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No. 21

Assessment of Iron Bioavailability in Humans Using Stable Iron Isotope Techniques

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FOREWORD

The prevalence of iron deficiency is unacceptably high globally, in particular among infants, children and women of childbearing age in developing countries. Effective, food based strategies to combat iron deficiency are, therefore, urgently needed. As only a fraction of dietary iron is absorbed and utilized, access to data on iron bioavailability from foods, diets and iron fortificants is crucial in the development of food fortification strategies and interventions based on dietary diversification.

The IAEA assists Member States in their efforts to develop evidence based interventions to combat iron deficiency by fostering the use of stable isotope techniques to evaluate iron bioavailability. During the past 20 years, the IAEA has contributed 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 via the technical cooperation programme and coordinated research projects addressing priority areas in nutrition. Based on stable isotope techniques, new data have been generated on, in particular, iron bioavailability from iron compounds used in food fortification programmes and information gained about dietary enhancers and inhibitors of iron absorption in infants and children.

This book is an important part of the IAEA's continued efforts to transfer technology and to contribute to capacity building in this field by providing information on the theoretical background and practical application of state of the art methodology for iron bioavailability assessment.

The 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 iron bioavailability. The primary contributor to the preparation of this book, K. O'Brien (United States of America), is gratefully acknowledged for generously sharing her technical expertise and experience in this field.

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

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1. INTRODUCTION

1.1. BACKGROUND

Iron plays an essential role in human health. Deficiency of this trace element is one of the leading causes of both mortality and disability worldwide [1]. The World Health Organization (WHO) has estimated that anaemia affects 47.4% of children under the age of 5 years, 25.4% of school age children, 30.2% of non-pregnant women, 41.8% of pregnant women, 12.7% of men and 23.9% of the elderly [2], and approximately 50% of these cases may be related to iron deficiency. Effective food based strategies are urgently needed to help combat iron deficiency. The success of such strategies is dependent on bioavailable sources of iron, i.e. iron that can be absorbed and utilized by the body. Stable iron isotope techniques offer an invaluable tool to assess the bioavailability of iron from different foods as well as to evaluate factors influencing bioavailability. For example, in the development of food fortification strategies, the bioavailability of iron compounds is an important consideration and much of the current knowledge about the potential usefulness of different iron compounds, based on bioavailability, has been generated from studies using stable isotope techniques.

1.2. OBJECTIVE

The aim of this publication is to provide an overview of stable isotope techniques to assess iron bioavailability in humans. The book is designed to serve as a practical guide for new users, for example nutritionists and other health professionals, who are interested in the application of stable isotope techniques to assess iron bioavailability as an important step in the development of food based strategies to combat iron deficiency.

1.3. SCOPE

A review of the main approaches utilized to assess iron bioavailability and the advantages and disadvantages of each approach are presented. It is recognized that the mass spectrometric instrumentation typically used for the analysis of stable iron isotope ratios (thermal ionization mass spectrometry (TIMS) or inductively coupled plasma mass spectrometry (ICPMS)) remains both expensive and limited in availability. Due to the high cost and technical skill needed to set up new analytical laboratories for stable iron isotope studies, the assumption is

made that, in most instances, the analyses will be undertaken by a well established external laboratory. The emphasis of this publication is, therefore, placed on the methodologies needed to design, implement and analyse the data generated from stable isotope studies for those who are relatively new to the techniques.

