and ageing [56]. A parameter derived from zinc stable isotope kinetic studies, which has received considerable attention, is the exchangeable zinc pool (EZP), defined as the labile portion of whole body zinc that exchanges with the plasma zinc within a period of 2–3 days [57, 58]. The EZP, which represents less than 10% of total body zinc [47] has been proposed as an indicator of zinc status, however it appears that the EZP declines less than plasma zinc concentration in response to severe zinc deficiency [53]. Although EZP appears to respond to a fall in dietary zinc intake, its response to marginal zinc deficiency and zinc supplementation is equivocal and requires further study. In children, the EZP is strongly associated with body weight, but in adults, EZP has been observed to vary with several factors, including absorbed zinc [50].

3.3.1. Compartmental modelling of the exchangeable zinc pool size

Compartmental modelling of the plasma isotopic tracer curve following an IV stable isotope dose can be used to determine the size of the EZP [54, 59]. This method requires frequent blood sampling (e.g. at 2, 5, 10, 20, 30, 45, 60 minutes; 2, 3, 6, 9, 12 hours; 1, 2, 4, 6, 8 days after stable isotope administration). The resulting plasma tracer data is fit to a multiple compartment model using a modelling programme such as SAAM II (simulation, analysis and modelling software). The EZP is determined as the sum of the masses of a three-compartment model that best describes plasma zinc kinetics in adults during an eight day period [56]. The detailed application of this complex method is beyond the scope of this publication.

3.3.2. Simpler exponential modelling of the exchangeable zinc pool size

As an alternative to the demanding sampling protocols necessary for creating full compartmental models of body zinc kinetics to calculate EZP size, a simpler method determines the EZP from plotting the isotope enrichment in plasma or urine after IV isotope administration [38, 60]. The logarithm of the plasma or urine enrichment is linear as early as 2 days, and for at least 9 [60] or even 24 days [38], after isotope administration; for practical application, sampling between days 3 and 9 is recommended [60]. This semi-logarithmic, or exponential, relationship can be extrapolated to time zero, the time of the dosing, to account for the rate of loss of the isotopic tracer out of the EZP. The EZP size is estimated by dividing the IV isotope dose by this urine or plasma tracer enrichment at time zero. Although the EZP nomenclature has been applied to both this simpler modelling method and the compartmental modelling method mentioned above, a comparison of both methods revealed that EZP results were 15–20% greater for the simpler method, compared with the compartmental method [59], which should be considered when comparing results between studies. An example of calculations using this method to determine EZP is given in Section 9.2.2.

In situations where the administration of an IV stable isotope is problematic (for example in infants) the EZP is estimated using a single orally administered stable isotope [58]. This method involves measuring the isotope enrichment of urine for 4–8 days after the dose. A plot of enrichment versus time is made in a similar way to that described above to find the intercept with the y-axis at time zero. An estimate of absorbed zinc, using faecal monitoring, is then used in the data analysis, replacing the IV dose.

3.4. ISOTOPE METHODS TO DETERMINE ENDOGENOUS FAECAL ZINC EXCRETION

EFZ is the zinc excreted in the faeces originating from gastrointestinal secretions rather than from unabsorbed dietary zinc. Zinc stable isotope studies are instrumental in identifying EFZ as important in homeostatic regulation, and the results are applied to derive current recommendations for human zinc intake [2–4].

Implementing EFZ protocols is more challenging than protocols for FZA or EZP. Although it is not impossible to conduct studies of EFZ in a community setting with volunteers living at home, studies of EFZ involve controlled diets as well as faecal (and urine) collections for several days, and are best conducted in

a clinical metabolic unit, where participants can reside and specimen collection procedures can be closely monitored.

EFZ cannot be measured directly using classic mass balance techniques, because the endogenously excreted zinc cannot be distinguished from the unabsorbed dietary zinc in faeces. Attempts to measure EFZ using a single oral stable isotope coupled with mass balance require assumptions that all of the excreted isotope was unabsorbed (not absorbed and re-excreted), and that fractional isotope absorption was similar to the FZA from the whole diet. These assumptions are not required when using an IV isotopic tracer.

3.4.1. Endogenous faecal zinc measurement

Stable isotope measurements of EFZ [38, 39, 61] involve the administration of an IV zinc isotope followed by an analysis of tracer enrichment in faecal samples as well as in samples containing zinc from endogenous origin (i.e. plasma, serum or urine). From these data, the amount of faecal zinc derived from endogenous sources can be calculated using a separate analysis of the total elemental zinc in one of these sample compartments, generally in faeces, as in Eq. (5), for EFZ based on total zinc in faeces:

$$\text{EFZ} = \frac{(\text{IV isotope enrichment in faeces})(\text{total Zn in faeces})}{(\text{IV isotope enrichment in urine, plasma or serum})(\text{time})} \quad (5)$$

where

EFZ is in mg/d;
total Zn is in mg;
IV isotope enrichments are in mg tracer/mg total Zn;

and time is in days representing a controlled diet period (see below).

3.4.2. Faecal markers

The measurement of EFZ is challenging, with the need to quantitatively collect faecal samples that represent a defined period of dietary intake and to estimate plasma or urine isotopic labelling at a time corresponding to the period of zinc isotope secretion into the intestine. Non-absorbable faecal markers can help improve the accurate collection and quantification of total zinc and zinc isotopes excreted in faeces. Readily visible faecal markers, such as dyes administered at the beginning and end of a specific diet period, can be used to identify the corresponding faecal samples [62], and markers that can be quantitatively

analysed can be administered throughout the controlled diet period to monitor and mathematically adjust for the completeness of the sample collection [38, 61, 62]. The similarity of the gastrointestinal transit rate for the marker compared to that for the ingested zinc isotope has been questioned, especially regarding plastic pellets, polyethylene glycol and coloured dyes. The excretion pattern of dysprosium, a rare earth element, has been found to be similar to that of zinc isotopes [61, 63–65]. Miller et al. [61] used brilliant blue dye to visually mark stools at the beginning and end of a controlled diet period and to estimate intestinal transit time; in addition, they administered dysprosium in proportion to dietary zinc with all meals throughout the controlled diet period to analyse and adjust for incomplete faecal collections. They tested the possibility of using single convenience faecal samples, but concluded that at least three well-labelled faecal samples were needed to ensure accuracy [61]. After six days of dysprosium administration, their example volunteers had only four to five such well-labelled faecal samples with dysprosium to zinc ratios exceeding 50% of the administered dose. This suggests that it may be most practical to plan for complete faecal collections using a visual marker, with use of the dysprosium marker to make mathematical corrections for incomplete collection.

If a non-absorbable, quantifiable marker is continually administered during the controlled diet period in proportion to meal zinc, Eq. (5) can be modified to account for incomplete sample collection as follows:

$$\mathrm{EFZ} = \frac{(\text{IV isotope enrichment in faeces})(\text{total Zn in faeces})}{(\text{IV isotope enrichment in urine, plasma or serum})(\text{time})} \times \frac{\text{marker dose}}{\text{total marker in faeces}} \tag{6}$$

where marker dose (mg) is the total weight of marker administered during the controlled diet period and total marker in faeces (mg) is the weight of marker in the faecal sample for the same time period. Total marker in faeces is determined by multiplying the analysed marker concentration in the faecal sample by the total faecal weight collected.

In Eq. (6), the total Zn in faeces, the marker, and the total marker in faeces all correspond to the same single or multiple day time period of the controlled diet, after adjusting faecal data for intestinal transit time. For calculations based on single days, Miller et al. [61] only included data from faecal samples that contained at least 50% of the ratio of marker to total zinc ingested.

Faecal samples can be analysed as pooled samples corresponding to 24 hour collection periods or a multiple day collection period, as indicated using unabsorbed markers administered at the beginning and end of the controlled

dietary period. Separate analysis of 24 hour samples may enable better evaluation of the timing and consistency of results, including interpretation of results using a continuous marker such as dysprosium. The following modification of Eq. (5) indicates how products of the faecal analyses are summed when analysing multiple 24 hour samples.

$$\text{EFZ} = \frac{\sum[(\text{IV isotope enrichment in faeces})(\text{total Zn in faeces})]}{(\text{IV isotope enrichment in urine, plasma or serum})(\text{time})} \quad (7)$$

3.4.3. Sample timing considerations

An additional component of measuring EFZ is estimation of the IV isotope enrichment in plasma or urine at the time corresponding to the secretion of the endogenous zinc isotope that is subsequently excreted in the faeces. Jackson et al. [38] obtained a plasma sample of metabolic balance at the midpoint of a four day controlled diet period. Using urine samples, Miller et al. [61] used a semi-logarithmic regression modelling method to estimate endogenous enrichment corrected for individual differences in gastrointestinal transit time (the time required for complete gastrointestinal transit of a visible, non-absorbable faecal marker, minus five hours to account for the marker passage through the stomach and upper small intestine), and the EFZ results were not significantly different from calculations that applied the mean urine enrichment during the controlled diet period of metabolic balance.

Yergey [39] recommended mathematical deconvolution analysis of the rates of excretion, assuming proportional rates of zinc excretion into the intestine and into the urine, and using complete urine collections for at least 48 hours. Total urinary zinc can then be applied to calculate EFZ, substituting for Eq. (5) as follows:

$$\text{EFZ} = \frac{(\text{IV isotope enrichment in faeces})(\text{total Zn in urine})}{(\text{IV isotope enrichment in urine})(\text{time})} \quad (8)$$

A study simultaneously comparing the methods of Jackson [38] and of Yergey [39] in female participants found no significant difference in the values obtained for EFZ (or FZA), although the latter method was somewhat more variable [35]. A procedure to measure EFZ is further detailed in Section 9.3.

3.4.4. Application of EFZ to measure zinc absorption from unlabelled food

As mentioned in the prior discussion of measurements of absorption, the determination of EFZ using a previously administered single IV isotope can be applied by using Eq. (2) with carefully collected balance data to determine zinc absorption. This is more difficult than the dual isotope method for determining absorption because it requires complete faecal collections and analyses. However, this method has the advantage of measuring zinc absorption from natural food sources without requiring the assumption that a zinc isotope added to food is absorbed as efficiently as the natural zinc in the food [40]. This assumption is discussed further in Section 5.3.

4. PLANNING AND PREPARING ZINC STABLE ISOTOPE DOSES

The dose of stable isotope administered should be sufficient to give a measurable enrichment (with sufficient accuracy and precision) of that isotope in the biological samples without substantially altering the usual zinc concentrations of the biological process. The amount of isotope administered will depend on the precision of the mass spectrometer; a less precise instrument will require a greater isotope amount administered to make an accurate measurement. This section will review considerations for isotope dosing in nutrition studies.

4.1. OBTAINING ISOTOPICALLY ENRICHED ZINC SOURCES

Enrichment of a zinc source with a specific stable isotope results in an increased proportion of that isotope and depleted proportions of other isotopes in comparison to the isotopic natural abundance. An isotopically enriched zinc source can be purchased commercially at varying levels of abundance from ~80 to >99%. Many such stable isotope sources can be produced to order, and most companies have highly enriched stable isotope sources in stock. Isotopically enriched zinc sources are usually available in the oxide or metal forms, which need to be converted into a soluble form prior to administration (see Section 4.3). It is important to purchase stable isotopes only from reputable suppliers with a reliable track record. Most commercial suppliers of stable isotopes state that the isotopes are not certified for use in humans because no special testing has been conducted to meet pharmaceutical standards.

Some characteristics of the five stable isotopes of zinc, including atomic mass, natural abundance, the abundance of commercially available enriched sources, relative costs, and typical amounts administered in recent human studies, are summarized in Table 1. Abundance of an isotope is expressed as a (molar) proportion of the total number of atoms of the element.

To minimize the amount of tracer administered, high enrichments of the isotopes with the lowest natural abundance (^{70}Zn and ^{67}Zn, followed by ^{68}Zn) are preferred. Although enrichments of zinc isotopes with the lowest natural abundance may cost the most (see Table 1 for relative prices), these isotopes tend to require lower amounts administered for detection in biological samples.

The assignment of isotopes will depend on the goals of the study. For dual or triple stable isotope tracer studies, the isotope of lowest natural abundance has often been chosen as the IV tracer. However, some studies designed to study zinc absorption from foods have used ^{67}Zn or ^{68}Zn as IV tracers and ^{70}Zn as the oral tracer, since the ^{70}Zn tracer can be detected when administering very low amounts, allowing minimal addition of extrinsic zinc to a test diet [66].

4.2. QUANTIFYING ISOTOPES AND PLANNING DOSES

Mass spectrometer analyses of stable isotopes are determined as isotope ratios representing molar ratios of the atoms detected of two different isotopes. Commonly, a tracer isotope (^{67}Zn, ^{68}Zn or ^{70}Zn) in the numerator is expressed relative to the more abundant ^{66}Zn isotope in the denominator. In contrast, nutrition study results are often reported on a weight basis (e.g. mg of zinc ingested, absorbed or excreted). In general practice, mass (weight) measurements are converted to amounts in moles for conducting calculations with isotope ratios, and later converted to weight units as needed for final expression of the results.

4.2.1. Converting from molar to weight units

For the naturally occurring element, molar units are converted to weight units by using the standard atomic weight. The standard atomic weight of natural zinc (65.38 g/mol or mg/mmol) is based on the atomic mass and abundance of all the naturally occurring zinc isotopes [67]. To convert amounts of natural zinc from molar (mmol) into weight (mg) units, multiply the mmol quantity by the atomic weight as in Eq. (9):

$$\begin{aligned} \text{Zn (in mg)} &= \text{Zn (in mmol)} \times \text{AW}_{\text{natZn}}\,\text{(in mg/mmol)} \\ &= \text{Zn (in mmol)} \times 65.38\ \text{mg/mmol} \end{aligned} \tag{9}$$

TABLE 1. CHARACTERISTICS OF ZINC STABLE ISOTOPE TRACERS USED IN NUTRITION STUDIES

Isotopes	Atomic mass (g/mol)[a]	Natural abundance (% of atoms) [33]	Commercially available abundance (%)	Relative cost/mg (US $)	Typical amounts administered[b]	
					Intravenous	Oral[c]
Zn-64	63.929	49.17	>99	n.a.[d]	n.a.[d]	n.a.[d]
Zn-66	65.926	27.73	>99	n.a.[d]	n.a.[d]	n.a.[d]
Zn-67	66.927	4.04	94	25	0.5–1.0 mg adult 0.3–0.8 mg infant	0.3–2.0 mg adult 0.3–1.5 mg infant
Zn-68	67.925	18.45	>99	11	0.5–1.0 mg adult 0.3–0.8 mg infant	1.5–3.0 mg adult 0.5–1.0 mg infant
Zn-70	69.925	0.61	85–96	42	0.3–0.8 mg adult 0.2–0.5 mg infant	0.3–1.2 mg adult 0.2–0.5 mg infant

[a] The term atomic mass refers to the mass of a specific nuclide, atom or isotope of the element, whereas atomic weight refers to the average mass of all the atoms or isotopes of a natural or isotope enriched sample. Both atomic mass and atomic weight are expressed in grams per mole (g/mol), based on the earth's gravitational force. The standard atomic weight of natural zinc, 65.38 g/mol, is based on the atomic mass and abundance of the naturally occurring zinc isotopes.

[b] The typical amounts administered as tracers in nutrition studies are shown for the three isotopes with low natural abundance. As further discussed in the text, the amount of isotope administered depends on the specific application and the precision of the mass spectrometer. These doses may need to be increased considerably when using instruments that measure less precisely. The doses in the table may have exceeded the instrumental limits of precision more liberally for ^{67}Zn and ^{70}Zn than for ^{68}Zn, depending on the applications used.

[c] The oral dose can be divided between multiple meals, in order to keep the tracer mass low (preferably <10% relative to the natural zinc ingested in the labelled food or supplement), and/or to better measure the results for a whole day's diet, which enables comparison to daily zinc requirements.

[d] n.a.: not applicable.

Similar conversions can be applied for specific isotopes, substituting the atomic mass for the respective isotope for the atomic weight (the term 'atomic mass' refers to a specific single isotope, whereas 'atomic weight' refers to the average mass of all isotopes of a natural or isotope enriched sample, see Table 1).

4.2.2. Calculating the average atomic weight of an isotopically enriched source

Once purchased, an isotope enriched product will be delivered by the supplier with specifications describing the isotope content. Using this information, it is possible to calculate an average atomic weight for zinc that is specific for the enriched source or product using Eq. (10). Note that the changed proportion of isotopes with different masses results in an average atomic weight that differs from that of naturally occurring zinc.

$$\begin{aligned} AW_S = &\left(AM_{64Zn} \times A_S^{64Zn}\right) + \left(AM_{66Zn} \times A_S^{66Zn}\right) \\ &+ \left(AM_{67Zn} \times A_S^{67Zn}\right) + \left(AM_{68Zn} \times A_S^{68Zn}\right) + \left(AM_{70Zn} \times A_S^{70Zn}\right) \end{aligned} \tag{10}$$

where AW is the average atomic weight (mg/mmol) of the multiple isotope source (S), AM is the atomic mass of a specific isotope indicated in the subscript (see Table 1) and A is the abundance (expressed as a mole fraction) of the isotope indicated by the superscript within the material indicated by the subscript. For example, A_S^{64Zn} is the abundance of ^{64}Zn in the multiple isotope source (S).

As an example, the following calculation yields the average atomic weight of zinc in a ^{70}Zn enriched zinc oxide (ZnO) product specified to contain the following mole fractions of isotopes: ^{64}Zn 0.0030, ^{66}Zn 0.0020, ^{67}Zn 0.0020, ^{68}Zn 0.108, ^{70}Zn 0.885. The following is the average atomic weight of zinc in the enriched commercial product, and the result reflects the high degree of enrichment of the ^{70}Zn isotope:

$$\begin{aligned} AW_S = &\left(63.929 \times 0.003\right) + \left(65.926 \times 0.002\right) \\ &+ \left(66.927 \times 0.002\right) + \left(67.925 \times 0.108\right) + \left(69.925 \times 0.885\right) \\ = &\ 69.677 \text{ mg Zn/mmol} \end{aligned} \tag{11}$$

4.2.3. Expressing the tracer dose as an enriched isotope source versus a single isotope

Since it is not possible to procure an enriched zinc isotope with 100% abundance, one needs to distinguish between a specific isotope (e.g. ^{70}Zn) and an enriched stable isotope source or material (e.g. a ^{70}Zn source with 88.5% abundance and smaller amounts of other zinc stable isotopes). This distinction becomes important in calculations relating the amount of tracer in samples to the amount administered. It is possible to base calculations on either the single

isotope or the entire isotope source as a tracer. However, especially when more than one tracer is used in a study, it is advisable to quantify both the dose and the sample enrichment based on the entire zinc isotope source, thereby accounting for the highly enriched zinc isotope as well as small amounts of other zinc stable isotopes in the same source.

Accordingly, in this publication, the tracer dose administered is defined as the total amount of all zinc isotopes from the enriched isotope source. Sample enrichment is the degree to which this tracer is incorporated into the biological sample, thereby exceeding the natural abundance. This is expressed as the amount of tracer zinc to total zinc in the sample, either as a ratio (mg Zn tracer/mg total Zn) or as per cent enrichment (mg Zn tracer/100 mg total Zn). The analyst needs to quantify the amount of enriched stable isotope tracer in a sample that contains the same stable zinc isotopes as the background, only in different proportions.

When evaluating the literature, please note that there are differences in the way that isotope doses and sample enrichment are defined and calculated. While this publication recommends calculation methods that account for all isotopes in a highly enriched tracer, some publications cite doses based on single isotopes. Comparisons between doses expressed in these different ways would require an adjustment for less than 100% enrichment of the isotope source. There are advantages and disadvantages to different methods of calculating and expressing tracer enrichment of biological samples from the isotope ratios measured by mass spectrometry. Such calculations are further discussed briefly in Section 8. Analysts need to keep in mind that results from different calculation methods (e.g. the tracer as a single isotope versus the whole isotope source), while similar, may not be directly comparable.

4.2.4. Assessing mass spectrometry precision

Instrumental precision is often described in terms of limits of detection (LOD) and limits of quantitation (LOQ). The LOD indicates the minimum amount that needs to be measured to detect a real difference from an unenriched or natural abundance baseline sample, and the LOQ is the minimum amount to reliably describe the magnitude of the measurement.

To determine the LOD and LOQ for a specific instrument and type of sample, the measurement variation is assessed using numerous (≥30) isotope ratio measurements of an unenriched baseline sample of interest (e.g. serum or urine sample). The standard deviation of these measurements describes the error when measuring a low enrichment of isotopic tracer in a sample. In work with stable isotope tracer enrichment, the LOD is commonly defined as three standard deviations above the mean and the LOQ as ten standard deviations above the

mean of the unenriched or natural abundance sample. The LOD and LOQ are determined using the same instrument, sample matrix, chemical analysis and calculation method used for calculating sample enrichment. The relative precision, or reproducibility of an instrument or method, is generally expressed using the relative standard deviation. Lower values are preferred when evaluating the precision of instruments or methods. (Calculation methods are further discussed in Section 8.)

Older mass spectrometry instrumentation used for zinc isotope analysis was commonly characterized by measurements with a relative precision of 0.2–1%, but modern, enhanced instrumentation can improve the relative precision to 0.02% or less. Such large differences in instrumentation precision would proportionally alter the tracer doses required for adequate detection in biological samples, and poor analytical precision may limit the types of studies that can be conducted. Analytical precision also differs between specific isotopes, and needs to be evaluated for each of the principal zinc isotopes used as highly enriched isotope sources (e.g. ^{67}Zn, ^{68}Zn and ^{70}Zn).

Tracer doses are planned so that the amount of the highly enriched isotope in a zinc isotope source is likely to yield sample enrichments that at least exceed the LOD, and preferably the LOQ, of the mass spectrometer for that specific isotope and analytical method, allowing as much leeway as feasible. The degree of error and uncertainty in isotope ratio measurements continues to decrease as the LOQ is exceeded, and analytical accuracy may be further improved by increasing doses to enrich samples to even higher levels. However, the analytical benefit of increasing the dose administered should be weighed against the benefit of minimizing the tracer dose to avoid disturbing the usual zinc intake and metabolic equilibrium (see Section 4.2.6).