1.4. STRUCTURE

Following the introduction, Section 2 presents a brief review of the global significance of iron deficiency and iron deficiency anaemia, and provides references and background reading for those interested in learning more about the indicators used to classify iron deficiency and iron deficiency anaemia. A basic understanding of the process by which iron is absorbed into the body and an understanding of key body iron pools is integral to the design of iron bioavailability studies. Section 3 reviews dietary haem and non-haem iron absorption, and whole body iron homeostasis. It is recognized that cellular iron trafficking is a rapidly evolving field and while significant advances have been made over the past 15 years many knowledge gaps remain, particularly in relation to the absorption of dietary haem iron. Section 4 introduces the concept of iron bioavailability, and defines stable and radioactive isotopes and the advantages and disadvantages of their use in human studies. There are three primary methodological approaches that have been used to assess iron bioavailability in human isotopic studies: faecal recovery of the ingested isotope, plasma appearance of the orally ingested isotope and red blood cell (RBC) incorporation of the iron isotope (following oral and/or oral and intravenous dosing with iron isotopes). These three approaches are presented in Section 5 as are the types of information that can be obtained using these differing study approaches. Section 6 discusses the basic principles of stable iron isotopes and general concepts of administration of stable isotopes, including the chemical form, dose selection, how to purchase and interpret isotopic enrichment of stable iron labels, distinctions between intrinsic versus extrinsic labelling and other aspects of study design that should be followed to ensure that the study design follows best practices. The most frequently used approach to assess iron bioavailability is the erythrocyte iron incorporation approach using single or multiple oral labels. Section 7 highlights this method and provides step by step procedures and examples of the calculations needed for each. Section 8 discusses special situations that may benefit from the use of both oral and intravenous isotopes to control for variability in the amount of absorbed iron that is incorporated into RBCs. The impact of this study design on the equations utilized is presented. It is often difficult to control for inter-subject variability in iron status when

undertaking studies on iron bioavailability and several approaches have been developed to deal with this problem. Section 9 provides information about these strategies and the equations used to normalize absorption data to a fixed index of iron status. Stable iron isotope ratios can be measured using TIMS or ICPMS. The specifics of each of these analytical approaches are presented in Section 10 along with supporting material should additional information be of interest. Many institutional human research review boards may not be familiar with stable iron isotope studies. Section 11 provides information on the ethical concerns associated with stable iron isotope use and provides web based templates and training course sites dealing with issues relevant to the process and resources to assist those new to clinical research. Statistical considerations that are necessary to inform sample size estimates along with useful web sites on this topic are presented in Section 12. Finally, suggestions for publishing data from iron bioavailability studies are provided in Section 13 and supporting ancillary material is provided in Appendices I–III.

2. IRON DEFICIENCY AS A PUBLIC HEALTH CONCERN

Iron deficiency is the most common nutrient deficiency in the world. It has been estimated that approximately 1.62 billion people globally are anaemic, representing 24.8% of the world's population [2]. Iron deficiency is thought to account for 50% of identified anaemia [2], and 800 000 deaths worldwide can be attributed to iron deficiency anaemia [3]. Deficiency of this trace element has adverse implications on health at all life stages. When iron deficiency occurs during critical windows of brain development, the cognitive deficits that occur may be irreversible and unresponsive to subsequent improvements in iron status [4, 5]. A growing literature highlights the relationships between the iron reserves of the newborn and the adverse impact of iron insufficiency on the neurochemistry, bioenergetics and morphology of the brain [4]. In adults, iron deficiency and iron deficiency anaemia can adversely impact physical work capacity and productivity, variables that may have a detrimental impact on economic potential. Anaemia often has the most significant impact on those that are most vulnerable. During the reproductive period, anaemic women have an increased risk of mortality and their infants also have an increased risk of dying in the perinatal period [2]. Clearly, the magnitude of adverse and often irreversible consequences associated with suboptimal iron status and iron deficiency anaemia

highlight the need to develop strategies to combat deficiency of this trace element.

To screen for anaemia at the population level, the indicator most often utilized is blood haemoglobin concentration and cut-off values have been established (Table 1).

Anaemia occurs as a result of dietary iron deficiency but it can also be due to a deficiency of other key nutrients (folate, vitamin B_{12} and vitamin A), infectious diseases (such as those caused by geohelminths, malaria or schistosomiasis), or inherited diseases that alter iron physiology (i.e. thalassaemia). To fully characterize iron status and classify anaemia as iron deficiency anaemia, additional biochemical indicators of iron status must also be analysed. A joint report by the WHO and the Centers for Disease Control and Prevention, United States of America provides a comprehensive review of methodologies used for the assessment of iron status along with benefits and disadvantages of individual iron status indicators [6]. This publication also provides an overview of the strengths and limitations of the methods available to control for inflammation and infections when defining iron deficiency and iron deficiency anaemia in those with concurrent infections. This resource may be useful for those interested in learning more about iron status indicators and selection of the best indicators to be used for the population group in question.