4.2.5. Reviewing similar studies to estimate the dose required

When calculating the tracer dose required, a useful starting point is knowledge of the approximate zinc content of the samples to be analysed, the expected zinc absorption and the sampling time post-enrichment. The dose will be affected by the size of the individual (i.e. the larger the person, the greater the volume into which an IV or oral dose will be dispersed), as well as the metabolic activity of the body compartments and rates of loss from these compartments. However, because studies with infants often involve zinc supplemented formulas or diets, investigators may choose to improve precision by using greater amounts of an oral isotope tracer without exceeding 10% of the total ingested zinc. A research participant with high deposition of zinc into specific tissues (i.e. during pregnancy) or high rates of loss may require larger doses to accurately detect serum or urine isotope enrichment. The dietary zinc level will also influence the

ability to detect the zinc stable isotope excreted in faeces, and greater doses may be needed for measuring isotopic enrichment in faeces as compared to serum or urine.

Guidance about doses and the resulting enrichment of samples can be obtained by reviewing the literature of similar studies and as well as the previous results acquired using the specific mass spectrometer that is available for the investigation. When establishing new methods or instrumentation, it may be useful to plan (with ethical approvals) a pilot study with a limited number of participants to practice procedures and check that a planned dose provides adequate sample enrichment.

As an example of estimating the dose, a previous result may have indicated that an oral dose of 1.0 mg of a source of highly enriched ^{70}Zn resulted in a 6% tracer enrichment of the urine samples of women after 5 days. A new study with similar participants will involve a mass spectrometer with a 1% LOQ for ^{70}Zn. The investigator will aim to lower the dose to reduce the amount of zinc in the tracer but to still exceed the LOQ sufficiently to accurately quantify the results even if they be may be lower or more variable than anticipated. The dose for a desired enrichment (e.g. 3% enrichment in Eq. (12)) can be estimated from a previous dose and associated enrichment by cross-multiplying:

$$\text{New dose (in mg)} = \frac{\text{new desired target \% enrichment} \times \text{previous isotope dose (in mg)}}{\text{previous \% enrichment in same sampling pool}} \quad (12)$$

Solving with the data in the example:

$$\text{New dose} = \frac{3\% \text{ enrichment} \times 1.0}{6\% \text{ enrichment in same sampling pool}} = 0.5 \text{ mg}$$

This indicates that a dose of 0.5 mg of a source of enriched ^{70}Zn can be expected to yield an enrichment of 3% in the sample under conditions similar to the previous study, which is still above the 1% LOQ for the mass spectrometer that will be used.

Because this publication expresses sample per cent enrichment as mg of all zinc isotopes in the tracer per 100 mg of total zinc in the sample, the sample enrichment would remain the same for any other highly enriched isotope, highly enriched ^{68}Zn for instance. However, the analytical LOQ based on this calculation method is usually greater for the zinc isotopes with higher natural abundance; for instance, the LOQ for ^{67}Zn may be approximately two times (and for ^{68}Zn, twenty

times) the LOQ for ^{70}Zn. Thus, the LOQ will probably increase, and the related dose will need to be increased accordingly, to ensure accurate measurement precision of the enriched samples when using different highly enriched tracers.

4.2.6. Minimizing and standardizing oral isotope doses

To minimize any effect of the isotope mass on a zinc absorption measurement, it is generally desirable to limit the oral dose of zinc stable isotope, preferably to less than 10% of the zinc content of the meal or diet that is labelled. Zinc added to the meal can reduce the fraction of zinc absorbed as well as alter the ratio of zinc to absorption enhancing or inhibiting food components. As indicated in Table 1, oral doses of 0.3–3.0 mg for adults have been used in recent zinc stable isotope studies, depending on the isotope. Since people commonly consume meals containing 2–5 mg zinc, the isotope doses can substantially increase the zinc content of a meal. As part of planning the isotope dose, the zinc content of the meal(s) to be labelled needs to be analysed. When it is not possible to improve the analytical technique or instrumentation, the amount of zinc isotope added in relation to the total dietary zinc can be kept minimal by:

(a) Dividing the isotope dose among multiple labelled meals (labelling a whole day's diet enables comparison of results to zinc requirements);
(b) Using only the isotope with the lowest natural abundance (^{70}Zn) so that a low dose can be accurately detected;
(c) Limiting studies to diets with relatively high zinc content (e.g. zinc fortified foods).

In general, the amount of zinc isotope needs to be controlled between experimental treatments. Unless the intent of the study is to test the influence of differences in the amount of zinc ingested, tests comparing zinc absorption under different conditions need to standardize the total zinc content of oral isotope doses by adding unenriched zinc. For example, based on differences in the LOQ for different isotopes, it may be determined that doses containing either 0.5 mg of ^{70}Zn or 1.5 mg of ^{67}Zn are required to achieve adequate measurement precision. If both isotopes were used to compare two different dietary treatments, the dose of total zinc for each absorption measurement could be standardized by using 1.5 mg of each isotope tracer, or by adding 1.0 mg natural Zn to the 0.5 mg ^{70}Zn isotope dose (and it may be best to divide these doses into multiple meals). Alternatively, the study could be designed to use only ^{70}Zn (0.5 mg) in each of two sequential absorption measurements, scheduling a new baseline assessment at the beginning of the second absorption measurement to account for the effect of the first measurement.

If the oral dose is divided among multiple non-identical meals, the distribution of the isotopic tracer needs to be the same as the natural zinc distribution in the meals. In other words, the ratio of zinc isotopic tracer to total zinc needs to be the same for each of the meals labelled. For example, for a full day of labelled meals, if 20% of the natural food zinc is at breakfast, then 20% of the zinc isotopic dose would be added to the breakfast. Error would be introduced if a zinc isotopic dose is divided equally between multiple meals that differ in zinc content and bioavailability.

4.2.7. Estimating the amount of isotope to purchase

The amount of isotope to purchase depends on the isotopic abundance of the enriched source, the chemical form, the size of the doses, the number of participants, and the number of doses per participant. Approximately 20–30% extra is required to allow for analyses of the prepared dose, sterility, pyrogenicity and fungal testing, and for the dosing of a few additional participants, if necessary. It is better to purchase all the stable isotope needed for an entire study in a single batch to ensure the same enrichment for all the isotope doses and simplify the study calculations.

As an example, a planned study will require 26 doses, each providing 0.5 mg of a highly enriched ^{70}Zn source. The dose has been estimated from previous study results of 1% sample enrichment associated with a 0.5 mg dose of a highly enriched source containing 98% ^{70}Zn. A commercially enriched source of the isotope has been identified that is 88.5% ^{70}Zn as ZnO. The sample enrichment of 1% from the previous study is well above the laboratory analytical LOQ of 0.2% for ^{70}Zn in this case, but if it were closer to the LOQ, the investigators may wish to increase the dose proportionally: by at least 10% = (98−88.5)/98 × 100 in this case to make up for the differences in product enrichment. To roughly estimate the amount of this enriched ^{70}ZnO to purchase, the calculation needs to account for the approximate fraction of the weight of the ^{70}ZnO contributed by ^{70}Zn, as follows:

$$\begin{aligned}\text{Enriched } {}^{70}\text{ZnO} &= 0.5 \text{ mg } {}^{70}\text{Zn source per dose} \times 26 \text{ doses} \\ &\times \frac{86 \text{ mg/mmol } {}^{70}\text{ZnO}}{70 \text{ mg/mmol } {}^{70}\text{Zn}} = 16.0 \text{ mg}\end{aligned} \tag{13}$$

Because this is a rough estimate for procurement purposes, the calculation has roughly estimated an atomic mass of 70 mg/mmol for the zinc in the highly enriched ^{70}Zn product, with an added mass of 16 mg/mmol for the oxygen atom, totalling 86 mg/mmol for the ZnO molecule. It would be technically more

precise to do the calculation on a molar basis, using the specific atomic weight of the isotopically enriched source as in Eqs (10) and (11), but this precision is not necessary for a rough estimate, and the information on the specific isotopic content may not be fully available before procurement. As indicated above, it is advisable to purchase at least 20–30% extra; accordingly, the above result can be rounded up to a purchase of 21 mg of enriched ^{70}ZnO.

4.2.8. Confirming the zinc content and isotopic abundance of the prepared dose

While the vender information on detailed isotopic composition that accompanies a purchase of enriched stable isotope is useful in planning the dose preparation, it is best practice to verify the isotopic composition of the final labelled material by measuring the isotopic ratios and the total zinc content. Such verification confirms the data provided by the isotope vendor and ascertains that no exogenous zinc contamination has been introduced to dilute the isotopic enrichment during the preparation of the dose solutions. Final verification of the zinc dose and isotopic abundance before administration can prevent errors that could jeopardize or ruin a study. The isotope abundance information provided by the supplier can be verified by measuring the isotope ratios using mass spectrometry.

Two methods for determining the total zinc content are (1) by applying mass corrections to the elemental concentrations obtained using either atomic absorption spectrophotometry (AAS) or inductively coupled plasma atomic emission spectrometry (ICP-AES), or (2) by a reverse isotopic dilution. Both have been described by Patterson et al. [68], and the first method will be further described here.

The AAS or ICP-AES instruments report weight concentrations (mg/mL) based on the atomic weight of the naturally occurring element that is used in the standards for these instruments. However, this is not the average atomic weight of the zinc in the enriched isotope solution. The light absorbance or emission readings of these AAS or ICP-AES instruments, respectively, are proportional to the number of atoms (millimolar content). Therefore, the weight based concentrations reported by the AAS or ICP-AES can be reconverted to mmol concentrations by applying the atomic weight of natural zinc (65.38 mg/mmol) that is associated with the zinc standards used for the analysis. This mmol concentration can then be converted back to weight using the specific atomic weight of the isotopically enriched zinc source, as calculated in Eqs (10) and (11) from the isotope abundance data provided by the supplier.

The calculation below demonstrates a correction of the mg concentration of a highly enriched zinc isotope solution when measured by AAS or ICP.

Using the same isotopic abundance data as the previous example, assume that the concentration of the dosing solution determined by regular AAS analysis is 0.097 mg/mL. This result needs to be corrected by converting to mmol/mL and then back to weight using the specific atomic weight of the isotope enriched product (69.677 mg/mmol from Eq. (11)), as follows:

$$\text{Corrected Zn conc.} = \text{Zn conc. from AAS or ICP} \times \frac{AW_S}{AW_N} \tag{14}$$

where Zn concentrations are expressed in mg/mL and AW is the average atomic weight (g/mol) of natural Zn (AW_N) or the enriched multiple isotope source (AW_S) as indicated in the subscript.

Applying the formula to the above example,

$$\begin{aligned} \text{Corrected Zn conc.} &= 0.097 \text{ mg/mL} \times \frac{69.677 \text{ g/mol}}{65.38 \text{ g/mol}} \\ &= 0.097 \text{ mg/mL} \times 1.0657 \\ &= 0.103 \text{ mg/mL} \end{aligned} \tag{15}$$

For each commercial batch of a highly enriched zinc isotope source, zinc analyses by AAS or ICP-AES expressed in weight units needs to be corrected for the difference in atomic weights between the natural zinc and the enriched source, in the case of this batch by an atomic weight correction factor of 1.0657.

4.3. PREPARING THE ISOTOPE DOSE

Zinc stable isotopes are most commonly purchased in the oxide form, but other forms such as chloride, carbonate salts or metal forms are also available. For oral or IV administration, the stable isotope can be solubilized with a small amount of sulphuric acid, converting it to zinc sulphate. If studying zinc absorption from zinc fortified foods, the oral zinc tracer needs to be in the same chemical form as the fortificant. For instance, zinc chloride has been converted to zinc sulphate or ZnO for such fortification studies [69]. A list of equipment and supplies is included in Appendix I.

4.3.1. Preventing zinc contamination in the laboratory

From preparation and administration of zinc isotopes to their analysis in biological samples, good quantitative results depend on avoiding zinc contamination in the laboratory [2, 70]. Zinc contamination is easily introduced and can ruin results, both by diluting the isotopic composition of a highly enriched tracer, and by inaccurately quantifying the total zinc administered or present in biological samples.

Sources of zinc contamination can include laboratory surfaces, dust or smoke in the air and sweat, fingernails, or saliva from sneezing or coughing. When a laminar flow class 100 clean room is unavailable, zinc contamination can be reduced by working in clean laboratories and as much as possible in a laminar flow hood. Samples held for short periods between analytical processes can be covered with plastic wrap or plastic paraffin film (e.g. Parafilm). Analysts should wear clean laboratory coats and disposable polyethylene gloves without talc or other coatings.

Laboratory supplies can also be a source of zinc contamination, and investigators need to consider all items that come in contact with samples. All chemicals used in the preparation of oral and IV doses and subsequent analyses need to be as pure as possible. A processing system is needed to distil and deionize water and ultrapure, double sub-boiled, quartz distilled acids are necessary and can be procured to avoid contamination with exogenous zinc.

Glassware, especially when used at low pH for extended periods, can be a source of zinc and other trace element contamination through cation exchange, and although glass can be used for some of the heating and volumetric procedures, plastic is preferred for extended storage of sample solutions. New plastic lab supplies can be used without further cleaning if purchased for one time use and pretested to confirm that samples are free of zinc (e.g. soak 2% hydrochloric acid (HCl) in the plastic and analyse for zinc content). Otherwise, all glass, polytetrafluoroethylene (PTFE) and previously used plasticware should be acid washed (soaked for a minimum of 3 hours in 10% standard grade nitric acid (HNO_3) in deionized water) and rinsed thoroughly (3–4 times) in deionized water.

Rubber is high in zinc and rubber sources, such as plunger tips in syringes or stoppers for vials, need to be avoided; siliconized stoppers or plungers can be used. Coloured pipette tips can be a source of zinc contamination. Anticoagulants and lubricants in blood collection tubes need to be pre-screened for zinc. Polypropylene heparinized syringes are advisable for blood collection to minimize zinc contamination. (See the list of equipment and supplies included in Appendix I.)

4.3.2. Sterility, pyrogenicity and fungal testing of IV doses

For IV administration, the solution needs to be filtered for sterility, and aseptically added to vials. Alternatively, filled, sealed vials can be sterilized in an autoclave. Any stable isotope solution for IV administration needs to be certified for sterility, which involves testing a designated fraction of the final prepared doses (currently 10% in the United States of America, following pharmacopoeia compounding standards). Testing and certification can be done by a hospital pharmacy or other certified compounding laboratory. Sterility, pyrogenicity and fungal testing may take several weeks, and can be expensive, so schedules and budgets need to be planned accordingly. Stable isotope doses prepared by a pharmacy often include an expiration date on the vials, and if prepared too far in advance, the sterility, pyrogenicity and fungal testing may no longer be valid. It is important to consider both the local pharmacopoeia and human research ethical review guidelines before initiating the study because the testing, certification, storage and recertification criteria may vary between countries and institutions. This may influence the amount of tracer that will need to be obtained for testing purposes.

4.3.3. Preparing IV stable isotope doses

It is advisable (and may be required by local human research ethical review committees) to work with a hospital or compounding pharmacy for the preparation and storage of the IV doses. Sterile techniques are necessary, including use of a laminar flow hood and 0.2 μm filtration of the solutions. Glass and plastic ware used for the preparation of IV doses is sterilized by autoclaving prior to use.

To prepare an IV infusion, the purchased isotope source is fully solubilized using a small amount of sulphuric acid, then diluted to a concentration of ~0.1–0.5 mg elemental Zn/mL (for comparison, clinical zinc infusions often contain 0.1 mg Zn/mL), and adjusted to pH6.0. For dilution, half-normal saline (commonly clinically labelled as 0.45% NaCl (w/v), which is 0.45 g NaCl/100 mL or 4.5 g NaCl/L) prepared for IV infusion is advised, as its infusion may be less irritating than deionized water. Such commercially prepared saline needs to be pretested to ensure low zinc content. For IV doses, volumes are generally <5 mL for adults, <3 mL for children and <1 mL for infants. The prepared IV zinc isotope doses are analysed for zinc content, which is expressed using appropriate atomic weight corrections specific to the batch of the enriched isotope (see Section 4.2.8). Before use, about 10% of the prepared doses are submitted to a certified laboratory for testing for sterility, pyrogenicity and fungal contamination. Once certified, the remaining doses can be stored securely at

refrigeration temperatures until use or expiration of the testing certification. Such certifications commonly expire after six months (see Appendix II for a detailed sample procedure for IV stable isotope dose preparation).

4.3.4. Preparing oral stable isotope doses

Preparation of oral isotope doses is similar to preparing IV isotope doses, with the following differences:

(a) Dose volume: In addition to choosing an accurately measurable dose, the desired volume will depend on the food to be labelled. Volumes of a few millilitres can be added to high liquid foods such as soup, porridge or beverages, but will need to be minimized to <1 mL for addition to drier foods, such as the inside of a roll or bun. It is a good idea to taste the food, and test food labelling procedures in advance of using elemental zinc.
(b) Diluent: The isotope is diluted with deionized water rather than with 0.45% NaCl saline.
(c) Sanitary procedures: Although it is preferred to pass the prepared solution through a 0.2 μm filter, it is not necessary to use strictly sterile containers, sterile technique or to conduct pyrogenicity, fungal and sterility testing. Sanitary laboratory procedures are followed.
(d) Storage as stock solution versus individual dose solutions: Depending on the scheduling of labelled meals and the facilities to accurately measure the isotope solution where foods will be isotopically labelled and served, investigators may choose to prepare and use the isotope solution in a single stock solution, securely capped, stored at 4°C and clearly labelled with concentrations (and with regular checks of the analytical concentrations). Measuring doses from the stock solution at the time of food labelling may facilitate using different amounts of the isotope label in meals with different zinc content and/or eliminate using extra fluid to quantitatively transfer pre-measured doses.

5. ADMINISTERING ZINC ISOTOPES TO STUDY PARTICIPANTS

This section will consider scheduling and methods for administering IV and oral zinc isotopes in nutrition studies, including discussions about labelling

the zinc naturally found in food, and controlling and measuring zinc in study diets. A list of equipment and supplies is included in Appendix I.

5.1. SCHEDULING ORAL AND IV DOSES TO MEASURE ZINC ABSORPTION

In theory, the dual isotope absorption method compares retention of oral and IV isotope tracers entering endogenous zinc pools simultaneously. Practically, the administration of the IV tracer is commonly scheduled directly after oral stable isotope administration or later in the same day. If the oral dose is divided into multiple meals, it may be ideal for the IV dose infusion to be administered in the same proportions as the oral dose is administered; however, it is generally more feasible to administer the total IV infusion after one of the meals. Investigators may consider scheduling the IV dose after a meal that is midway through the oral dosing or after the meal with the greatest zinc content. Compartmental modelling of zinc absorption kinetics [49] may be used to estimate timing of the IV relative to the oral isotope administration. Although it is common to administer a ~5 mL IV isotope dose during a five minute period, some investigators suggest that, to mimic the gradual plasma appearance of an oral tracer, the IV tracer may be best administered slowly, over a period of 10–30 minutes.

When using three different zinc isotopic tracers to assess zinc absorption from two different dietary treatments, each with a different oral tracer, the third (IV) tracer may be administered after completion of the meals from one treatment and before starting meals for the next treatment [71]. The oral treatments with respective tracers would generally be administered in reverse order for half of the participants, as an experimental control for treatment sequence. A literature review can reveal several different schedules for the timing of oral and IV stable isotope administration to address the needs of specific study designs.

5.2. ADMINISTERING IV ZINC ISOTOPES

Intravenous isotope administration needs to be done by a medical professional qualified to administer IV infusions (an IV nurse or medical doctor). Packaging all ancillary supplies needed for dosing helps standardize methods and facilitates implementation of studies in field settings. Quantitatively accurate IV isotope administration is challenging, but essential for quality measurement results. The prepared IV isotope solution from a single dose storage vial can be quantitatively infused into a peripheral forearm vein with the use of a catheter infusion set attached to a 3-way stopcock. The procedure enables both the

emptied dose vial and syringe to be flushed with sterile saline (0.45% NaCl) that is subsequently infused to ensure that all of the dose has been delivered. Appendix III describes this procedure in detail, as well as an alternative procedure without a 3-way stopcock that requires less administered saline, but has possible disadvantages that may increase error in quantifying the exact dose administered.

5.3. ORAL ISOTOPES: INTRINSIC VERSUS EXTRINSIC LABELLING OF FOODS

Depending on the purpose of the study, oral isotope doses can be incorporated into food intrinsically (during growth of the food), or extrinsically (by addition in the final stages of food preparation). This publication describes primarily extrinsic labelling methods. Extrinsic labelling requires much less time and fewer resources, but validation is necessary to determine if absorption and metabolism of the extrinsic zinc label is similar to that of the zinc naturally present.

5.3.1. Intrinsic labelling

Intrinsic labelling involves incorporating the stable isotope into the plant or animal food during the biological formation of the food so that the form and distribution of the zinc isotope closely resemble that of the natural zinc in the food. For plant foods, the stable isotope can be incorporated using hydroponic culture or stem injection techniques. For animal foods, the stable isotope is administered to the animal orally or parenterally and time is allowed for natural zinc incorporation before the animal product is collected (meat, milk, eggs). The goal of intrinsic labelling is for the stable isotope consumed to be indistinguishable from natural zinc in the way it is presented to the digestive tract (e.g. bound to proteins) so that it is absorbed and metabolized in the same way as naturally occurring zinc. Unfortunately, intrinsic labelling requires relatively large quantities of stable isotopes, and the time and resources required to grow the food will significantly add to the cost of the study [72]. Intrinsic labelling with stable isotopes may not be feasible for all foods of interest because of a low zinc content of the food, or because of a low label recovery in the plant or animal component.

5.3.2. Extrinsic labelling

Extrinsic labelling involves adding the stable isotope to food in the final stages of preparation. It is assumed that the stable isotope (extrinsic label)

exchanges completely with, and is absorbed and metabolized identically to, the naturally occurring zinc. To facilitate exchange of the extrinsically added isotope with the naturally occurring dietary zinc in the labelled foods, the isotope is added to the best source(s) of zinc in the test meal(s). Time (commonly overnight) may be allowed for the isotope to equilibrate with the native zinc present in the food, but the precaution of an extensive equilibration time prior to consumption may be unnecessary, as full exchangeability of zinc from the tracer and from the food in the same chemical form (i.e. common pool) for absorption is unlikely to occur before mixing occurs in the gastrointestinal tract. One study reported no significant difference in zinc absorption whether the zinc label was added (a) directly to bread 16 h prior to consumption, (b) immediately before serving, or (c) into a solution used to make the bread dough [73].

However, extrinsic labelling of only the liquid portion of a meal can be problematic, because of different gastric emptying times between the liquid and solid phases that reduce the opportunity for exchange of the zinc isotope with the native zinc in the meal. Studies with iron isotopes have demonstrated that the addition of the isotope to only the liquid portion of the meal increased isotope absorption relative to mixing the isotope into the solid foods of the meal [74, 75]. A finding that absorption of zinc in the chloride form slightly exceeded absorption of zinc in the oxide form was unfortunately confounded because the ZnO tracer was added directly to porridge, whereas the zinc chloride form was provided in a separate beverage along with the meal [69]. Despite these problems with the labelling of the liquid portion of the meal, some investigators prefer to serve the isotope as a liquid to enable easier quantitative control of the isotope delivery. In this case, investigators need to carefully supervise the meal to ensure that the isotope solution is consumed in small portions during the last half of the meal.

5.3.3. Validity of extrinsic labelling

Intrinsic and extrinsic labelling have been compared using zinc stable isotopes or radioisotopes to measure zinc absorption by humans from a limited number of foods: beef [64], poultry [76–78], milk [79–81] and beans [82]. Most studies found good agreement between absorption measurements using intrinsically and extrinsically administered isotopes of zinc, although both the bean study and one of the three poultry studies [76] reported 10–20% lower absorption with extrinsic, compared to intrinsic, labelling (the results from the two labelling methods were significantly correlated). Many of the above cited studies to validate extrinsic labelling were done decades ago with radioisotopes that are less frequently used now, but had the advantage of using nearly mass-free

quantities of isotopic tracer, without disturbing the amount of natural zinc in the food.

Rather than comparing absorption of single foods that were intrinsically versus extrinsically labelled, Sheng et al. [40] compared zinc absorption from controlled mixed diets using independent stable isotope methods for the intrinsic (naturally occurring) and extrinsic zinc absorption measurements. The extrinsic measurements were conducted using the dual isotope absorption method, comparing the relative biological retention of orally and intravenously administered zinc isotopes. Extrinsic labelling was accomplished by administering the oral zinc isotope as a solution during the last half of each meal for six days; the IV isotope was administered once, midway through this period. The intrinsic measurements relied on the zinc naturally contained in the diets, together with determination of faecal excretion of endogenous zinc, which was measured using another zinc isotopic tracer previously administered intravenously. This allowed for determination of intrinsic zinc absorption as total diet zinc minus unabsorbed zinc, the latter determined by subtracting EFZ from the total faecal zinc, as in Eq. (2). This careful comparison of intrinsic and extrinsic zinc absorption in 21 women, using independent methods, provided evidence for validation of the commonly used extrinsic labelling technique for measuring zinc absorption from composite diets with a variety of foods.

5.4. CONTROLLING ZINC IN STUDY DIETS

Many zinc isotope studies require quantitatively controlled diets with known zinc content. This is true whether controlling intake for just one to two meals to measure food zinc bioavailability, for a full day to determine daily zinc absorption from a particular diet, or for several days to determine endogenous zinc excretion. Working with a local dietician may be helpful to develop test meals or whole diets with menus that both meet the study objectives and optimize compliance by being palatable to the study population and easy to eat completely. Pilot testing of palatability and portion sizes with individuals from the study population, or even as part of informing the study applicants, can help avoid problems at the time the isotope labelled meals are administered.

5.4.1. Diet preparation

Diet preparation should be in a research setting in a kitchen equipped to support sanitary preparation, holding and service of foods with weighed ingredients for each participant in a controlled, reproducible manner. To keep foods as consistent as possible within a study, foods that can be stored should

be purchased in a single commercial lot or batch. Foods with a relatively homogenous composition (e.g. bread, or a well-blended soup or porridge) can be prepared from the same batch of ingredients, divided into individual portions and then frozen. A top-loading digital scale that can easily tare a weigh paper or light container should be used to weigh, with 1% accuracy, all ingredients served to individual participants. Food preparation practices should be modified to avoid all sources of potential zinc contamination. Kitchen workers should wear disposable gloves (Fig. 2). When the goal is to evaluate zinc absorption from food as commonly consumed, it may be advisable to use a water source confirmed to be consistently low in zinc for all dish cleaning, food preparation and cooking. Deionized water should be used, as feasible, when the study goal is to minimize zinc intake. Dishes and cooking utensils should be selected to be low in zinc, and use of plastic, glass or PTFE and minimizing the use of metal utensils is advisable (Fig. 3). Dishes or utensils can be checked for potential zinc contamination by soaking them in dilute HCl that is subsequently analysed for zinc. Although some investigators use plastic cutlery (knives, forks and spoons) exclusively, others have concluded that clean, stainless steel cutlery can be used without substantial zinc contamination.

5.4.2. Analysis of zinc in the test meals or diet

The zinc content of the test meals should be analysed as part of the study planning. This will help ensure that research goals are met and, if zinc absorption is being tested from multiple different meals, will provide information needed

FIG. 2. Preparing study meals; note gloves and PTFE coated pan.

FIG. 3. Preparing study meals; note digital scale and clean plastic or glass containers for food.

to distribute the isotopic label in proportion to the natural zinc content of the meals. Prior to analysis, the foods are completely prepared with the same cooking methods, utensils, and ingredient sources as the meals that will be served to the participants. The water source for ingestion is pre-checked to ensure a relatively constant, low zinc concentration, or deionized water may be used. After the meals have been prepared for ingestion, deionized water, as well as acid washed containers, should be used for all analytical procedures so as not to contribute additional zinc that was not served. If deionized water is not available, a single batch of bottled water can be useful, together with zinc analysis of the water and records of the amounts of water added during sample preparation. Dietary composites are prepared for analysis using a blender with stainless steel blades in an acid washed glass or plastic blender container (Fig. 4). After homogenous blending, an aliquot for analysis is taken immediately, before separation occurs.

It is important to check by chemical analysis that any supplemental zinc added to the meals will provide the amounts intended by the researchers. The zinc content of a meal influences fractional absorption. Unintentional differences in zinc content between meals when comparing bioavailability from different zinc sources can confound the absorption results and make them difficult to interpret. In addition to analyses during study planning, it is best to analyse dietary zinc from samples collected during implementation of the study to

FIG. 4. A fully prepared diet is blended for subsequent zinc analysis.

provide confirmation for the final data analysis and reveal any seasonal changes or other variations from the original analysis.

5.4.3. Dietary controls before or after the isotope labelled meals

Depending on the research protocol, a constant weighed diet may be served for several days before and after isotope administration, especially when the research results are to be associated with a specific diet (e.g. when assessing EFZ). For short term measurement of zinc absorption from labelled meals, providing a standardized meal before and/or after the isotopic tracers are administered can be a relatively simple procedure (perhaps involving pre-packaged commercial foods that can be consumed away from the research centre) that might help reduce measurement variation.

5.5. PROCEDURES FOR ORAL ISOTOPE ADMINISTRATION

5.5.1. Administering the oral isotope by extrinsically labelling food

Labelled meals are carefully planned with foods, containers and preparation or reheating procedures that enable quantitative ingestion of the labelled food, while minimizing risks of splatters or spills. The dilution of the isotope dose

is planned to avoid adding an undesirably large amount of fluid to a solid labelled food.

Foods are often labelled with extrinsic zinc isotopes the day before administration, mainly to avoid longer holding times while facilitating work flow, and potentially to allow better equilibration of zinc isotope with the zinc naturally present in the food. Ideally, labelling is done in a research kitchen with equipment for quantitative measurement, while using sanitary food handling techniques and holding the labelled foods at refrigeration temperatures, as appropriate.

Serving containers for the labelled food should be chosen to facilitate quantitative measure of consumption and to avoid leaks or spills of the stable isotope solution. Dishes or containers with smooth sides can be easily and scraped and rinsed by the participants. If the food is to be heated, it may be advantageous to consider containers that will allow pre-cooking, adding the label, and reheating in a microwave oven without needing to transfer the food.

For isotopic labelling, a precisely measured oral dose can be added to the food using a syringe (with pre- and post-weighing of syringe), or by pipetting with an automatic pipette using a clean, mineral-free pipette tip. The accuracy of the measurements can be checked throughout the procedure using a sensitive digital scale (by weighing the labelled food before and after, or if the weight of the food plus container is too great, by testing the pipetting process separately). The dose can be distributed in the food in multiple locations, such as small indentations in the food, while ensuring that the dose is contained within the food (not on the edges of the dish). Spills do happen, so it is best to be prepared by arranging for the food to be consumed over a tray with sides and absorbent paper to facilitate collection of any spills containing the isotope (any spills can be saved frozen in a plastic bag for subsequent analysis). Serving the food with spatulas and rinse bottles can facilitate complete ingestion of the weighed, labelled meal. The participant can be asked to eat all the food and then to rinse the container that held the labelled food three times with deionized water, which would then also be consumed. As a quality control, dose aliquots of the isotope solution measured at the time of food labelling can be periodically saved for subsequent zinc analysis.

5.5.2. Administering the oral isotope in a beverage with the meal

As discussed in Section 5.3.2 on extrinsic labelling, extra caution is needed if administering the isotope in a liquid served with the meal because differences in stomach emptying between liquids and solids may result in greater fractional absorption than if the isotope had been added to the solid food. If this technique is chosen for the isotope delivery, this potential problem can be minimized by having the participant consume the isotope solution in multiple sips during the last half of the meal. For quality control, this procedure is closely supervised

and documented by the research staff, making the isotope solution available only after half the meal has been eaten, and encouraging small sips of isotope solution between bites of food for the rest of the meal. After the isotope solution has been consumed, the container is rinsed three times with deionized water that is also consumed. Losses can be accounted for by using ashless filter paper to collect any spills containing the isotope (Fig. 5).

FIG. 5. Zinc isotope solution administered during the last half of a meal using a syringe; ashless filter paper can be used to collect any spills containing the isotope, for subsequent analysis.

6. INSTRUMENTATION FOR ZINC STABLE ISOTOPE DETERMINATION

Mass spectrometry is the principal analytical technique used to measure zinc stable isotopes [83]. Inductively coupled plasma mass spectrometry (ICP-MS) [84] is the method most frequently employed for zinc stable isotope studies, but thermal ionization mass spectrometry (TIMS) is also used. Further information and diagrams describing ICP-MS and TIMS are in the IAEA

publication on nutrition studies using iron stable isotopes [85]. Both ICP-MS and TIMS have the following components:

(a) An inlet for sample introduction;
(b) A source for vaporizing the sample and producing focused, charged ions;
(c) A mass analyser that separates the generated ions according to their mass to charge ratio as they pass through a magnetic or electrostatic field;
(d) A detector to quantify the number of ions with a specific mass to charge ratio, representing a specific isotope;
(e) An analyser to process and collect the data generated.

Before introduction into either system, the zinc from the sample is first chemically purified and isolated; for most biological samples, this involves acid digestion and ion exchange chromatography, as is further discussed in Section 7.

6.1. THERMAL IONIZATION MASS SPECTROMETRY

Developed and used in geochemical and nuclear isotope applications, TIMS is known for its high relative precision (<0.01%). With TIMS, the zinc purified from a biological sample is loaded in a multistep process onto an ultrapure metal filament for ionization at a high temperature under vacuum. The positive zinc ions generated are focused through an electric field and then separated according to mass using either a quadrapole mass analyser in older models, or a magnetic sector mass analyser in newer, more precise models (further diagrammed in Ref. [85]). The high precision of TIMS can enable investigators to reduce the isotope doses in human studies. However, TIMS requires extensive sample preparation and this can limit sample throughput to as few as 10–15 samples per day, a rate that may be impractical for some laboratory programmes [37]. The TIMS sampling process can be accelerated by using automated filament loading systems, larger sample carousels and newer vacuum systems to re-establish a vacuum more quickly after changing the sampling carousel. TIMS instrumentation works well for measuring zinc stable isotope ratios. When using two zinc tracers with TIMS, isotopic fractionation corrections can be made very precisely using two other non-administered zinc isotopes. The use of three zinc tracers is possible with either ICP-MS or TIMS. With TIMS, this involves iterative correction for isotopic fractionation using the other two non-administered isotopes. Despite the greater initial cost, operator expertise and sample analysis times, the extremely high accuracy and precision of TIMS, with minimal isobaric and spectral interferences, make it a valuable instrument for nutritional studies that seek to minimize doses of zinc isotopic labels.

6.2. INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY

The ICP-MS with a quadrupole analyser has been the most widely used mass spectrometry technique for isotopic zinc analysis. The quadrupole ICP-MS is lower in cost compared with TIMS (see Table 2), and is generally simpler and easier to use than TIMS with respect to operator training. It enables higher sample throughput, and still yields sufficient precision for many zinc stable isotope applications (relative precision <1.0%) [37, 84]. With ICP-MS, the isolated zinc samples are volatilized and ionized in a high temperature argon plasma produced by electrical discharge.

The resulting ions are separated by mass and charge, commonly using a quadrupole mass analyser. The quadrupole mass analyser, composed of four rods to which a combination of voltages and radiofrequencies are applied, selects and focuses passing ions with a specific mass to charge ratio. Although only one mass to charge ratio value at a time follows a stable trajectory to reach the detector, multiple mass to charge ratio values can be detected by cycling the voltage and frequency settings. ICP-MS can be characterized by spectral interferences, and this can be especially limiting for analysis of some elements, such as calcium or iron, but are not as problematic for zinc, and can be managed with appropriate sample preparation and use of isotope standards. Interferences with zinc isotopes can include isobaric interference with isotopes of other elements that have the same

TABLE 2. APPROXIMATE RELATIVE COSTS OF MASS SPECTROMETRY INSTRUMENTS USED FOR STABLE ISOTOPE STUDIES

Instrument	Approximate cost (US $)
TIMS (sector field)	650 000
ICP-MS:	
Quadrupole	100 000–200 000
Time of flight	175 000
Sector field (double focusing):	
High resolution single-detector	400 000
Multicollector	750 000

mass, as well as polyatomic interferences from species produced instrumentally (with argon, water or air) or during sample preparation (with analytes; see Table 3) [84, 86]. The sample preparation procedures are devised to extract zinc from interfering substances in the biological sample [84], and instrumentation such as ultrasonic nebulizers for sample introduction and dynamic reaction cell and collision cell technologies help to reduce mass interferences.

Since the mid-1990s, high resolution ICP-MS instruments, including single-detector sector field or multicollector sector field instruments, have become available with improved measurement capabilities and performance. These commercially produced ICP-MS instruments separate ions using a magnetic field, and feature very high resolution with claims of analytical precision close to that achieved using TIMS as well as very rapid analysis times (about five minutes per sample) [87].

The multicollector ICP-MS instruments measure several isotopes simultaneously, further improving the relative precision of stable isotope ratio measurements (<0.05%) [88]. The combination of an ICP ion source, a sector field mass spectrometer and a multiple array of Faraday detectors, provides a very powerful technique to determine stable isotope ratios [84]. Compared to the quadrupole ICP-MS, the high resolution magnetic sector instruments

TABLE 3. ZINC STABLE ISOTOPES AND SPECTRAL INTERFERENCES (*based on Ref.* [84])

Isotope	Isobaric interferences	Polyatomic interferences		
		M^{2+}	Argon based	Molecular ions
^{64}Zn	^{64}Ni		$^{40}Ar^{24}Mg^{+}$, $^{36}ArCO^{+}$	$^{32}SO_2^{+}$, $^{27}Al^{37}Cl^{+}$, $^{48}TiO^{+}$, $^{48}CaO^{+}$
^{66}Zn		$^{132}Ba^{2+}$	$^{40}Ar^{26}Mg^{+}$	$^{49}TiOH^{+}$, $^{34}SO_2^{+}$
^{67}Zn		$^{134}Ba^{2+}$		$^{35}ClO_2^{+}$
^{68}Zn		$^{136}Ba^{2+}$, $^{136}Ce^{2+}$	$^{40}Ar^{28}Si^{+}$, $^{40}Ar^{14}N_2^{+}$	$^{36}SO_2^{+}$
^{70}Zn	^{70}Ge	$^{140}Ce^{2+}$	$^{40}ArNO^{+}$, $^{40}Ar^{14}N^{16}N^{+}$	$^{35}Cl_2^{+}$, $^{54}FeO^{+}$

have lower LOD, and much less interference. For instance, the high resolution magnetic sector ICP-MS instruments, unlike the quadrupole ICP-MS, can spectrally separate interfering masses, such as the mass signal of ^{56}Fe and the ArO^+ produced from the argon plasma, enabling analyses of iron stable isotopes. As with the quadrupole ICP-MS, the high resolution magnetic sector ICP-MS instruments still require extensive sample preparation [87]. In the field of zinc research, accessibility to multicollector, high resolution ICP-MS instruments has allowed improved precision for analysing naturally occurring differences in zinc isotopic fractionation in biological samples, including plant, animal and human samples [89–96]. This data can help answer additional questions regarding zinc metabolism in nature.

In summary, both TIMS and ICP-MS instruments have advantages and disadvantages for analysis of zinc stable isotopes in nutrition studies. Greater precision, which is associated with a greater initial cost of both the TIMS and magnetic sector ICP-MS, enables studies using smaller amounts of isotopic labels. Sample preparation and throughput may be simpler for ICP-MS than for TIMS, but greater expertise may be needed to understand interferences and apply appropriate quality control protocols for accurate measurement of isotope ratios by ICP-MS. Correction of data for mass dependent fractionation is important for ICP-MS as well as TIMS. Both types of instruments require extensive operator training and maintenance.

7. COLLECTING AND PREPARING BIOLOGICAL SAMPLES FOR ZINC ISOTOPE RATIO ANALYSIS

7.1. BIOLOGICAL SAMPLES: PLASMA, SERUM, URINE AND FAECAL

Samples are collected in zinc-free polypropylene containers, with care to prevent zinc contamination, including the acid washing of supplies and containers as described in Section 4.3.1. A list of equipment and supplies is included in Appendix I.

Either plasma or serum samples can be used for assessing zinc concentration [2] and isotopic enrichment. For blood plasma collection, a polypropylene heparinized syringe is used to minimize zinc contamination. The heparin needs to be tested for possible zinc contamination and syringes with rubber plungers need to be avoided because rubber is a source of zinc contamination [2]. Alternatively, a winged (butterfly needle) phlebotomy set

with a high gauge (small diameter) needle attached through flexible tubing to a heparinized vacuum tube may be preferred. Compared to a needle attached directly to an evacuated tube, the winged needle allows the phlebotomist greater flexibility to accurately draw blood at a shallow angle.

Blood sampling is scheduled at a consistent time of day after an eight hour fast, as plasma or serum zinc is reduced following a meal. For plasma, drawn blood samples are immediately placed on ice and centrifuged within 30 minutes to separate the plasma and minimize movement of zinc from the blood cells; haemolysis will falsely increase the apparent plasma zinc value. For serum, drawn blood samples are allowed to clot for 30–40 minutes on ice or under refrigeration, and are centrifuged to separate the clot [2]. The plasma or serum samples can be separated into aliquots in labelled polypropylene tubes for storage at −20°C; 2 mL plasma or serum is then designated for the zinc isotope analysis.

For spot urine collections, first morning voids may be preferred to obtain a relatively concentrated sample. The urine can be collected and frozen in a polypropylene container at −20°C without further treatment. Faecal samples can be individually collected and frozen in sturdy, resealable, polyethylene bags.

7.2. SAMPLE STORAGE

Isotopically labelled biological (blood, urine, milk, faeces) and diet samples collected during a zinc stable isotope study can be stored at −20°C or below. After sample collection is complete for some or all of the participants, frozen samples can be transported to the analytical laboratory that will prepare them for stable isotope analysis.

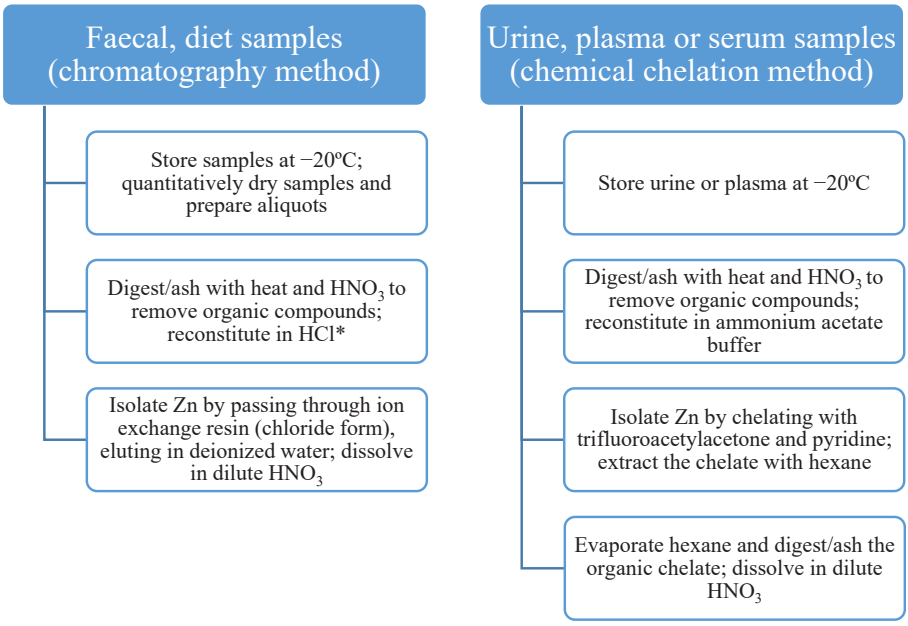
Alternatively, some investigators reduce the bulk of diet or faecal samples that need to be transferred between labs by quantitatively preparing the sample aliquots before transfer. This is done by homogenizing samples with deionized water in a blender and taking several smaller aliquots for freezing and transfer. Careful records need to be kept of original and aliquot weights (including water added and container weights), to enable subsequent determination of the total (daily) amount of zinc and zinc isotope in the original samples (the procedure is further described in Appendix IV).

Data variation can be minimized by grouping each participant's samples from an entire study together for laboratory processing in a single batch, to control for potential differences in reagents used. When working with noxious chemicals or heating with strong acids, the use of a fume hood is essential. Exposure to external metal contamination can be minimized by using a metal-free fume hood when available, and by using plastic wrap or plastic paraffin film (e.g. Parafilm) to cover samples whenever possible (see Section 4.3.1). It is good practice to

include reagent blanks (measuring at least an order of magnitude less than the sample dilutions being analysed) as well as spike blanks in the sample preparation to monitor possible contamination (e.g. put a small amount of the enriched zinc tracer through the sample procedure and measure the amount of zinc introduced using isotope dilution).

7.3. ISOLATION OF ZINC FROM BIOLOGICAL SAMPLES

Biological samples contain organic compounds and other minerals that need to be removed before mass spectrometry analysis for zinc isotopes. Samples can be digested or ashed to remove the organic material, after which zinc is isolated from the other minerals by using ion exchange chromatography and/or chelation. These methods are summarized in Fig. 6 and the text below, and detailed procedures using ICP-MS are included in Appendices IV and V. The zinc isolation method needs to be selected and tested during the planning stages



*Analysis of total elemental zinc in the same samples by AAS or ICP-AES is conducted separately, and may use aliquots resulting from the acid digestion.

FIG. 6. A summary of chemical procedures to prepare biological samples for mass spectrometry analysis of zinc isotope ratios.

of the study in collaboration with the analytical laboratory that will be doing the zinc isolation.

7.3.1. Diet or faecal samples

Prior to the digestion process, representative diet or faecal samples are quantitatively dried by lyophilization in a freeze drier, or by drying to a constant weight at 100°C in a drying oven or muffle furnace. The weighed samples are then digested or ashed with concentrated ultrapure HNO_3 on a hot plate with or without additional heating in a muffle furnace (at not more than 450°C to prevent zinc volatilization), or in a specialized microwave system and then evaporated to dryness, repeating as necessary (especially with high fat or high fibre samples) to form a white ash [97]. Digests are brought into solution, commonly using HCl. After such a digestion, some sample aliquots can be assayed for elemental zinc content by AAS or ICP-AES, and other aliquots of the digest are further prepared for mass spectrometry analysis of isotope ratios by isolating the zinc from other minerals in the digest using ion exchange chromatography [43, 48]. This involves preparation of an anion exchange resin in the chloride form, using concentrated HCl, which binds the zinc ions while eluting other minerals, including copper and iron at an intermediate HCl concentration, and leaving zinc to elute with dilute HCl or water. The procedure is described in detail in Appendix IV. Analysis of total elemental zinc in the same samples by AAS or ICP-AES is conducted separately, and may use aliquots resulting from the acid digestion.

7.3.2. Urine, plasma or serum samples

Because urine and plasma or serum can be relatively low in zinc content, and chlorine can partially interfere with the subsequent isotope analysis by mass spectrometry, a chelation chemistry method has been developed to isolate zinc from the digested sample without adding HCl [44, 98]. Without prior acidification, urine, plasma or serum samples are dried and ashed using heat and HNO_3 (and hydrogen peroxide in some procedures) [98], then dissolved in ammonium acetate buffer, chelated with trifluoroacetylacetone with added pyridine, and extracted into hexane, followed by further digestion with concentrated HNO_3 to destroy the chelate and any other remaining chemicals, and final dissolution in dilute HNO_3 [98]. Compared with methods that introduce chlorine into the sample, this chelation method may improve detection of the zinc isotopes, which could be important, depending on the limitations of the specific mass spectrometer. Details of the procedure are in Appendix V.

Urine samples are also relatively low in organic matter, so some alternative urine sample procedures do not use a complete HNO_3 digestion. One procedure

involves acidifying the urine after collection and before storage at −20°C to prevent microbial growth and mineral precipitation or adhesion to the storage surface (0.4 mL concentrated HCl (trace element grade)/100 mL urine). After thawing, the solids are removed from the urine by centrifugation. Note that the assumption of negligible amounts of zinc in the centrifuged urinary precipitate may need to be tested under specific study conditions.

As a further alternative to chloride-free chemical chelation, a chelating cation exchange resin can be applied to separate some of the divalent transition metals and monovalent cations, followed by zinc isolation using the same chloride form anion exchange resin described above for faecal samples [35, 43]. Especially depending on the final HCl concentrations used, this method may introduce some chlorine into the sample. To help volatilize the chloride ions introduced by such isolation procedures, the sample may be evaporated to dryness and redissolved in dilute HNO_3 prior to mass spectrometry analysis.

7.4. CHEMICAL CONSIDERATIONS FOR MASS SPECTROMETRY

For determination of zinc stable isotope ratios using ICP-MS, all zinc samples are diluted to approximately the same zinc concentration in dilute HNO_3. Reconstituting with dilute HNO_3 [61, 98] avoids additional introduction of chloride, which may hinder isotope ratio analysis (the chlorine dimer $^{35}Cl_2^+$ has a similar mass/charge ratio and can interfere with ICP-MS analysis of ^{70}Zn; see Table 3). The desired zinc concentration depends on the sensitivity of the ICP-MS as well as the sample introduction system. Zinc natural abundance standards, diluted to the same concentration as the enriched samples, are analysed as standards during the ICP-MS runs, usually every 5–10 samples, and can be used to apply corrections for the natural abundance ratios as necessary in the final data analysis. Reagent blanks (dilute HNO_3) are also measured periodically, approximately every 10–12 samples. The mass spectrometer can be set to optimize the count rate of mass 66 (^{66}Zn) in the zinc natural abundance standards, and minimize the count rate in the blank samples.

There is a possibility of mass discrimination using the older quadrupole based ICP-MS instruments for zinc stable isotope ratios, and this bias can change with time in response to variations in instrumental conditions. Roehl et al. [99] used gallium as an isotope ratio internal standard ($^{71}Ga/^{69}Ga$) to correct ICP-MS zinc stable isotope ratio determinations for mass bias drift. Gallium is suited for mass drift correction in the case of zinc because it is a rare element not present in biological samples, its mass is close to that of zinc, its stable isotopes are comparable in abundance and these gallium isotopes do not have isobaric interferences from singly charged ions.

TIMS instrumentation works well for zinc stable isotope ratios because there are enough zinc isotope masses to generate a very precise correction for isotopic fractionation using non-administered zinc isotopes. For any type of mass spectrometry instrumentation, high quality data will require operators with extensive training and experience.

8. CALCULATION OF STABLE ZINC ISOTOPE DATA

Whichever mass spectrometry method is used to measure the zinc masses, the final output is in the form of an isotope molar ratio, and the reference isotope is usually ^{66}Zn. The isotope ratio data need to be converted to mass units for nutrition applications such as determining the amounts of zinc absorbed or endogenously excreted, or for kinetic modelling of the amounts of zinc moving between different biological compartments or exchangeable pools. Some mathematical approaches commonly used in current literature for conversion of isotopic ratios to mass units will be discussed. First to be discussed is the simplified isotope ratio calculation that assumes 100% enrichment of an isotope source and does not account for cross-contamination by other isotopes when more than one incompletely enriched isotope source is administered. The second mathematical approach to be discussed involves more complex calculations that account for cross-contamination when as many as three incompletely enriched sources are administered.

8.1. INITIAL CORRECTIONS OF ISOTOPE RATIOS FOR INTERNAL STANDARDS

As a first step, the isotope ratios from the instrumental measurements can be corrected based on the results from the natural zinc solution that was regularly measured as an internal standard during sample measurements. For each specific isotope ratio determined in the same analytical batch, a correction factor can be calculated by dividing the natural isotope ratio as appears in the literature (these can be calculated from the most recent natural abundance data as appear in Table 1) by the isotope ratio measured for the natural zinc solution. These correction factors can then be applied to all the sample isotope ratios measured at the same time.

8.2. CONVERTING ISOTOPE RATIOS TO ISOTOPE ENRICHMENT: A SIMPLIFIED APPROXIMATION

To determine the amount of the tracer that has been incorporated into a biological sample, the isotope ratio is converted to mass units and expressed as tracer enrichment. A relatively simple mathematical approximation of the isotope enrichment of a sample is obtained from the difference between isotope ratios in the enriched and baseline samples, expressed relative to the baseline isotope ratio [100]. To convert to the mass units needed for the applications in this publication (mg tracer/mg total zinc in the sample), this fractional excess of isotope is multiplied by the natural abundance of the isotope, and can be further corrected for weight differences between the specific isotope and natural zinc. Equation (16) provides an example, using ^{70}Zn as the isotopic label and ^{66}Zn as the reference isotope for the ratio measurements:

$$^{70}\text{Zn enrichment} = \frac{R_{70/66}\ \text{enriched} - R_{70/66}\ \text{baseline}}{R_{70/66}\ \text{baseline}} \times A_N^{70Zn} \times \frac{AM_{70Zn}}{AW_{natZn}} \quad (16)$$

where

^{70}Zn enrichment is the amount of the administered ^{70}Zn isotope from the tracer incorporated into the biological sample, expressed as mg tracer/mg total Zn;
$R_{70/66}$ enriched is the ^{70}Zn:^{66}Zn ratio measured in the enriched sample;
$R_{70/66}$ baseline is the ^{70}Zn:^{66}Zn ratio measured in the baseline sample;
A_N^{70Zn} is the natural abundance of ^{70}Zn = 0.0061 mole fraction (Table 1);
AM_{70Zn} is the atomic mass of ^{70}Zn = 69.925 g/mol (Table 1);

and AW_{natZn} is the atomic weight of natural Zn = 65.38 g/mol (Table 1, footnote a).

Equation (16) can similarly be applied with other isotopic labels such as ^{67}Zn or ^{68}Zn instead of ^{70}Zn, with appropriate substitutions for the respective isotope ratios, natural abundance and atomic mass of the isotope. The equation provides only an approximation of sample isotope enrichment, because it does not account for any of the other zinc isotopes that may be present in a highly enriched isotope material, and it assumes no alteration in the amount of the ^{66}Zn reference isotope in the denominator of the isotope ratio. It is most accurate when applied to the administration of only one highly enriched isotope. Please also note that, because this calculation refers to the sample enrichment of a single isotope, the associated tracer dose is quantified as the amount of the single highly

enriched zinc isotope (e.g. ^{70}Zn), rather than as the amount of all zinc isotopes in the isotopically enriched material.

The enrichment calculation in Eq. (16) becomes less accurate:

(a) As the commercially available enrichment of the isotope product decreases below 100%;
(b) With tracer isotopes that have higher natural abundances;
(c) With increases in the administered isotope dose relative to the zinc mass of the biological sample [100].

Additional potential inaccuracy is added by the use of more than one isotopic tracer in a study, each contributing minor amounts of other zinc isotopes, resulting in cross-contamination.

8.3. CROSS-CONTAMINATION PROBLEMS WHEN ADMINISTERING MORE THAN ONE ENRICHED ISOTOPE SOURCE

Especially when using more than one zinc isotopic tracer, as in the dual isotope method to determine zinc absorption, Eq. (16) provides only an approximation based on the untenable assumption that the isotopically enriched stable zinc source is 100% enriched. Although highly enriched stable isotope products are available, none are 100% pure, as listed in the commercially available abundance data in Table 1. Table 4 provides examples of the isotopic composition of commercially enriched zinc stable isotope products, demonstrating that they

TABLE 4. EXAMPLES OF COMMERCIALLY AVAILABLE ZINC STABLE ISOTOPES AND THEIR ENRICHMENTS (*expressed as % of the total zinc atoms*)

Zinc isotope	^{64}Zn	^{66}Zn	^{67}Zn	^{68}Zn	^{70}Zn
Natural abundance	49.17	27.73	4.04	18.45	0.61
^{67}Zn enriched product	0.74	1.56	94.2	3.46	0.04
^{68}Zn enriched product	0.01	0.08	0.43	99.4	0.09
^{70}Zn enriched product	0.01	0.06	0.05	4.39	95.5

can be highly enriched (94.2% ^{67}Zn, 99.4% ^{68}Zn and 95.5% ^{70}Zn for the three enriched products, respectively), but also contain measurable percentages of the other zinc isotopes.

When enrichments of two different zinc isotopes are used in a single experiment, additional error occurs from cross-contamination. Cross-contamination error occurs, for instance, when the same participant is administered an enriched ^{70}Zn source containing a small amount of ^{68}Zn, as well as an enriched ^{68}Zn source containing a small amount of ^{70}Zn. The error can be reduced by purchasing isotope products with very high enrichment, while relying on the approximate calculation of enrichment in Eq. (16). However, more accurate and complex calculations account for all sources (natural and one to three tracers) of zinc isotopes influencing both the numerator and denominator of the measured isotope ratios [34, 48, 68]. Computers can facilitate the routine use of more complex mathematical calculations that account for cross-contamination, and such calculations are advisable when more than one enriched zinc isotope is administered.

8.4. MATHEMATICAL APPROACHES FOR USING MORE THAN ONE ENRICHED ZINC ISOTOPE

At least two mathematical approaches have been applied that minimize cross-contamination when multiple zinc isotope sources are administered by accounting for all the isotopes in each isotopically enriched source. Although these calculation methods are complex, they can be incorporated into computer spreadsheets for routine use. Both methods are presented here to compare the previously published tracer to tracee ratio (TTR) method, designed for compartmental kinetic analysis using two zinc tracers, to the enrichment ratio method, derived in Appendix VI to quantify up to three zinc tracers.

8.4.1. The tracer to tracee ratio calculation

The tracer to tracee ratio method was first applied to the analysis of zinc stable isotope studies in humans by Lowe et al. [48]. The method as applied for the administration of two zinc stable isotope tracers is mathematically derived in an appendix to that paper [48], and the concept is more generally explained for isotopic tracers elsewhere [32, 101].

It is important to note that both the calculation methods that account for all the isotopes in an isotopically enriched source define the term ‘tracer’ not as a single zinc isotope, but as the mixture of all the isotopes administered from an isotopically enriched source (in this case, all five stable isotopes of zinc).

The 'tracee' is defined as the mixture of all the isotopes naturally present in the sample, without any tracer. Accordingly, when using the TTR approach, the dose is the total mass of zinc (all zinc isotopes) in the administered isotope preparation rather than the amount of the single enriched zinc isotope (i.e. ^{70}Zn or ^{67}Zn). For example, if a dose prepared from an incompletely enriched ^{70}Zn product (88.5% of atoms) contains 0.444 mg of ^{70}Zn and a total zinc content of 0.500 mg when including all zinc isotopes, the administered dose would be 0.444 mg ^{70}Zn when using the simplified isotope enrichment calculation method described in Section 8.2 above, but 0.500 mg when using the TTR calculation method.

For the TTR calculation method [48], the isotopic ratios of the tracer(s), tracee (baseline or natural abundance) and enriched sample are used to calculate the TTR, defined for zinc as:

$$\text{TTR} = \frac{\text{amount of Zn derived from tracer}}{\text{amount of Zn derived from tracee}} \tag{17}$$

The TTR is an expression of the isotopic labelling in the biological sample that minimizes cross-contamination error by accounting for all zinc isotopes in the tracer. This calculation method was developed so that the TTR data (expressed in molar units) can be directly applied in established kinetic compartmental models that were originally developed using radiotracer data [47, 102].

For other nutritional applications, such as determining EFZ excretion or exchangeable plasma zinc pools, the calculated TTR is converted from molar to weight ratios using the average atomic weights of the enriched isotopic tracer(s) and the natural zinc tracee [48]. However, because the denominator of the TTR is the tracee, rather than the total zinc in a sample including the administered tracer(s), further calculation is needed to determine the mass (mg) of zinc tracer in the biological sample [48]. The following equation uses the TTR to calculate the amount of tracer in a sample that originated from one of two administered isotope sources [48].

Calculation of the tracer mass in the sample from TTR is as follows:

$$\text{Zn derived from tracer}_a = \text{TTR}_a \times \frac{\text{total Zn in sample}}{1 + \text{TTR}_a + \text{TRR}_b} \tag{18}$$

where

Zn derived from tracer and total Zn in sample are in mg;
the subscripts *a* or *b* represent two different tracer sources administered (e.g. the oral and IV tracers);
total Zn in the sample is measured separately (e.g. by AAS or ICP-AES);

and the TTRs have already been converted from molar to weight ratios.

8.4.2. The enrichment ratio calculation

An alternative complex calculation converts isotope ratios to an enrichment ratio, which is defined as all zinc in the sample from an isotopically enriched source divided by the total amount of zinc in the sample, as represented by the equation below.

$$\begin{aligned}&\text{Enrichment ratio}\\&= \frac{\text{all sample Zn derived from an isotopically enriched source}}{\text{total amount of Zn in the sample}}\\&= \frac{\text{tracer in sample}}{\text{tracee + all adminstered tracers in sample}}\end{aligned} \tag{19}$$

where the amounts of zinc are expressed in mg.

Similar to the TTR method, this enrichment ratio method minimizes cross-contamination error by accounting for all zinc isotopes in the tracer, and considers the administered dose to be the total amount of all zinc isotopes in the enriched isotope source that is administered. However, because the ratio uses as a denominator the total zinc in the sample, and because it is expressed in weight units, the results can be more directly applied in other nutrition calculations. The tracer mass (mg) in the sample can be directly determined by multiplying the enrichment ratio by the total sample zinc measured by AAS or ICP-AES. If desired, the enrichment ratio can be adjusted to the TTR format before application to kinetic compartmental modelling, or it can be applied directly without adjustment to such modelling [49]. Although some investigators prefer the TTR units for such modelling, that discussion is beyond the scope of this publication. As described in Appendix VI, this calculation method has been derived for administration of up to three different enriched zinc isotope sources, and calculation software can be accessed on the Internet.

8.4.3. Comparing the tracer to tracee and enrichment ratio calculations

These two complex calculations produce similar results for determinations of FZA and EFZ excretion. For EFZ, the TTR data needs to be converted to express the amount of tracer in relation to the total sample zinc as described in Eq. (18), essentially deriving the same information as the enrichment ratio. Without such a conversion, the two calculation methods give similar, but not

identical results for the size of the EZP. At the low isotopic enrichment levels that are commonly employed, the resulting difference is about 1%.

Conversion between the two methods is described by Eq. (20), which is derived from Eqs (18) and (19), after all component ratios have been expressed as weight in mg/mg.

Conversion between the tracer to tracee ratio and enrichment ratio is as follows:

$$\text{Enrichment ratio}_a = \frac{\text{TTR}_a}{1 + \text{TTR}_a + \text{TRR}_b} \tag{20}$$

where the subscripts *a* or *b* represent two different tracer sources administered and the TTRs are expressed in weight ratios.

For the applications in this publication (determinations of absorption, endogenous faecal zinc excretion and EZP), the enrichment ratio calculation can be directly applied, or the TTR calculation can be less directly applied to express tracer enrichment in mg tracer per mg total zinc. These units are employed in the examples for the next section.

8.4.4. Applying calculations to nutrition studies, spills corrections, and LOD and LOQ determinations

Both of the above methods that account for all isotopes in a highly enriched isotope source can be routinely applied with computer spreadsheet programs. Each program requires data entry for:

(a) The abundance of all zinc isotopes in each highly enriched isotope source (specific for each purchased batch);
(b) The measured sample isotope ratios corresponding to the highly enriched isotope sources administered (e.g. $^{67}Zn/^{66}Zn$, $^{68}Zn/^{66}Zn$ and $^{70}Zn/^{66}Zn$, where ^{66}Zn is used as the reference isotope for highly enriched ^{67}Zn, ^{68}Zn and ^{70}Zn, respectively).

The program calculates data that directly (for enrichment ratio calculation) or with further modification (for TTR calculation) yield the isotope tracer enrichments, in mg tracer per mg total sample zinc. Essentially, given the abundance pattern of zinc isotopes in each tracer and in nature (e.g. zinc in the unenriched sample), the calculation determines the amount of each tracer relative to the total sample zinc. These data can then be applied to the equations for determining zinc absorption, exchangeable pool size, and endogenous zinc excretion in faeces.

Table 5 indicates data entered for isotope abundance and the measured sample isotope ratios as described in (a) and (b) above, as well as the resulting calculated per cent enrichments. In this example, only two enriched tracers (^{67}Zn and ^{70}Zn) were administered, as would occur in the dual isotope zinc absorption method.

The measured isotope ratios all use ^{66}Zn as the reference (denominator) isotope. The units for per cent enrichment are mg tracer/100 mg total zinc. In Table 5, only ^{67}Zn and ^{70}Zn enriched tracers were administered, and the abundance values for the ^{68}Zn tracer have been included as place holders for the calculation. See Appendix VI for details of the calculation, and on-line reference to the spreadsheet.

As an additional handy application, the same calculation can similarly determine the amount of each tracer contained in any unintentional spills that may have occurred during tracer administration to a specific participant. Any spills collected from administration of the oral and/or IV tracers for that participant can be combined for a single chemical analysis, and again, from the abundance pattern of zinc isotopes in each tracer and in nature, the program calculates the mg of each tracer per mg total zinc in the sample from the (combined) spills. Total zinc is analysed separately from the same sample digestate produced for the isotope analysis. The mg of tracer spilled can then be subtracted from the originally intended tracer dose to correct the dose amount.

As indicated in Section 4.2.4, evaluation of the precision of the isotopic analysis involves determination and comparison to the LOD and LOQ for the specific sample type, chemical procedure, mass spectrometer and calculation procedure. To determine the LOD and LOQ using this calculation procedure, an unenriched biological sample (e.g. baseline urine) is analysed multiple times ($n \geq 30$) and the isotope ratios for each analysis are entered into the spreadsheet

TABLE 5. EXAMPLE CONVERSION OF SAMPLE ISOTOPE RATIOS TO PER CENT ENRICHMENT

Enriched tracer	Abundance (mole fraction)					Ratio	% Enrichment (mg tracer/ 100 mg Zn)
	^{64}Zn	^{66}Zn	^{67}Zn	^{68}Zn	^{70}Zn		
^{67}Zn	0.0253	0.0145	0.9500	0.00977	0.0003	0.151267	0.165883
^{68}Zn	0	0	0	1	0	0.665849	0.000933
^{70}Zn	0.0013	0.0009	0.0004	0.0418	0.9556	0.032929	0.337184

program to determine sample enrichment. Since the sample is not enriched, the mean enrichment is expected to be close to zero, and the standard deviation can be multiplied by 3 or 10 to estimate the LOD or LOQ, respectively. These LOD or LOQ values (calculated for ^{67}Zn, ^{68}Zn and ^{70}Zn enriched tracers) are then compared to the respective sample enrichment results to ensure that the tracer dose administered was adequate for accurate detection and quantitation under the specific study conditions.

8.5. CHOOSING AND CHECKING ISOTOPE CALCULATION METHODS

Multiple methods have been developed and applied to calculate the results from zinc stable isotope studies in humans. Care should be taken to use the appropriate units and definitions for the specific calculation method. As recommended by Patterson and Veillon [68], calculation methods should be tested using theoretical experimental conditions to calculate expected isotope ratios in the samples and validate by back-calculating to show that the original theoretical isotope dose can be accurately determined. When administering more than one enriched zinc isotope, it is preferable to use one of the more complex computerized calculations that correct for cross-contamination by accounting for all the zinc isotopes in an enriched isotope source.

9. STUDY PROTOCOL IMPLEMENTATION

This section provides examples of protocol and participant scheduling, as well as final data calculations.

9.1. EXAMPLE: ZINC ABSORPTION (DUAL ISOTOPE METHOD)

This example outlines the protocol and data analysis for a single determination of fractional absorption using the dual isotope method.

9.1.1. Sample protocol for zinc absorption

The timeline for the protocol for a single determination of fractional zinc absorption using the dual isotope method is summarized in Fig. 7.

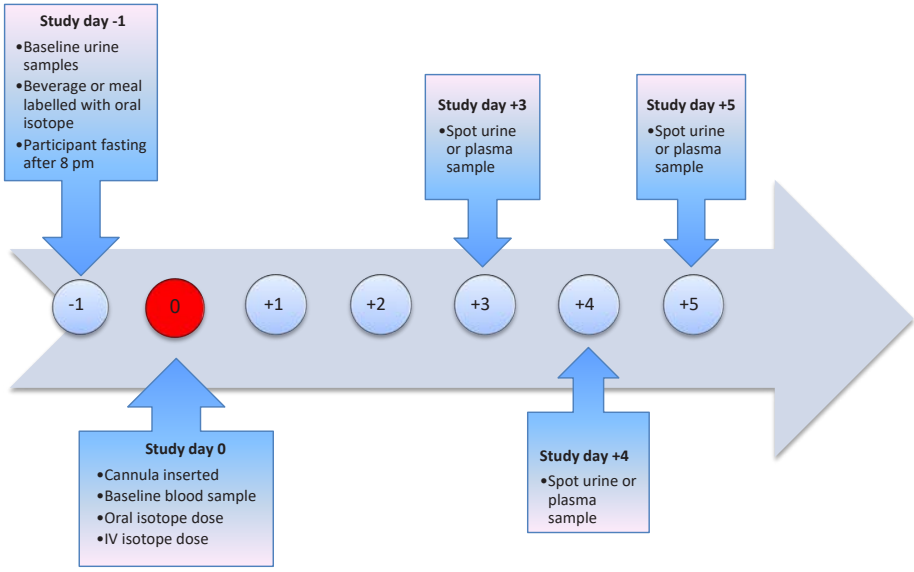


FIG. 7. Study timeline for the measurement of zinc absorption.

9.1.1.1. Study day –1

Preparations are made for stable isotope administration, including the meal(s) in which the isotope will be consumed. If urine samples are to be used for the determination of FZA, a baseline sample is collected from the participant. This enables the participant to practice sample collection and the urine can be used as a baseline control during analyses. Participants are asked to fast and consume only water from 8 p.m. of the evening before and until the isotope administration the next morning.

9.1.1.2. Study day 0

Fasting blood (4 mL) is collected for baseline measurements, including plasma or serum zinc and zinc isotopes. Depending on the specific research protocol, additional blood for other possible indicators of zinc, iron, protein or inflammatory status (e.g. haemoglobin, ferritin and C-reactive protein) may also be collected. The sample tube is placed immediately onto ice and centrifuged within 30 minutes to separate the plasma, or after 30–40 minutes to separate serum. The participant consumes the test meal(s) with the oral isotope dose quantitatively administered, as described in Section 5.5. If any of the isotope dose is spilled, it is blotted with ashless filter paper and saved in a labelled, sealed

plastic bag for subsequent analysis. The IV stable isotope dose is quantitatively administered by suitably trained personnel, as described in Section 5.2.

9.1.1.3. Study days 3–5

Participants are provided with trace mineral-free plastic containers to collect at least the first morning void on each of days 3, 4 and 5 after isotope administration. As a backup for the analyses, and especially with children (who may be more likely to have contaminated samples), it is useful to schedule urine collection morning and evening on these days. The morning samples can be analysed initially, and the evening samples held for additional use only if needed. The volume of urine required is 10–20 mL of each sample. Alternatively, a blood sample (4 mL) can be taken daily on days 3, 4 and 5.

9.1.2. Calculation of zinc absorption

Typical results are shown in Fig. 8 for the testing of zinc absorption by a female, measured from three identical vegetarian meals. The meals were served after an overnight fast at breakfast, mid-day and early evening, and contained a total of 10 mg of zinc from foods labelled with a total of 1 mg of enriched ^{67}Zn

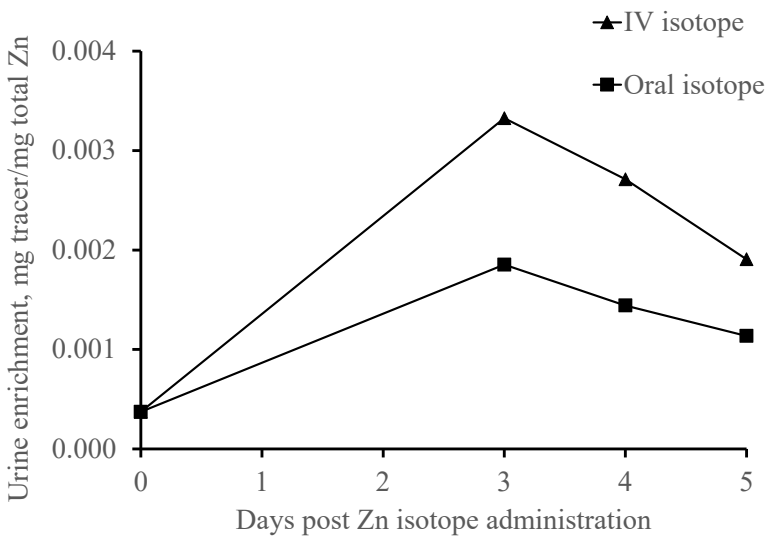


FIG. 8. Sample plot of urinary enrichment with zinc tracer versus time following oral and IV stable isotope administration.

stable isotope source (note that labelling three meals rather than just one reduces the contribution of the oral isotope tracer to about 9% rather than 23% of the total zinc ingested). At the end of the first meal, a single dose of 0.5 mg ^{70}Zn was administered intravenously. Initial morning urine samples were collected on days 3, 4 and 5 after the labelled meals.

By applying Eq. (3) to the resulting data, FZA can be calculated from each of the three days of urine samples. From the example results in Table 6, the average FZA for these data is 0.281, or 28.1% absorption, with a coefficient variation of 5.7% for these samples taken on days 3, 4 and 5 for a single zinc absorption measurement from a single participant and set of labelled meals. As the relative amounts of the oral and IV tracer remain quite stable by day 3 after the dosing [43], studies could be planned with an increased number of urine samples (e.g. samples twice daily or for an extended number of days) as desired to further reduce the measurement variability.

Applying the assumption that the fractional absorption is similar for both oral tracer and the labelled foods (i.e. that the extrinsic labelling was valid), the total amount of zinc absorbed from the three meals can be calculated by multiplying the total zinc ingested by the fractional absorption as described in Eq. (4). That is, multiplying the 11 mg ingested (10 mg from food plus 1 mg from the label) by 0.281 provides the result that 3.09 mg zinc was absorbed from the three test meals by this participant.

TABLE 6. EXAMPLE RESULTS FROM CALCULATION OF FRACTIONAL ZINC ABSORPTION (*based on urinary tracer enrichment data depicted in Fig. 8*)

Day	Oral enrichment (mg/mg total)	IV enrichment (mg/mg total)	Oral tracer dose (mg)	IV tracer dose (mg)	FZA
3	0.0019	0.0033	1	0.5	0.279
4	0.0014	0.0027	1	0.5	0.266
5	0.0011	0.0019	1	0.5	0.298

9.2. EXAMPLE: EXCHANGEABLE ZINC POOL (SIMPLER METHOD)

This example outlines the protocol and data analysis for a single determination of the EZP, which could be scheduled both before and after an

intervention such as zinc supplementation. The simpler EZP method requires the administration of an IV stable isotope of zinc, and subsequent measurement of stable isotope enrichment in the plasma, serum or urine between 3–9 days after the dose. The EZP sampling protocol can be combined with the protocol to measure zinc absorption, permitting the determination of both fractional absorption and EZP from the same set of samples. During the week leading up to stable isotope administration and during the sampling period, most protocols ensure that participants are consuming a standard diet of constant zinc intake to ensure a relatively steady state and known dietary intake during the collection period.

9.2.1. Sample protocol for exchangeable zinc pool measurement

An example of a study protocol is illustrated in Fig. 9.

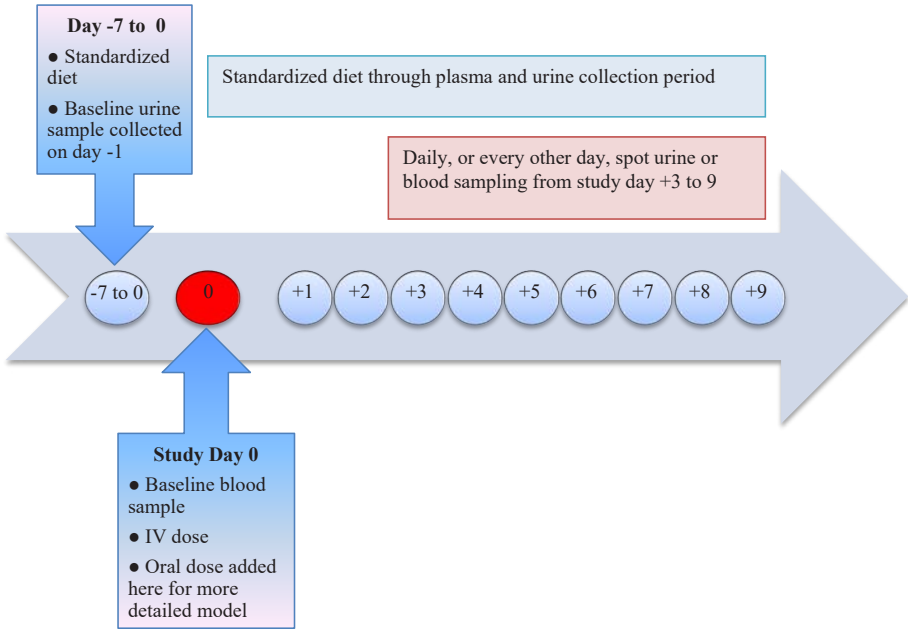
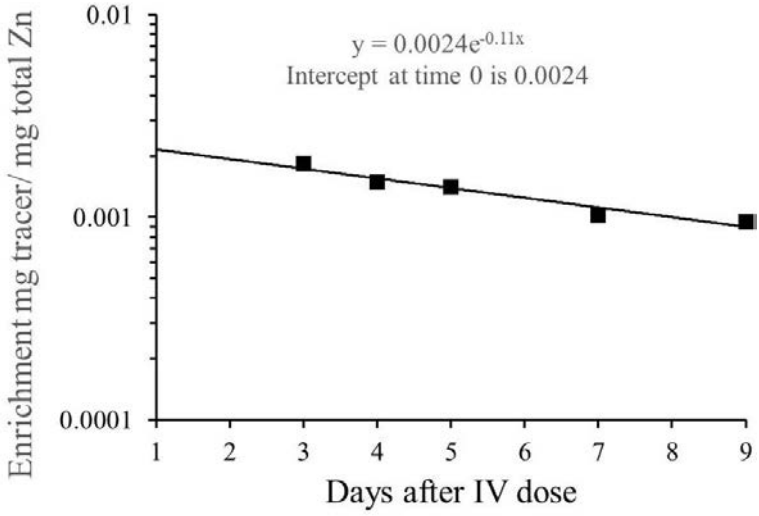


FIG. 9. Study timeline for the measurement of EZP.

9.2.2. Calculation of exchangeable zinc pool

Plasma, serum or urine samples are processed and analysed and isotope enrichment is calculated as indicated in earlier sections, and the data is used to determine the size of the pool into which the stable isotope has been distributed. For days 3–9 after the dose, sample enrichment decreases in an exponential fashion, appearing straight when plotted as an exponential trend line on a semi-logarithmic plot (Fig. 10). Alternatively, the data can be plotted as a linear trend line between ln (sample enrichment) versus time. By extrapolating this line back to t=0, the sample enrichment can be estimated before any of the label was lost from the EZP. The EZP equals the dose of the IV tracer (mg) divided by the y-intercept value for t=0.

The result indicates an EZP size of 213 mg, which can be further expressed in relation to body weight or lean body mass and analysed for relationships to other zinc and health variables.



y intercept = 0.0024
Dose IV Zn isotope = 0.511 mg
EZP = IV dose / y intercept
= 0.511 / 0.0024 = 213 mg Zn

FIG. 10. Sample IV tracer enrichment plot and EZP calculation.

9.3. EXAMPLE: ENDOGENOUS FAECAL ZINC

9.3.1. Sample protocol for endogenous faecal zinc

This example outlines a protocol and data analysis for a single determination of EFZ, which could be scheduled both before and after an intervention. The timeline in Fig. 11 illustrates a sampling strategy for one measurement.

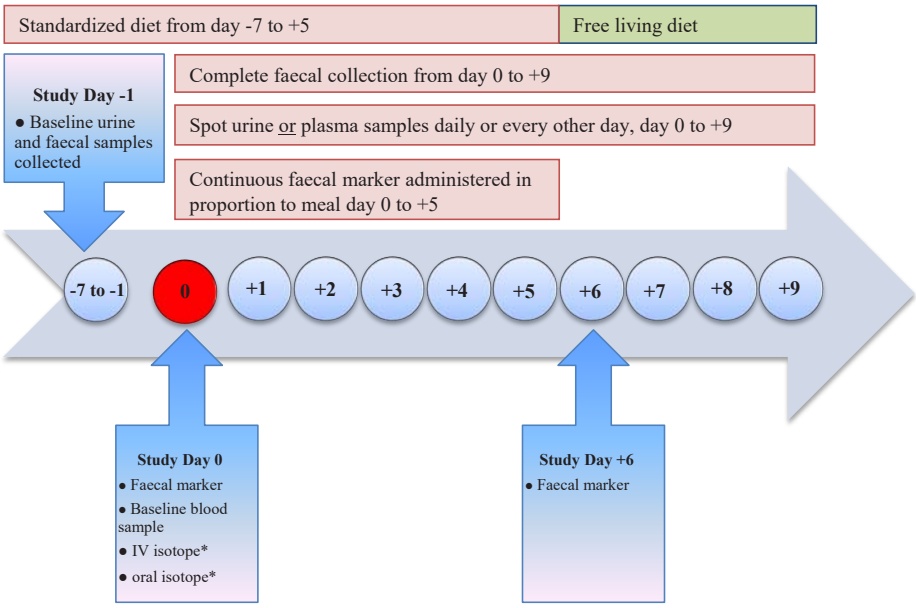
Provision of a standardized diet for 1 week prior to stable isotope administration allows time for equilibration. The controlled diet or balance period for EFZ determination is scheduled for six days in this sample protocol, allowing time for collection of several labelled faecal samples to quantify total faecal zinc and zinc isotope enrichment, as well as the endogenous isotope enrichment of plasma, serum or urine corresponding to the time of endogenous zinc secretion into the intestine. Faecal samples are collected for this period plus an additional four days, allowing for a delay in excretion of all faecal samples associated with the six day controlled diet period, which can be identified using a non-absorbable faecal marker, such as brilliant blue dye. The use of an additional non-absorbable faecal marker, such as dysprosium, administered with meals in proportion to zinc content, will allow further quantitative correction for incomplete faecal samples.

Investigators should confirm with the participants that a complete faecal collection has been conducted, and no faecal samples are missing. Although EFZ can be determined with collection of at least three adequately marked spot faecal samples [61], a participant's data may need to be excluded if samples are incomplete or the faecal markers are not sufficiently excreted in the time allowed for collection.

As noted in the footnote to Fig. 11, the oral isotope dose is included only if FZA is being measured concurrently. If only EFZ is measured, it may be preferred to schedule the IV isotope administration 4–7 days prior to beginning the faecal collection period to further ensure stabilization of the label in the endogenous zinc pool before faecal collections begin. However, if both FZA and EFZ are measured from the same IV isotope administration, both the IV and oral isotopes are administered at similar times (day 0 in this example), and measurement of the urine or blood sample enrichment begins no sooner than day 3.

9.3.2. Calculation of endogenous zinc excretion in faeces

The faecal and urine, plasma or serum samples are prepared and analysed and isotope ratios are converted to isotope enrichment as already described. EFZ is calculated by applying the sample data to Eq. (5). When applying this equation, the faecal data correspond to the controlled diet or balance period. If only non-quantitative faecal markers are administered at the beginning and end



*The oral isotope dose is included only if FZA is being measured concurrently.

FIG. 11. Study timeline for the measurement of EFZ.

of the period, the data can be summarized for all samples excreted between these markers, representing six days between administrations of the markers in this example. The chemical analyses can be performed on individual samples or a pooled sample that is prepared by combining constant fractions of individual faecal samples.

In calculating EFZ using Eq. (5), the IV isotope enrichment in urine, plasma or serum corresponds to the time that the EFZ was secreted into the intestine. This can be determined from a blood sample scheduled midway through the balance period (the beginning of day 3 for the six day balance in this example). Alternatively, spot urine samples can be used for (exponential) modelling of the logarithm of zinc isotope enrichment versus time, followed by calculation of the enrichment midway through the balance period. Using the example data from Fig. 12, the enrichment at day 3 would be 0.0017 mg tracer per mg total Zn. Such modelling can also be used to estimate enrichment of urine, plasma or serum at other specific time points to correspond with single or daily faecal samples, after adjustment for faecal transit time.

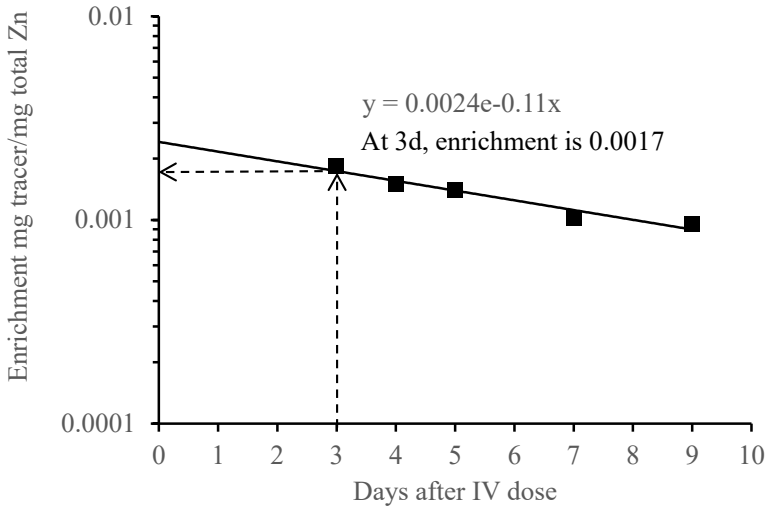


FIG. 12. Estimating endogenous zinc enrichment with isotope at a specific time point using urine, plasma or serum data.

Applying data on the faecal and urinary enrichment and the total faecal zinc to Eq. (5) for a six day balance period:

$$\mathrm{EFZ} = \frac{(0.000344 \text{ mg tracer/mg total Zn})(6.43 \text{ mg total Zn})}{(0.0017 \text{ mg tracer/mg total Zn})(6 \text{ d})} \tag{21}$$

$$= 2.16 \text{ mg/d}$$

Such data can be further applied to associations with zinc intake or dietary bioavailability, and have been used to estimate human zinc requirements, as discussed earlier.

10. ADDITIONAL CONSIDERATIONS FOR STUDY DESIGN AND PLANNING

A human study using zinc stable isotope tracers requires extensive and careful planning. The study design and application of stable isotope techniques will vary depending on the nature of the research question. Data obtained with stable isotopes before and after interventions can provide information on the

effectiveness of programmes such as zinc supplementation, zinc fortification, or dietary modifications such as reducing dietary phytate. Studies can determine zinc absorption from various foods or zinc absorption differences among population groups (young, elderly, pregnant, lactating). Study results can inform public health officials and policy makers responsible for determining appropriate amounts and forms of zinc for supplementation and fortification, and for making dietary recommendations.

The specific research questions will influence whether one, two or three stable isotopes are administered, whether they are given orally or intravenously, whether foods are intrinsically or extrinsically labelled, and the time frame for scheduling isotope administration and subsequent sampling.

10.1. RESEARCH COLLABORATIONS

Contact potential collaborating researchers at an early stage. Many international studies have two groups of collaborators with one providing access to the population to be investigated and with facilities appropriate for accurately preparing and serving controlled meals and collecting the biological samples, and the other providing experience and mass spectrometry equipment for conducting zinc stable isotope studies in humans. Determination of the mass spectrometry laboratory that will be analysing the samples for zinc stable isotope ratios will impact the planning of the doses given and the collection and handling of samples, as well as the data analysis. Staff training to conduct trace element work is an important part of the planning stage. It is best for collaborators to agree in advance on the division of work, including how biological samples will be prepared before transfer between the laboratories. Advance planning includes discussion of the procedures for international transfer of biological samples (diets, blood, urine and/or faeces), which may require ethical and/or government approvals at both sites, and special precautions for transportation of biohazard samples or samples shipped with dry ice. Preparation of a data analysis plan in consultation with a statistician is an important part of the plan, including agreements about the roles of collaborators in the reporting of results. A possible timeline for study planning is presented in Fig. 13, and additional planning considerations are discussed below.

10.2. SITE SELECTION

In addition to the research question being asked, the design of the study will be influenced by practical consideration of the available methodology, the access

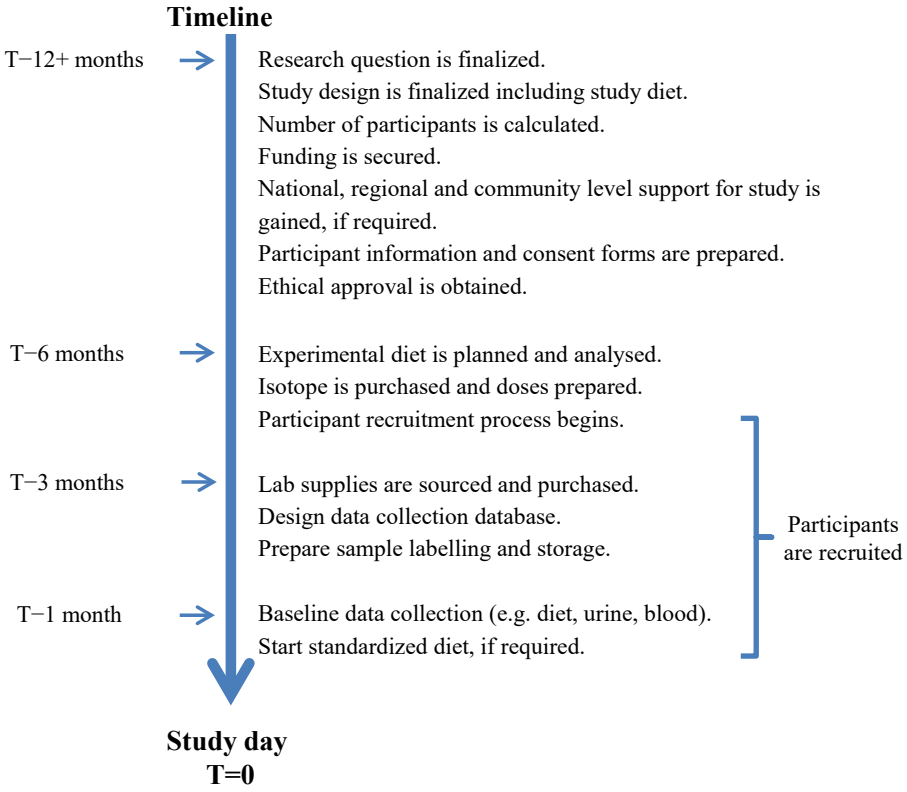


FIG. 13. Sample timeline for stable isotope study planning.

to community and research facilities, the difficulty for participants to comply with study procedures and logistical management of time, people and resources. Depending on geographic locations, study planning may need to consider the influences of religious and/or cultural practices, political situations and seasonal differences in food availability that may affect study logistics.

Participant testing and sample collection in zinc stable isotope studies generally requires facilities that can support quantitative administration of isotopes and of weighed, controlled meals or diets, as well as appropriate collection and short term storage of biological samples. Studies involving complete faecal and urine collections for several days are best conducted in a clinical metabolic unit facility where participants can reside and receive careful training and close monitoring of specimen collection procedures. Such complete sample collection is more challenging and less certain, but not impossible in a community setting or a participant's home if participants and staff are well trained and closely

supervised. For designs involving fewer controlled meals and only urine or blood sampling, it may work best to study 'free living' participants, who reside in their homes and come to the study site as scheduled for research procedures and sample collections. Protocols that require multiple blood sampling over a short period of time on one day may be best undertaken with participants attending a day clinic with medical staff on hand to insert IV catheters and maintain blood flow through the IV catheter for accurately timed sampling.

10.3. SAMPLE SIZE DETERMINATIONS AND STATISTICAL ANALYSIS PLANNING

Plans for the number of participants and the data analysis can benefit from consultation with a statistician. For small human studies, statistical power can often be improved by repeating observations (more than one experimental treatment, or pre- and post-intervention assessments) in the same individuals to account for interindividual variation. The sample size should be determined based on the statistical power desired and the minimal detectable difference that is considered biologically meaningful to the research question. As in any power analysis, existing relevant data from similar studies is used to estimate the mean and standard deviations expected for the outcome measurements.

Multiple resources are available to assist with sample size determinations, minimal detectable differences and power calculations.[1] Once calculated for statistical power, it is advisable to increase the estimated sample size to allow for potential dropouts and possible loss of data due to field or laboratory errors. Anticipating the final statistical analyses of the data is also helpful in study planning.

10.4. SELECTION OF PARTICIPANTS

It is important to define the study population with respect to age, sex, socioeconomic status, and physiological status. Because of the expense and intensity of isotope studies, typically the study population will be a subset of a population targeted for larger scale interventions, and thus is likely to be as representative of the target population as possible.

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[1] https://homepage.divms.uiowa.edu/~rlenth/Power/index.html
http://hedwig.mgh.harvard.edu/sample_size/size.html
http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize
http://statpages.info

The current state of health and medical history of potential study participants should be ascertained, under the guidance of a physician. Participant screening may include obtaining a blood sample to determine basic clinical chemistry and haematology parameters. Testing for iron status may be important, if the study involves extensive blood sampling. Screening for common illnesses that may affect the study results is important. For example, children and adults in low income countries are often treated for helminth infections prior to the beginning of a study.

Applicants for the study (including their affected parents or caregivers) need to be willing and able to comply with the experimental diet and other study conditions, including the demands of the sample collections. Exclusion criteria will include conditions or medications that may alter zinc homeostasis, unless such conditions are relevant to the question under investigation. The use of dietary supplements also should be considered, and, if possible, discontinued for at least one month prior to the beginning of the study protocol.

10.5. SAFETY, ETHICAL CONSIDERATIONS AND INSTITUTIONAL APPROVAL OF HUMAN RESEARCH

Good research governance requires that, before implementation, all research in humans should first be approved by human research ethical review boards at local as well as collaborating institutions. All procedures used in the design, implementation and consenting process should follow existing ethical and best practice guidelines (see Refs [103–105] for examples of human research guidelines).

In keeping with these guidelines, research participants should have a clear understanding of the study design, procedures that will be followed, amount of time the study will require of them, what benefits and/or risks might be associated with the research and whom to contact if they have any questions. They should not feel coerced into participating and should understand that they are free to withdraw from the study at any time without any adverse consequences. All of the participant data collected are confidential and plans should be in place to protect the confidentiality of all identifying information.

For zinc stable isotope studies, it is important that the protocol submitted for approval explains what a zinc stable isotope is and justifies why it is used. The administration of oral and IV zinc isotopes and sample collection should be clearly described, anticipating any special concerns of the population to be

studied. The protocol should indicate that there are no known risks associated with zinc stable isotopes, administered orally or intravenously, while also indicating the discomfort and possible risk of infection that may occur from the blood collections and infusions (see below). If the review committee has never been involved in review of other stable isotope research, it may take longer to provide the committees with the necessary information for them to fully evaluate the protocol. As such, it is advisable to begin this process or discuss this with the appropriate contact from the research ethical review committee during the planning stages to facilitate implementation of the research and minimize the time required for the approval process.

In discussing the safety of administering zinc stable isotopes with human research review committees, the following points may be helpful. Stable isotopes are not radioactive and are present naturally in the environment, including in foods and the human body. Stable isotopes of many elements (carbon, oxygen, hydrogen, nitrogen, iron, zinc) have been used extensively in biomedical and nutritional research. Enriched stable isotopes of zinc contain the same kinds of atoms, but in different proportions, as natural zinc. There are no concerns for safety with ingestion or infusion of enriched zinc stable isotopes in the amounts discussed in this publication. As can be calculated from the natural abundance of the isotopes, typical doses of highly enriched ^{67}Zn (3 mg) or ^{70}Zn (0.5 mg) employed with these stable isotope measurements are commonly consumed within ten days by adult women ingesting zinc in recommended amounts (8 mg/d) from ordinary foods. The primary safety concern in these stable isotope methods is related to the IV infusion procedure, as there may be pain with the needle stick, pain with the infusion, bruising at the site of the infusion or a risk of infection, which can be minimized by following aseptic procedures.

The protocol should indicate the method of obtaining informed consent from the study participants or their legal guardians before any study procedures begin. Informed consent should be obtained from adults and assent from children old enough to provide assent. Parental consent is needed for paediatric studies that are undertaken in age groups that are not yet old enough to assent. The age cut-offs and preferred means of obtaining assent often vary between research institutions due to local customs. Local requirements for the consent/assent process need to be respected while following accepted guidelines.

The consent must be written or translated into the language(s) spoken by the participants enrolled in the study and a witness to the consent process may be required if the participant is under the legal age or is unable to give approval. In some instances the population group of interest may not be literate. Verbal

informed consent scripts may be required and an alternative means of verifying consent (thumbprint) should be put into place.[2]

Full disclosure to participants of all potential risks is mandatory. In zinc stable isotope studies, these risks primarily include the pain, discomfort and possible risks of bruising and infection associated with the venipuncture used to collect blood or to insert a catheter for intravenous dosing. Remuneration for participants' time and inconvenience is typically provided to individuals participating in zinc stable isotope studies. The amount and type of compensation should be given at a level that provides compensation for the amount of personal effort required, but is not so high that it would coerce people into participating when they otherwise would not be willing. The type of remuneration given has also to be compatible with local cultural practices and regulations. The contact information of local representatives should be provided to each study participant in case they have any questions or concerns at any stage of the study.

10.6. STUDY DURATION

The duration of study depends on the research question and possible intervention. Shorter studies may measure zinc absorption from foods consumed for 1–2 days, together with 6–8 days of sample collection, while longitudinal dietary or supplementation interventions may last several weeks or months to allow time for adaptation and response to the interventions. Peer reviewed journals generally require that manuscripts reporting the results of human studies include statements that they have complied with an appropriate ethical approval process. Such journals also increasingly require advanced registration of clinical trials in a public trials registry approved by the International Committee of Medical Journal Editors.

[2] For guidelines in preparing consent forms and obtaining informed consent, refer to the below web sites:
www.who.int/rpc/research_ethics/Process_seeking_IF_printing2.pdf
www.who.int/rpc/research_ethics/informed_consent/en

Appendix I

EQUIPMENT AND SUPPLIES

This appendix lists the equipment and supplies needed for the assessment of zinc metabolism in humans using stable zinc isotope techniques.

I.1. LABORATORY EQUIPMENT

- Fume hood, acid resistant.
- Microwave digestion system with digestion vessels and accessories. Digestions can also be conducted using a hot plate with or without a controlled temperature ashing oven/muffle furnace. Both microwave and muffle furnace systems are used in the sample procedures in this publication.
- ICP-AES or AAS (or collaborator).
- ICP-MS or TIMS (or collaborator).
- Hot plate with heating block for test tubes.
- pH meter and associated supplies.
- Balance (0.1 mg).
- Freezer (−20°C), non-frost-free.
- Refrigerator.
- Deionized water supply.
- Personal protective equipment: laboratory coat, safety glasses.
- Freeze drier (lyophilizer).
- Vortex mixer.
- Calculator.
- Label maker.

I.2. LABORATORY SUPPLIES

- Graduated cylinders (100, 1000 mL);
- Glass beakers;
- Glass test tubes (e.g. 16 × 100 mL rimless culture tubes);
- Erlenmeyer flasks of heat resistant (e.g. Pyrex) glass (100 mL) for muffle digestions;
- Volumetric flasks (20, 50 mL);
- Plastic test tubes (11 mL) with stoppers;

— Plastic bottles for acid and buffer solutions;
— Racks for sample processing;
— Large plastic containers for acid washing;
— Automated pipettes (50, 200, 1000 and 5000 μL pipettes and tips, trace-metal free);
— Glass or polypropylene columns for ion exchange;
— Column rack;
— Large glass pans for use with the hot plate;
— Polypropylene tubes and small bottles.

I.3. LABORATORY CONSUMABLES

— Powder-free, non-latex gloves;
— Pipette tips;
— Transfer pipettes (3.5, 6 mL);
— 1.5 mL microtubes;
— Plastic wrap or plastic paraffin film (e.g. Parafilm);
— Absorbent ashless paper;
— Plastic backed absorbent paper or pads for catching spills;
— Tissues;
— Resealable bags;
— Permanent markers;
— Labels.

I.4. LABORATORY CHEMICALS

— HNO_3, high purity;
— HCl, high purity;
— Ammonium acetate;
— Ammonium hydroxide (NH_4OH);
— Sodium hydroxide;
— Hexane;
— 1, 1, 1-Trifluoro-2, 4-pentanedione;
— Pyridine;
— Analytical grade anion exchange resin, chloride form, 8% cross-linkage, 100–200 dry mesh size, 106–180 μm wet bead size, ~1000 mol. wt limit (e.g. AG1-X8 resin).

I.5. CLINICAL SUPPLIES

— Scale for body weight;
— Syringes;
— Vacutainers;
— Trace-element free heparinized syringes;
— Alcohol;
— Tourniquet;
— Ashless filter paper to collect possible spills during isotope administration;
— Ziplock bags;
— Sterilized half-normal saline (0.45% NaCl);
— Powder-free gloves;
— 0.2 μm filters;
— Needles;
— Adhesive bandages;
— IV infusion supplies with 3-way stopcock;
— Urine collection containers, polypropylene;
— Polypropylene containers for urine storage;
— Faecal collection containers: plastic bags or faecal buckets, acid washed.

I.6. RESEARCH KITCHEN EQUIPMENT AND SUPPLIES

— Refrigerator;
— Freezer (−20°C);
— Sink;
— Microwave oven;
— Stove or hot plate;
— Balance (0.1 g);
— Oven;
— Blender with multiple containers;
— Plastic cutting boards;
— Stainless steel knives;
— Stainless steel or PTFE cooking utensils;
— PTFE coated cooking pots and pans;
— Plastic or glass food containers;
— Supply of deionized water;
— Spatulas;
— Rinsing bottles;
— Labels;
— Permanent markers.

I.7. FIELD WORK SUPPLIES

— Personal protective equipment (laboratory coat, safety glasses, latex-free gloves);
— Cooler and ice packs for transporting biohazard samples;
— Portable centrifuge, if needed;
— Resealable bags;
— Laboratory book for recording sample collections (data forms/consent forms);
— Timepiece.

I.8. OFFICE SUPPLIES

— Computer with word processing, database and graphics software;
— Calendars for subjects with study instructions;
— Standard operating procedures for all study protocols;
— Filing cabinet for locking personal identifiable information.

Appendix II

PREPARING THE IV ISOTOPE DOSE: A DETAILED EXAMPLE

The following sample procedure prepares individual doses of an isotopically enriched zinc product for IV administration. For this example, the objective is to provide 0.5 mg of a highly enriched ^{70}Zn source per dose.

II.1. CALCULATIONS

Using the specifications provided with the commercially enriched product, calculate the average atomic weight of the enriched zinc isotope product. Assume that 21 mg ZnO was purchased with the following zinc isotope composition in mole fractions: ^{64}Zn 0.0030; ^{66}Zn 0.0020; ^{67}Zn 0.0020; ^{68}Zn 0.1080 and ^{70}Zn 0.885. The average atomic weight of this enriched zinc is 69.677 mg/mmol (see Eq. (11)). Record the specific batch and average atomic weight for this enriched product.

Plan the approximate volume for each dose. For IV doses, volumes are generally <5 mL for adults, <3 mL for children and <1 mL for infants, with elemental zinc concentrations of 0.1–0.5 mg/mL. For this example, providing the dose of 0.5 mg Zn from the enriched ^{70}Zn source in 4.0 mL will result in a concentration of about 0.125 mg Zn/mL. The exact volume will be determined after zinc analysis of the prepared solution.

Estimate the final volume after dilution of the 21 mg of the purchased ^{70}Zn enriched ZnO to ~0.125 mg Zn/mL, accounting for the proportion of Zn in the ZnO as well as the average atomic weight of the ^{70}Zn enriched source (69.677 mg/mmol):

$$21\ \text{mg ZnO} \times \frac{\text{mmol ZnO}}{(69.677 + 16)\ \text{mg ZnO}} \times \frac{69.677\ \text{mg Zn}}{\text{mmol Zn}} \times \frac{1\ \text{mL}}{0.125\ \text{mg Zn}} = 137\ \text{mL} \quad (22)$$

Estimate the number of doses resulting from the planned dilution. A 137 mL solution divided by 4.0 mL per dose is 34 doses, allowing for the needed 26 doses, with ~8 extra.

II.2. PROCEDURE

(1) Accurately weigh the ^{70}Zn enriched ZnO into a tared, sterile plastic cup or beaker of sufficient size to easily hold the expected final volume. Record the weight.

(2) Completely dissolve the ZnO by adding 1–3 mL 0.5M H_2SO_4 and shaking gently. Solubility can be facilitated by warming on a low heat source. If the ZnO is not completely dissolved, add 1–2 mL additional H_2SO_4. Check that solubility is complete without visible residue.

(3) Using a balance, bring the stock solution up to the calculated final volume with 0.45% NaCl saline. For practical purposes, the measurement is by weight, assuming that 1 mL solution weighs 1 g; although not strictly correct, this assumption enables the use of a balance to precisely and reproducibly adjust and record the final amount of solution. It is also common to measure the individual doses by weight, using the same assumption.

(4) Using a pH meter along with 1M NH_4OH and 1M H_2SO_4, titrate the dose solution to a pH of 6.0. Be sure to clean the pH meter probe with alcohol prior to use and with deionized water during use to minimize contamination.

(5) Under a laminar flow hood, transfer the solution through a 0.2 μm filter into a sterile container.

(6) Take a small sample to prepare for total zinc analysis, by drawing about 0.5 mL solution into a sterile syringe and placing it in a clean Eppendorf tube.

(7) From the sample of isotope solution, prepare triplicate dilutions with 0.1M HCl (e.g. combine 20 μL solution and 2 mL of the HCl for a 1:101 dilution), and measure the total zinc concentration by AAS or ICP-AES.

(8) Calculate the final concentration of the isotope solution by multiplying the concentration from the zinc analysis by the dilution factor and by correcting for the atomic weight of the isotope enriched zinc source. This atomic weight correction adjusts for the AAS or ICP-AES measurement assumption of a natural isotope abundance, an assumption that does not apply to this isotope enriched sample (see Section 4.2.8). Remember that the atomic weight correction factor is specific for each batch of isotope.

(9) Using the results of the zinc analysis, calculate the desired volume of individual dose aliquots. In this example, the desired dose is 0.5 mg total Zn; if the measured isotope solution has a corrected concentration of 0.13 mg/mL, the desired volume of each dose aliquot would be 0.5/0.13 = 3.85 mL.

(10) Pre-weigh and number the sterile vials with stoppers for individual IV doses. Record weights and ID numbers.
(11) Using sterile techniques and working under the laminar flow hood, filter the solution again while transferring the desired amount of solution into the pre-weighed sterile vials. This procedure includes drawing the desired volume (3.85 mL in this example) into a syringe, to which is attached a 0.2 μm filter followed by a sterile needle. The needle is placed through the stopper into the vial for injection of the filtered solution.
(12) Weigh each sterile vial after adding the isotope solution. Record weight.
(13) Clean the vial stopper with alcohol, then cover tightly with plastic wrap or plastic paraffin film (e.g. Parafilm).
(14) Calculate the weight of the solution by subtracting the pre-weight from the post-weight.
(15) Calculate the amount of isotope enriched zinc in each vial by multiplying the corrected zinc concentration in Step 9 by the weight of the solution in Step 14.
(16) Record the preparation date, the amount of total (isotope enriched) zinc in the vial, the batch number and the dose number on labels and place on the vial. Depending on the choice of subsequent calculation methods, and to avoid confusion, it may be useful to include both the amount of the stable isotope and of the total zinc.
(17) Keep a record of isotope enriched zinc, batch number, concentration of isotope solution, tube ID number, pre-and post-weights of the vials, volume of solution in vials, and amount of isotope enriched zinc (mg) in the vials for future reference.
(18) Arrange for a certified laboratory to conduct sterility pyrogenicity, and fungal testing on 10% of the vials (4 vials in this example). Do not administer the isotope solution unless the results are negative for all tests. When the results are available, add labels to the vials indicating the certification and corresponding expiration date.

The vials containing IV solutions can be stored in a secure setting at refrigeration temperatures, usually 4°C, until the expiration of the sterility, pyrogenicity and fungal testing, commonly six months.

II.3. SUMMARY

The above procedure has prepared 26 doses of ~0.5 mg isotope enriched ^{70}Zn (containing 0.885 mole fraction ^{70}Zn), plus extra for isotope analyses; sterility, pyrogenicity and fungal testing; possible additional participants; or

later verification of analytical testing, as needed. The final data analyses for each participant will use the specific zinc content of their isotope solution, based on the pre- and post- weights of the prepared dose vials.

Appendix III

ADMINISTERING THE IV ISOTOPE DOSE: QUANTITATIVE CATHETER INFUSION USING A 3-WAY STOPCOCK

This procedure describes the quantitative infusion of a prepared IV isotope solution from a single dose storage vial into a peripheral forearm vein with the use of a catheter infusion set attached to a 3-way stopcock. The procedure enables both the emptied dose vial and syringe to be flushed with sterile saline that is subsequently infused to ensure that all of the dose has been delivered. Note that the procedure needs to be conducted by a medical professional qualified to administer IV infusions (an IV nurse or medical doctor).

III.1. PROCEDURE

(1) Record isotope dose information (zinc isotope batch, dose ID number and amount of dose) together with participant ID, date, and time.
(2) Place indwelling catheter or infusion set into the antecubital vein of the participant using standard medical procedures and zinc-free materials. A lidocaine cream can be used to numb the area prior to insertion of the catheter if desired. Attach a 3-way stopcock to the infusion set or catheter. (Note: if taking a blood sample, do this before attaching or infusing any isotope.)
(3) Working over ashless filter paper and using a sterile syringe, withdraw the entire pre-measured isotope dose from the sterile vial, being extremely careful not to spill any of the dose. Leave an air space in the inverted syringe, remove it from the needle, leave the needle in the stopper of the emptied dose vial.
(4) Place the syringe with the isotope dose on one open port of the 3-way stopcock. Use special care when attaching the syringe with the isotope in order not to lose any dose from the syringe. Place filter paper under the participant's arm in case of dose spills or leaks. If leaks occur, collect on filter paper, save paper in labelled, sealable plastic bag for subsequent analysis.
(5) Using a second syringe and needle, load the syringe with 10 mL of sterile 0.45% NaCl saline. Detach the saline loaded syringe from the needle, and attach it to the first needle that remains in the stopper of the dose vial. Transfer the sterile saline into the dose vial, swirl gently to mix with any

remaining isotope solution and then remove saline from vial with same syringe.

(6) Place this second syringe with 0.45% NaCl saline on the other open port of the 3-way stopcock.
(7) Repeat Step 5 with a third syringe and set aside.
(8) Record the time, and then begin slowly infusing the isotope into the vein at a rate of ~1 mL/min. Observe closely if the vein has ruptured or isotope is leaking out of the vein. If any leakage is observed, blot with filter paper and save the filter paper for future analysis.
(9) Once all of the isotope is infused, close the port to the vein. Record the time.
(10) Push a small amount of 0.45% NaCl saline (~ ½ the volume of the dose solution) into the dose syringe. Close the port to the saline syringe. Push the saline in the dose syringe into the vein.
(11) Repeat this transfer of saline from the saline syringe to the dose syringe to the vein a second time with a volume of saline equal to the original dose solution volume.
(12) Repeat a third time with remaining saline. Record the time.
(13) Remove the infusion set from the participant's arm.

III.2. AN ALTERNATIVE PROCEDURE

Some investigators prefer an alternative method for IV zinc isotope administration, to reduce the total rinse volume required with the above 3-way stopcock system. Briefly, the alternative method uses either a butterfly needle attached to a small length of tubing with a Luer lock ending that can make a tight connection with a Luer lock syringe or a heparin lock catheter with only one hub. Then, instead of attempting to quantitatively draw, administer and rinse a pre-weighed isotope amount from a prepared vial, the isotope is drawn from the vial as completely as possible into a syringe that is pre-weighed (with cap in all cases) before the isotope is drawn (empty), weighed after the isotope is drawn into the syringe, and weighed again after the isotope has been injected through the catheter. The exact weight of the administered isotope is determined from the difference in the syringe weights, and another syringe filled with saline is used to flush the catheter tubing. Possible disadvantages of this method are that obtaining an accurate weight of the dose requires (a) a highly sensitive balance at the site of isotope administration, (b) no change in initial isotope concentration from possible evaporative loss, and (c) no back-flow of blood into the syringe.

Appendix IV

ZINC PURIFICATION FROM FAECES TO MEASURE ISOTOPE RATIOS

IV.1. OBJECTIVE

In this procedure, faecal samples are digested to ash with a muffle furnace and further heating in HNO_3 on a hot plate. After this digestion, total elemental zinc can be analysed by AAS or ICP-AES. For isotope ratio analysis, the zinc is further isolated from the digestate by ion exchange chromatography, and the concentration adjusted prior to mass spectrometry analysis.

IV.2. MATERIALS

— 100 mL Erlenmeyer flasks or 250 mL beakers of heat resistant, borosilicate glass (for digestion vessels when starting with dry or wet samples, respectively);
— 20, 50 mL volumetric flasks;
— Disposable transfer pipettes;
— AAS or ICP-AES tubes (polypropylene);
— Polypropylene test tubes (11 mL) with stoppers;
— Glass chromatography columns with glass wool for resin support (or polypropylene column with built-in resin support) providing at least 5 mL volume for the resin bed and another 25 mL of additional column and/or reservoir (e.g. glass column dimensions of 24 cm height, 0.8 cm internal diameter and 25 mL upper reservoir);
— Column rack and eluent containers;
— 50, 200, 1000 and 5000 µL pipettes and tips, trace-metal free;
— Plastic bottles, acid washed;
— Plastic wrap or plastic paraffin film (e.g. Parafilm);
— Permanent marker;
— Labels.

IV.3. EQUIPMENT

— Freeze dryer (lyophilizer);
— Muffle furnace;

— Laboratory scale;
— Vortex mixer;
— Acid resistant vent hood;
— Hot plate;
— AAS or ICP-AES;
— ICP-MS.

IV.4. REAGENTS AND SOLUTIONS

— Check published Material Safety Data Sheets for safety and disposal information;
— Deionized water;
— Analytical grade anion exchange resin, chloride form, 8% cross-linkage, 100–200 dry mesh size, 106–180 μm wet bead size, ~1000 mol. wt limit (e.g. AG1-X8 resin);
— Concentrated HNO_3, ultrapure grade;
— 2% HNO_3, ultrapure grade;
— 0.125, 0.5, 6M HCl, ultrapure grade.

IV.5. METHOD

IV.5.1. Initial preparation of faecal samples

Two alternative homogenization procedures are described, using either dried or wet faecal samples. Selection may depend on the amount of sample that needs to be transferred, and the time and equipment available for drying samples at the initial laboratory collection site. The dry homogenization procedure enables aliquots to be obtained with less concern about representative sampling from a rapidly separating liquid slurry, and is less odorous to implement. The wet homogenization procedure can enable transfer from the initial laboratory of smaller frozen aliquots rather than entire frozen samples (or dried samples that require additional time and drying equipment).

IV.5.1.1. Dry homogenization

Faecal samples collected in sturdy, resealable polyethylene bags and then frozen can be freeze dried to a constant weight with a freeze dryer and ground to homogeneity by using a closed blender/mixer or by manually applying a rolling pin and mixing the finely powdered sample from outside of the closed

bag. Depending on the research protocol, faecal samples can be analysed as pooled 24 hr samples or multiple day pooled samples prepared to represent a specific dietary period (usually as indicated using non-absorbed faecal markers) by combining a consistent proportion of the total dry weight of each faecal sample to be included. Record the total dry faecal weight of the homogenate to enable subsequent calculation of total zinc and zinc isotope excretion per day. The prepared homogenous samples can be stored in polypropylene bottles in a desiccator.

IV.5.1.2. Wet homogenization

The faecal samples are homogenized in 24 hr pools in a glass blender, saving multiple aliquots of the homogenates (~50 g wet weight) in weighed polypropylene bottles. To enable calculation of the total amount of zinc and zinc isotope in the original samples, it is essential to record the total weight of the homogenate (blender container with and without sample and added deionized water) and the aliquots (bottles with and without the aliquots of the homogenate). Sample aliquots can be frozen and stored.

IV.5.2. Sample digestion

This step eliminates organic matter, leaving the mineral ash.

(1) Assemble samples. If possible, all faecal samples for one or more study participants are processed in a single analytical batch. Include blank samples and faecal standards, which may include purchased faecal standards with certified zinc concentrations and/or a prepared faecal pool sample that can serve to check the consistency of results between analytical batches.

(2) Use digestion containers of heat resistant glass, labelled with a short, heat proof identification by etching, that have been acid washed and dried:
   — For sample aliquots from a dry homogenate, a 100 mL Erlenmeyer flask/beaker can serve as the digestion vessel. Re-dry the faecal samples as necessary in a freeze drier (lyophilizer) or drying oven to a constant weight. Tare the digestion vessel on a lab scale, add an aliquot of the dried faecal sample (~1–1.5 g), and record the dry aliquot weight.
   — For sample aliquots from a wet homogenate, a 250 mL beaker can serve as the digestion vessel. Thaw the previously weighed wet sample aliquot and transfer it quantitatively to the digestion vessel using deionized water to rinse as necessary. Dry the sample at 100°C in a drying oven until it reaches a constant weight.

(3) Heat samples in a muffle furnace at 200°C for 3 hours, then increase temperature to 300°C for 3 hours, then 450°C for 24 hours.
(4) When cool, remove beakers from muffle furnace. In a ventilation hood, add enough concentrated HNO_3 to just cover the ashes. Place on hot plate at a very low temperature (not boiling) until sample is dry again. Do not let the sample char.
(5) Return the sample to the muffle furnace and heat again at 450°C for 24 hours.
(6) If the sample looks well ashed (mostly white or yellowish, not dark coloured), continue to the next step. If not, repeat steps 4 and 5.
(7) Quantitatively reconstitute the ashed sample in 6M HCl. Add a small amount of acid to beaker, washing bottoms and sides of glass to remove any zinc from them. Using a transfer pipette, transfer the acid to a 20 mL volumetric flask (50 mL flask may be needed if high zinc content is expected, as resulting from a zinc supplemented diet). Repeat, adding small amounts of acid to the beaker and transferring it to the volumetric flask at least two more times, and carefully bring the flask up to the total marked volume.
(8) Vortex sample well and transfer without further dilution to a zinc-free plastic container for storage until AAS or ICP-AES analysis.

IV.5.3. Determination of total zinc in faecal sample

(1) Vortex the digested sample well.
(2) Dilute 50 or 100 µl of sample with 0.125M HCl to obtain a zinc solution <1.00 µg/L for analyses by AAS. Start with 100 µL of sample added to 10 mL of acid (a 1:101 dilution factor) for AAS or ICP-AES analysis.
(3) Vortex sample immediately prior to AAS or ICP-AES analysis.
(4) Analyse samples using AAS or ICP-AES standard procedures. Dilutions of the reconstituted sample should be within the interval of confidence for the calibration curve of the atomic absorption spectrophotometer. If the solution is outside the standard curve (<0.1 or >1.2 µg/L; or expressed in decilitre volumes, <10 or >120 µg/dL), repeat the dilution with a more appropriate dilution. Blanks can be analysed without dilution.
(5) Perform these dilutions in duplicate. Duplicate results should be within 1% relative standard deviation of one another. If they have a greater variability, repeat dilutions to obtain agreeable results. Once they agree, the sample is ready for purification using anion exchange columns. Save a small aliquot of the solution in a polypropylene Eppendorf tube for repeat analyses of total zinc, if needed.

Total faecal zinc is calculated for each aliquot using Eq. (23) adjusted as necessary for alterations in dilution of the digestate. If the faecal aliquot was weighed dry, then dry weights are used for both the aliquot and total homogenate weights, but if the aliquot was weighed wet, then wet weights are used for both.

$$\text{TFZ} = \text{Zn in } \mu\text{g/100 mL} \times \frac{\text{10.1 mL dilution}}{\text{0.1 mL digestate}} \times \frac{\text{20 mL digestate}}{\text{Aliquot wt}} \times \text{Total homogenate wt} \quad (23)$$

Mean total faecal zinc content is calculated by averaging the above results for the two duplicate samples that were digested. If duplicates are not within 2% relative standard deviation, repeat dilutions and AAS (or ICP-AES) analyses.

One of the duplicate digested faecal samples is selected to purify for zinc stable isotope analysis using mass spectrometry by the procedure in IV.5.4.

IV.5.4. Zinc isolation and measurement of isotope ratios from sample digestates

IV.5.4.1. Column preparation

(1) Prepare glass column by placing pea sized glass wool or filter in column. Glass wool can be cleaned in 0.125M HCl prior to use. Check that deionized water runs easily through column with glass wool.

(2) To prevent bubbles forming in column, use degassed deionized water. Water can be degassed by boiling for five minutes; let cool before using. Alternatively, water can be degassed by allowing it to sit at room temperature for several days before using.

(3) Mix a slurry of the degassed, deionized water with anion exchange resin (3:1 water to resin). Slowly add a small amount of the resin slurry to column. Keep resin in column wet and covered with about 1 cm of deionized water at all times. It is important that the resin never goes dry. Add more resin, then water, resin, water, etc., until the desired column height (~8 cm for column with 0.8 cm internal diameter) is reached. The slower the resin is packed, the tighter the resin is, and the higher the recovery of zinc will be.

(4) (OPTIONAL) The resin occupies ~4 mL volume of the column. However, the volume of the mobile phase that runs through the resin is approximately 60–70% of the empty column space, or ~2.5 mL. This can be measured more accurately as follows:

— Add a small amount of iron solution, which is bright yellow in colour.

— Add small increments of 0.5M HCl until the iron band has moved through the resin.
— Record the volume used, either by collecting and weighing acid or keeping track of how much volume is added to the column.

(5) Columns can be stored at this time. Make sure that the resin is covered with degassed, deionized water (Fig. 14). For storage, cover the top of the column with plastic wrap or plastic paraffin film (e.g. Parafilm), and place the bottom in a test tube, with plastic paraffin film wrapped around the top of the tube and column. (Alternatively, a capped test tube can be modified by drilling a hole in the cap to just fit the external diameter of the column.)

IV.5.4.2. Column cleaning

(1) Remove the storage tube and let the water drain out (just to the top of the resin).
(2) Fill the column to the top (~20–30 mL) with 6M HCl and let drain. Add solutions slowly so as not to disturb resin bed.

FIG. 14. Chromatography columns for purification of zinc from other minerals in digested samples.

(3) Fill the column to the top (~20–30 mL) with 0.5M HCl and let drain.
(4) Fill the column to the top (~20–30 mL) with deionized water and let drain.
(5) Add ~ 5 mL of 6M HCL and let drain.
(6) Add ~ 5 mL 0.5M HCl and let drain.
(7) Add ~ 5 mL of deionized water and let drain.
(8) Repeat steps 5–7 twice more.
(9) Add 1 mL deionized water and collect the solution in a test tube. Check this on AAS or ICP-AES for zinc contamination. If no contamination is evident (<2 µg Zn/dL), the column is ready for sample addition. If the column still contains zinc, repeat Step 8 washing procedures and check again for zinc contamination.
(10) Columns can be capped and stored with degassed deionized water at this point, if desired. Immediately prior to use, check for cleanliness again (Step 9). Do not allow the resin to dry out or use the columns if bubbles of gas are present in the resin.

IV.5.4.3. Sample addition and zinc elution

(1) Once the columns are clean and have been checked for any zinc contamination, add ~5 mL of 6M HCl to charge the column. After this has passed into the resin, the sample can be added by pipette, trying not to disturb resin. This will probably use all of the reconstituted sample from the digestate. A maximum of 1000 µg Zn is ideal for recovery and timeliness; a minimum of 200 µg Zn is advisable.
(2) Let the sample drain through the column (no need to collect unless absolute recovery is essential or needs to be quantified).
(3) Fill the column to the top with 0.5M HCl and let drain out (no need to collect eluent).
(4) Place 2.4 mL of deionized water in the column and collect this eluent. This column will contain the end of the 0.5M HCl and will be contaminated with iron, sodium, potassium, among others.
(5) Add 7.5 mL of deionized water to column and collect this eluent in a separate 10 mL polypropylene test tube.
(6) Determine the zinc concentration of this final eluent by diluting a small aliquot (50 µL) with 5 mL 0.125M HCl and measure the zinc concentration by AAS or ICP-AES, adjusting the dilution as necessary to fit the standard curve for the instrument.

IV.5.5. Measurement of zinc isotope ratios by inductively coupled plasma mass spectrometry

(1) Based on the measured zinc concentration of the final column effluent, dilute the effluent containing the isolated zinc with 2% HNO_3 to obtain 8 mL of a 50 µg Zn/L solution. This dilution can be determined with the following formulas, modified for the dilution used to measure zinc concentration in Step 6 of Section IV.5.4.3.

$$\text{vol. of effluent}(\mu\text{L}) = 8\ \text{mL} \times \frac{50\ \mu\text{g Zn}}{1000\ \text{mL}} \times \frac{100\ \text{mL}}{\text{measured}\ \mu\text{g Zn}} \times \frac{50\ \mu\text{L effluent}}{5.05\ \text{mL dilution}} \quad (24)$$

$$\text{vol. of 2\% HNO}_3(\text{mL}) = 8\ \text{mL} - \left(\text{vol. of effluent}(\mu\text{L}) \times \frac{1\ \text{mL}}{1000\ \mu\text{L}}\right) \quad (25)$$

(2) Operating the ICP-MS in ion counting detection mode, optimize the ICP-MS on mass 66 to obtain the highest possible (^{66}Zn) count rate while analysing 50 µg Zn/L natural zinc solution and the lowest count rate while analysing 2% HNO_3. Arrange purified, diluted biological samples in the auto sampler. Include an internal standard (natural abundance zinc solution) after every six samples and a blank (2% HNO_3) after every 12 samples. Begin automatic sampling and the instrumental program to determine the following zinc isotope ratios (as needed when using three tracers): ^{67}Zn/^{66}Zn, ^{68}Zn/^{66}Zn and ^{70}Zn/^{66}Zn.

Appendix V

ZINC PURIFICATION FROM URINE, PLASMA OR SERUM TO MEASURE ISOTOPE RATIOS

This method to purify zinc from urine, plasma or serum uses acid digestion to eliminate organic compounds, leaving only the mineral ash. Chemical chelation and extraction are then applied to isolate zinc, followed by further acid digestion to destroy the chelate. The zinc concentration is adjusted in preparation for analysis of the zinc isotope ratios by mass spectrometry.

V.1. EQUIPMENT AND SUPPLIES

— Microwave digestion vessels and related accessories;
— 100, 1000 mL graduated cylinders;
— Glass beakers;
— Glass test tubes (e.g. 16 × 100 mm rimless culture tubes);
— Plastic test tubes (11 mL) with stoppers;
— 3.5 mL transfer pipettes;
— 6 mL fine tip transfer pipettes;
— Pipette tips (1–200 µL, 200–1000 µL, 1–10 mL);
— 1.5 mL microtubes;
— Microwave digestion oven for biological samples;
— Muffle furnace;
— Fume hood;
— pH meter;
— Laboratory scales;
— Hot plate with heating block for test tubes;
— ICP-AES or AAS;
— ICP-MS.

V.2. REAGENTS AND SOLUTIONS

— Check published Material Safety Data Sheets for safety and disposal considerations.

V.2.1. Ammonium acetate buffer preparation

(1) Measure 710 mL deionized water using a 1 L graduated cylinder and add to a 1 L polypropylene bottle.
(2) In a fume hood, measure 70 mL NH_4OH solution in a 100 mL graduated cylinder and add it to the polypropylene bottle.
(3) In a fume hood, measure 50 mL acetic acid in a 100 mL graduated cylinder and add it to the polypropylene bottle.
(4) Mix the solution thoroughly.
(5) Measure pH of the buffer using the pH meter and adjust the pH to 5.6 using acetic acid and NH_4OH solution.

V.2.2. Other chemicals

— Hexane;
— 1, 1, 1-Trifluoro-2, 4-pentanedione;
— Pyridine;
— HNO_3, trace-metal grade or ultrapure;
— HCl, trace-metal grade.

V.3. PROCEDURE

(1) Thaw urine, plasma or serum samples at room temperature. If possible, include a complete set of urine, plasma or serum samples for one or more study participants to be processed in a single analytical batch.
(2) Prepare the digestion unit vessels, liners, caps, lids, etc. for digestion. The PTFE liners and caps should be acid washed and dried.
(3) In each microwave digester vessel, pipette 5.5 mL urine or 2 mL plasma or serum, mixing the sample prior to adding to vessel. Add 1.2 mL concentrated HNO_3. Place the covers and caps on the vessels, and complete the preparation of the vessels for microwave digestion.
(4) Digest samples using a programmed protocol established for the digestion of urine, plasma or serum in the specific microwave instrument. Digestion may require about 1.5 hours.
(5) Pour digested samples into clean, labelled glass beakers. Samples should be clear.
(6) Dry samples on the hot plate at 240°C. Make sure bottom and sides are dry and that samples are white, not black. Remove the beakers from the hot plate when they are dry.

(7) Prepare for the zinc chelation and extraction of the digested urine, plasma or serum samples. For each sample, place plastic test tubes in a rack, labelled using a permanent marker and place a 3.5 mL transfer pipette into each tube, being careful to not touch the tips of the pipettes.
(8) Dissolve samples in 2 mL ammonium acetate buffer. Rinse the sides of the beaker completely and use the transfer pipette to loosen any solids off the beaker. Transfer samples into the prepared plastic test tubes, discarding the pipette into the waste.
(9) Add 100 µL of 1, 1, 1-trifluoro-2, 4-pentanedione and then 500 µL of hexane to the test tubes. Mix well with a 6 mL fine tip transfer pipette and suction up the sample. Discard only the upper layer into a hexane waste container.
(10) Add another 500 µL of hexane, mix well with the transfer pipette, suction up the sample, and again discard the upper layer into a hexane waste container.
(11) Add 25 µL of 1, 1, 1-Trifluoro-2, 4-pentanedione, 25 µL of pyridine and 500 µL of hexane to each sample. Mix well with the transfer pipette and pipette only the upper layer into a clean glass test tube.
(12) Add another 500 µL of hexane, mix well with transfer pipette and pipette the upper layer into the glass test tube.
(13) Set the heating block to a low temperature. Place the glass tubes in the heating block to evaporate the hexane. Meanwhile, transfer the ammonium acetate, pyridine and 1, 1, 1-Trifluoro-2, 4-pentanedione mixture to the appropriate waste container and dispose of the tubes and pipettes into the waste.
(14) Using 250 mL beakers to hold the glass test tubes, burn the samples in a muffle furnace at 450°C for at least 12 hours.
(15) Prepare samples for ICP-MS analysis by adding 6.5 mL of 2% ultrapure HNO_3 to each glass test tube. Wait at least 30 minutes before transferring samples into new 11 mL plastic test tubes.
(16) Pipette 500 µL of each sample into a clean microcentrifuge tube.
(17) Measure the zinc concentration using AAS or ICP-AES (only one reading is necessary; this is used to adjust all samples to similar zinc concentrations before ICP-MS analysis).
(18) Based on the zinc concentration determined above, prepare a solution with the zinc concentration appropriate for the mass spectrometer to be used. For

example, to prepare a 6 mL solution containing 50 μg Zn/L, the volumes to be used can be calculated as follows:

$$\text{vol. of sample (in mL)} = 6\ \text{mL} \times \frac{50\ \mu\text{g Zn}}{1000\ \text{mL}} \times \frac{1}{\text{Zn measured in } \mu\text{g}/100\ \text{mL}} \tag{26}$$

(19) Operating the ICP-MS in ion counting detection mode, optimize the ICP-MS on mass 66 to obtain the highest possible (^{66}Zn) count rate while analysing 50 μg Zn/L natural zinc solution and the lowest count rate while analysing 2% HNO_3. Arrange the purified, diluted biological samples in the auto sampler. Include an internal standard (natural abundance zinc solution) after every six samples and a blank (2% HNO_3) after every 12 samples. Begin automatic sampling and the instrumental program to determine the following zinc isotope ratios (as needed when using three tracers): ^{67}Zn/^{66}Zn, ^{68}Zn/^{66}Zn and ^{70}Zn/^{66}Zn.

Appendix VI

CALCULATION OF SAMPLE ENRICHMENT FROM STABLE ISOTOPE RATIOS USING THREE ZINC ISOTOPE SOURCES

The following calculation procedure accounts for cross-contamination when using up to three isotopically enriched zinc sources.[3] The reader should note that for this method the term 'enrichment' has been specifically defined for this calculation.

VI.1. CALCULATION OF ENRICHMENT FROM STABLE ISOTOPE RATIOS

Enrichment is defined to be all zinc in the sample from an isotopically enriched source divided by total amount of zinc in the sample, both expressed in mg. Enrichment is calculated from the measured (and corrected for instrumental mass bias) isotope ratios by an algorithm that takes into account the isotope abundances and average atomic weights of both the natural and the isotopically enriched zinc contained in the samples. Since the enriched zinc preparations used as tracers are not isotopically pure, the use of multiple tracers requires that the calculation of each tracer isotope enrichment takes into account the contributions to that isotope ratio from all tracers present in the samples. Since as many as three

[3] This calculation of enrichment from stable isotope ratios is based on the work of L.V. Miller, K.M. Hambidge, and P.V. Fennessey, University of Colorado School of Medicine, Denver, Colorado, USA (presented here with permission).

isotopically enriched tracers (^{67}Zn, ^{68}Zn and ^{70}Zn) may be used, the enrichment values are calculated using the following equations:

$$R_{67/66} = \frac{\frac{\left(1-E^{67}-E^{68}-E^{70}\right)\times A_N^{67}}{AW_N} + \frac{E^{67}\times A_{67}^{67}}{AW_{67}} + \frac{E^{68}\times A_{68}^{67}}{AW_{68}} + \frac{E^{70}\times A_{70}^{67}}{AW_{70}}}{\frac{\left(1-E^{67}-E^{68}-E^{70}\right)\times A_N^{66}}{AW_N} + \frac{E^{67}\times A_{67}^{66}}{AW_{67}} + \frac{E^{68}\times A_{68}^{66}}{AW_{68}} + \frac{E^{70}\times A_{70}^{66}}{AW_{70}}} \quad (27)$$

$$R_{68/66} = \frac{\frac{\left(1-E^{67}-E^{68}-E^{70}\right)\times A_N^{68}}{AW_N} + \frac{E^{67}\times A_{67}^{68}}{AW_{67}} + \frac{E^{68}\times A_{68}^{68}}{AW_{68}} + \frac{E^{70}\times A_{70}^{68}}{AW_{70}}}{\frac{\left(1-E^{67}-E^{68}-E^{70}\right)\times A_N^{66}}{AW_N} + \frac{E^{67}\times A_{67}^{66}}{AW_{67}} + \frac{E^{68}\times A_{68}^{66}}{AW_{68}} + \frac{E^{70}\times A_{70}^{66}}{AW_{70}}} \quad (28)$$

$$R_{70/66} = \frac{\frac{\left(1-E^{67}-E^{68}-E^{70}\right)\times A_N^{70}}{AW_N} + \frac{E^{67}\times A_{67}^{70}}{AW_{67}} + \frac{E^{68}\times A_{68}^{70}}{AW_{68}} + \frac{E^{70}\times A_{70}^{70}}{AW_{70}}}{\frac{\left(1-E^{67}-E^{68}-E^{70}\right)\times A_N^{66}}{AW_N} + \frac{E^{67}\times A_{67}^{66}}{AW_{67}} + \frac{E^{68}\times A_{68}^{66}}{AW_{68}} + \frac{E^{70}\times A_{70}^{66}}{AW_{70}}} \quad (29)$$

where

R is the molar isotope ratio specified by the subscript;
E is enrichment of the isotope specified by the superscript, expressed as a weight ratio (mg/mg);
A is the abundance of the isotope specified by the superscript in the material specified by the subscript (e.g. denotes the abundance of ^{67}Zn in the ^{70}Zn enriched Zn, expressed as an atom fraction);

and AW is the average atomic weight for the material indicated by the subscript (mg/mmol).

Equations (27–29) are rearranged to the general form:

$$\beta_1 = \alpha_{11}\times E^{67} + \alpha_{12}\times E^{68} + \alpha_{13}\times E^{70} \quad (30)$$

$$\beta_2 = \alpha_{21}\times E^{67} + \alpha_{22}\times E^{68} + \alpha_{23}\times E^{70} \quad (31)$$

$$\beta_3 = \alpha_{31}\times E^{67} + \alpha_{32}\times E^{68} + \alpha_{33}\times E^{70} \quad (32)$$

The coefficients of the general equations (30–32) are defined as follows:

$$\alpha_{11} = \mathrm{R}_{67/66} \times \left(\frac{\mathrm{A}_{\mathrm{N}}^{66}}{\mathrm{AW}_{\mathrm{N}}} - \frac{\mathrm{A}_{67}^{66}}{\mathrm{AW}_{67}} \right) + \frac{\mathrm{A}_{67}^{67}}{\mathrm{AW}_{67}} - \frac{\mathrm{A}_{\mathrm{N}}^{67}}{\mathrm{AW}_{\mathrm{N}}} \tag{33}$$

$$\alpha_{12} = \mathrm{R}_{67/66} \times \left(\frac{\mathrm{A}_{\mathrm{N}}^{66}}{\mathrm{AW}_{\mathrm{N}}} - \frac{\mathrm{A}_{68}^{66}}{\mathrm{AW}_{68}} \right) + \frac{\mathrm{A}_{68}^{67}}{\mathrm{AW}_{68}} - \frac{\mathrm{A}_{\mathrm{N}}^{67}}{\mathrm{AW}_{\mathrm{N}}} \tag{34}$$

$$\alpha_{13} = \mathrm{R}_{67/66} \times \left(\frac{\mathrm{A}_{\mathrm{N}}^{66}}{\mathrm{AW}_{\mathrm{N}}} - \frac{\mathrm{A}_{70}^{66}}{\mathrm{AW}_{70}} \right) + \frac{\mathrm{A}_{70}^{67}}{\mathrm{AW}_{70}} - \frac{\mathrm{A}_{\mathrm{N}}^{67}}{\mathrm{AW}_{\mathrm{N}}} \tag{35}$$

$$\beta_1 = \frac{\mathrm{R}_{67/66} \times \mathrm{A}_{\mathrm{N}}^{66} - \mathrm{A}_{\mathrm{N}}^{67}}{\mathrm{AW}_{\mathrm{N}}} \tag{36}$$

$$\alpha_{21} = \mathrm{R}_{68/66} \times \left(\frac{\mathrm{A}_{\mathrm{N}}^{66}}{\mathrm{AW}_{\mathrm{N}}} - \frac{\mathrm{A}_{67}^{66}}{\mathrm{AW}_{67}} \right) + \frac{\mathrm{A}_{67}^{68}}{\mathrm{AW}_{67}} - \frac{\mathrm{A}_{\mathrm{N}}^{68}}{\mathrm{AW}_{\mathrm{N}}} \tag{37}$$

$$\alpha_{22} = \mathrm{R}_{68/66} \times \left(\frac{\mathrm{A}_{\mathrm{N}}^{66}}{\mathrm{AW}_{\mathrm{N}}} - \frac{\mathrm{A}_{68}^{66}}{\mathrm{AW}_{68}} \right) + \frac{\mathrm{A}_{68}^{68}}{\mathrm{AW}_{68}} - \frac{\mathrm{A}_{\mathrm{N}}^{68}}{\mathrm{AW}_{\mathrm{N}}} \tag{38}$$

$$\alpha_{23} = \mathrm{R}_{68/66} \times \left(\frac{\mathrm{A}_{\mathrm{N}}^{66}}{\mathrm{AW}_{\mathrm{N}}} - \frac{\mathrm{A}_{70}^{66}}{\mathrm{AW}_{70}} \right) + \frac{\mathrm{A}_{70}^{68}}{\mathrm{AW}_{70}} - \frac{\mathrm{A}_{\mathrm{N}}^{68}}{\mathrm{AW}_{\mathrm{N}}} \tag{39}$$

$$\beta_2 = \frac{\mathrm{R}_{68/66} \times \mathrm{A}_{\mathrm{N}}^{66} - \mathrm{A}_{\mathrm{N}}^{68}}{\mathrm{AW}_{\mathrm{N}}} \tag{40}$$

$$\alpha_{31} = \mathrm{R}_{70/66} \times \left(\frac{\mathrm{A}_{\mathrm{N}}^{66}}{\mathrm{AW}_{\mathrm{N}}} - \frac{\mathrm{A}_{67}^{66}}{\mathrm{AW}_{67}} \right) + \frac{\mathrm{A}_{67}^{70}}{\mathrm{AW}_{67}} - \frac{\mathrm{A}_{\mathrm{N}}^{70}}{\mathrm{AW}_{\mathrm{N}}} \tag{41}$$

$$\alpha_{32} = \mathrm{R}_{70/66} \times \left(\frac{\mathrm{A}_{\mathrm{N}}^{66}}{\mathrm{AW}_{\mathrm{N}}} - \frac{\mathrm{A}_{68}^{66}}{\mathrm{AW}_{68}} \right) + \frac{\mathrm{A}_{68}^{70}}{\mathrm{AW}_{68}} - \frac{\mathrm{A}_{\mathrm{N}}^{70}}{\mathrm{AW}_{\mathrm{N}}} \tag{42}$$

$$\alpha_{33} = R_{70/66} \times \left(\frac{A_N^{66}}{AW_N} - \frac{A_{70}^{66}}{AW_{70}} \right) + \frac{A_{70}^{70}}{AW_{70}} - \frac{A_N^{70}}{AW_N} \tag{43}$$

$$\beta_3 = \frac{R_{70/66} \times A_N^{66} - A_N^{70}}{AW_N} \tag{44}$$

The values of the coefficients are calculated by plugging in the known and measured values for all the variables. Equations (30–32) are then solved for the three enrichment results using matrix determinants and Cramer's Rule as shown by the following equations:

$$E^{67} = \frac{\begin{vmatrix} \beta_1 & \alpha_{12} & \alpha_{13} \\ \beta_2 & \alpha_{22} & \alpha_{23} \\ \beta_3 & \alpha_{32} & \alpha_{33} \end{vmatrix}}{\begin{vmatrix} \alpha_{11} & \alpha_{12} & \alpha_{13} \\ \alpha_{21} & \alpha_{22} & \alpha_{23} \\ \alpha_{31} & \alpha_{32} & \alpha_{33} \end{vmatrix}} \quad E^{68} = \frac{\begin{vmatrix} \alpha_{11} & \beta_1 & \alpha_{13} \\ \alpha_{21} & \beta_2 & \alpha_{23} \\ \alpha_{31} & \beta_3 & \alpha_{33} \end{vmatrix}}{\begin{vmatrix} \alpha_{11} & \alpha_{12} & \alpha_{13} \\ \alpha_{21} & \alpha_{22} & \alpha_{23} \\ \alpha_{31} & \alpha_{32} & \alpha_{33} \end{vmatrix}} \quad E^{70} = \frac{\begin{vmatrix} \alpha_{11} & \alpha_{12} & \beta_1 \\ \alpha_{21} & \alpha_{22} & \beta_2 \\ \alpha_{31} & \alpha_{32} & \beta_3 \end{vmatrix}}{\begin{vmatrix} \alpha_{11} & \alpha_{12} & \alpha_{13} \\ \alpha_{21} & \alpha_{22} & \alpha_{23} \\ \alpha_{31} & \alpha_{32} & \alpha_{33} \end{vmatrix}} \tag{45}$$

A sample Microsoft Excel spreadsheet for these calculations can be downloaded from the IAEA Human Health Campus under the nutrition tab. Click on 'Iron and Zinc Bioavailability' and look under 'Additional resources'.

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GLOSSARY

abundance. The proportion of atoms of a specific isotope as compared with the total number of atoms of that element, expressed as a fraction (mole fraction) or per cent (%).

apparent zinc absorption. Zinc absorption determined by subtracting the amount of zinc excreted in faeces from the amount of zinc in the diet, without taking into account endogenous zinc excretion into the gut.

bioavailability. The degree to which a nutrient can be absorbed and used from a specific source, usually an ingested source such as a food, fortificant or supplement, for normal metabolic function or storage.

biohazard. An organism, or substance derived from an organism, that poses a threat to (primarily) human health. This can include medical waste or samples of a microorganism, virus or toxin (from a biological source) that can impact human health.

complete urine collection. A complete 24 hour urine collection, starting after the first void in the morning and finishing with the first void the following morning.

endogenous faecal zinc excretion. Endogenous zinc (zinc in the body) that is secreted into the intestine and subsequently excreted from the body.

enrichment. The abundance of an isotope above the baseline (natural abundance) level for that element.

exchangeable pools. Also known as miscible pools. Atoms or molecules in a chemical matrix or system (such as a part of the diet or the body) that interchange rapidly and completely. A tracer for a pool of zinc atoms reveals the metabolism and proportional distribution of the rest of that pool or form of zinc. In physiological systems, exchangeable pools are not necessarily defined organs or organ systems.

extrinsic labelling. A measured quantity of isotopic tracer is added to the test meal in the final stages of food preparation, rather than during the biological growth of the plant or animal.

fat free mass. The term used in body composition studies to refer to the part of the body that is not fat; fat free mass includes water, protein and minerals.

fractional zinc absorption. The zinc absorbed by the gastrointestinal tract, expressed as a fraction of the dietary zinc consumed.

fractionation. Isotopic fractionation is the term used to describe the fact that molecules containing different isotopes display slightly different reaction rates. Fractionation results in an observed change in isotopic signature in different portions or fractions of a chemical system. This can occur during chemical or enzyme-catalysed reactions, exchange reactions or during physical changes such as evaporation.

homeostasis. Homeostasis refers to the body's ability to regulate physiologically its inner environment to ensure its stability in response to fluctuations in the outside environment.

inductively coupled plasma mass spectrometry. A type of mass spectrometry that uses inductively coupled plasma to ionize the sample of interest before it enters the mass spectrometer.

intrinsic labelling. The zinc isotopic tracer is incorporated into the food of interest in a way that is as similar as possible to the way that zinc is normally incorporated into the food during growth of the plant or animal.

isobaric interference. Interference in the mass spectrometry measurement of an isotope caused when a sample contains an isotope of a different element with the same mass. For example, ^{64}Ni can cause isobaric interference for ^{64}Zn.

isotope dose. The mass, usually in mg for stable isotope studies, of an isotopically enriched source administered as a tracer in the body.

isotope ratio. The output from inductively coupled plasma mass spectrometry or thermal ionization mass spectrometry is often expressed as an isotope ratio, which is a derived ratio of the amount of the minor isotope compared to the amount of the major isotope with certain instrumental corrections applied during data reduction.

isotopes. Atoms of an element that have the same number of protons and different numbers of neutrons. Isotopes can be stable or radioactive. The change

in the number of neutrons changes the atomic mass, but has minimal or no effect on the chemical properties of the element. Zinc has five stable isotopes:

^{64}Zn has 30 protons and 34 neutrons
^{66}Zn has 30 protons and 36 neutrons
^{67}Zn has 30 protons and 37 neutrons
^{68}Zn has 30 protons and 38 neutrons
^{70}Zn has 30 protons and 40 neutrons

kinetics. A study of the rates of a chemical process; in the present context, the rates at which the mineral zinc is absorbed, distributed, metabolized and excreted in the human body.

limit of detection. The minimum amount that needs to be measured to detect a real difference from a baseline sample.

limit of quantification. The minimum amount that needs to be measured to reliably describe the magnitude of the measurement.

major isotope. The most abundant stable isotope of an element is sometimes referred to as the major isotope. For zinc this is ^{64}Zn.

mass spectrometry. An instrumental technique used to determine the elemental composition of a sample or molecule according to the mass to charge ratio of its ions in an electric or magnetic field under vacuum.

minor isotope. The least abundant stable isotope of an element is sometimes referred to as the minor isotope. For zinc this is ^{70}Zn.

natural abundance. The proportion of atoms of a stable isotope relative to the total atoms of the same element as found naturally on earth. The natural abundance is expressed as a fraction (mole fraction) or per cent (%) of the total amount of zinc atoms (all isotopes). Note that abundance is expressed in atoms or moles (not weight). The natural abundance of zinc isotopes is detailed in Table 1.

phytate. Phytic acid, or inositol hexaphosphate, is the principal storage form of phosphorus in plant tissues, especially bran and seeds, and is a strong chelator of important minerals such as calcium, magnesium, iron and

zinc, therefore making them less bioavailable for absorption in the gastrointestinal tract.

plasma. (In physics.) An ionized gas.

plasma. (In physiology and medicine.) The straw coloured liquid component of blood, in which blood cells are suspended. Plasma is the supernatant that forms after whole blood centrifugation when whole blood is collected in the presence of an anti-coagulant.

polytetrafluoroethylene. A heat resistant polymer known for slippery, non-sticking properties, which may be used for containers including labware (e.g. commercial Teflon).

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

pyrogen. A substance capable of producing a fever. Stable isotope solutions that are used intravenously need to be tested for pyrogenicity to ensure they are safe for use in human studies.

quadrupole mass analyser. A type of mass analyser that filters ions based on their mass to charge ratio. It is comprised of four circular rods that separate ions based on the stability of their trajectory as they pass through the oscillating electric fields that are applied to the rods by alternating radio frequency and direct currents.

radioactive isotopes. 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. Zinc has five radioactive isotopes: ^{62}Zn, ^{63}Zn, ^{65}Zn, ^{69}Zn, ^{72}Zn.

relative standard deviation. The standard deviation expressed relative to the mean of the measurements (% RSD = SD/mean × 100); also known as the coefficient of variation (% CV).

sector mass analyser. A type of mass spectrometer that focuses the ions using a static electric or magnetic sector or a combination of the two. Double

focusing sector instruments focus the ion beam according to both direction and velocity. Lighter ions are deflected more than the heavier ions.

serum. The straw coloured liquid component of blood with all clotting factors removed. Serum is the supernatant that forms after whole blood is collected without an anti-coagulant, allowed to clot, and then centrifuged.

spot urine. A small urine sample collected at any time during the day.

stable isotopes. Stable isotopes have stable nuclei that do not decay or produce radioactive emissions. Stable isotopes occur naturally and are found in our body and in the foods we eat. The fraction of each stable isotope of an element that is found in nature is known as the 'natural abundance' of the isotope. Zinc has five stable isotopes: ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{70}Zn.

sterility. Tracer solutions should be sterile (aseptic) so that they do not contain any infectious microorganisms that would be capable of introducing infections when administered.

thermal ionization mass spectrometry. A type of mass spectrometry that uses heat to ionize the sample of interest off of metal filaments before the sample enters into the mass spectrometer.

tracee. The same substance (e.g. element) as the tracer that is already present in the system to be labelled. Before an enriched isotopic tracer has been introduced, the tracee is expected to be present in natural abundance.

tracer. (Also known as a label or tag). An identifiable substance, such as a dye or an isotope, that can be introduced into a biological system and followed through the course of a process. The tracer provides information on the process or on the redistribution of the substance. A tracer needs to be metabolized in the same way as the substance that it represents and the amount of tracer used should not alter the biological process or distribution.

true zinc absorption. Uptake and transfer of dietary zinc across the mucosal cells in the gastrointestinal tract, with consideration for zinc that is endogenously secreted back into the gut and excreted in the faeces.

ABBREVIATIONS

AAS	atomic absorption spectrometry
EDTA	ethylenediaminetetraacetic acid
EFZ	endogenous faecal zinc
EZP	exchangeable zinc pool
FZA	fractional zinc absorption
ICP-AES	inductively coupled plasma atomic emission spectrometry
ICP-MS	inductively coupled plasma mass spectrometry
IV	intravenous
LOD	limit of detection
LOQ	limit of quantification
PTFE	polytetrafluoroethylene
TIMS	thermal ionization mass spectrometry
TTR	tracer to tracee ratio
ZnO	zinc oxide

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This publication is part of the IAEA's continued effort to transfer technology and to contribute to capacity building for improved understanding of absorption, dietary bioavailability and nutritional requirements of all populations, particularly those at risk of zinc deficiency. The aim of this publication is to provide theoretical background information and to detail the practical application of state of the art methodologies to assess zinc metabolism. Based on stable isotope techniques, new data have been generated concerning absorption of zinc from indigenous diets and foods biofortified with zinc through selective agricultural breeding or fortified with zinc compounds. This information is essential for the design of effective food fortification programmes and to evaluate nutritional interventions for infants and children.

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