TABLE 1. HAEMOGLOBIN THRESHOLDS USED IN THE ASSESSMENT OF ANAEMIA [2]

Group	Age	Threshold (g/L)
Children	0.5–4.99 years	110
Children	5.00–11.99 years	115
Children	12.00–14.99 years	120
Non-pregnant women	15.00 years or older	120
Pregnant women		110
Men	15.00 years or older	130

3. IRON ABSORPTION AND BODY IRON POOLS

An understanding of basic iron absorption and body iron pools is vital to the design of stable isotope studies. This knowledge informs the selection of the form of iron isotope to administer (i.e. ferric versus ferrous, haem versus non-haem), the amount of iron to administer, the timing of sample collection and the selection of the biological sample to use for post-dosing assessment of stable isotope enrichment. For most minerals and trace elements in the human body, homeostatic mechanisms exist to respond to states of deficiency and excess by altering urinary losses, rates of intestinal absorption and secretion of the nutrient into the gastrointestinal tract (endogenous faecal secretion). These adaptive processes protect the body from both deficiency and the toxic consequences that can be caused from excess exposure. However, very little iron is excreted in the urine and sweat. Moreover, these and other routes of loss, such as endogenous faecal secretion, cannot be regulated to rid the body of excess iron once it has accumulated. As there are no physiologically regulated routes of iron excretion from the body, humans have evolved complex mechanisms to control the amount of iron absorbed from the diet. Regulation of intestinal iron absorption allows iron stores to be replenished when iron is lost from the body through bleeding, parasitic infections or when physiological needs increase during various life stages, such as rapid growth during childhood and adolescence or pregnancy.

Iron in the diet is obtained from both plant based (non-haem iron) and animal based (containing both haem and non-haem iron) sources. Additional iron can also be ingested in the form of supplements or fortified foods. Haem and non-haem iron are very different with respect to their bioavailability and absorption. A significant increase in the understanding of proteins involved in iron absorption has occurred over the past 10–15 years and the hormone integral to the control of whole body iron homeostasis (hepcidin) has now been identified [7–10]. To date, the majority of what is known about iron bioavailability has focused on non-haem iron as this typically comprises the majority of dietary iron.

3.1. NON-HAEM IRON ABSORPTION

Non-haem iron is ingested in the diet as either ferrous iron (Fe^{2+}) or ferric iron (Fe^{3+}). Non-haem iron is ingested from plant sources, animal tissues, iron fortificants or from contamination of foods, such as may occur from environmental sources such as cast iron cookware or soil.

The non-haem iron that enters the gastrointestinal tract is exposed to the apical side of the enterocytes lining the lumen of the small intestine. A specific iron transporter on the luminal surface of the enterocyte (divalent metal transporter 1 (DMT1)) facilitates iron entry into the cell. In order for non-haem iron to be transported by DMT1, it must be in the ferrous state. A ferrireductase (duodenal cytochrome b (Dcytb)) is present on the luminal cell membrane to help ensure that ferric (Fe^{3+}) iron reaching the small intestine is converted into ferrous (Fe^{2+}) iron that can be transported into the intestinal cell by the DMT1 protein. Expression of DMT1 is upregulated during iron deficiency to assist in absorption of dietary iron [11]. As iron must be in the ferrous state to be absorbed, reducing substances in the diet (such as ascorbic acid) may increase iron absorption by facilitating conversion of ferric to ferrous iron. Ascorbic acid is also a cofactor for Dcytb [12].

Once iron enters the enterocyte, it can either be stored as ferritin (and may subsequently be lost from the body when the enterocyte is sloughed off into the gut lumen), or it can be exported across the basolateral membrane of the enterocyte via iron export proteins. The basolateral exporter responsible for the extrusion of iron from the enterocyte is ferroportin (FPN). As iron is exported from the cell, the ferrioxidase, hephaestin (HEPH), assists in the conversion of ferrous iron into ferric iron. These proteins may also help maintain FPN on the cell surface [13]. Together, FPN and HEPH allow the iron in the enterocyte to be exported into the circulation where iron is picked up and bound by transferrin to be delivered to body tissues. In iron-replete individuals, approximately 20–40% of circulating transferrin is saturated with iron.

3.2. HAEM IRON ABSORPTION

To date, the process of haem absorption and the fate of the haem that enters the enterocyte remain unclear. Haem bioavailability is known to differ from that of non-haem iron and is not impacted by many of the inhibitors and enhancers known to affect bioavailability of non-haem iron [14]. Proteins thought to import haem have been identified [15, 16] but much of this process remains uncharacterized. Once haem enters the enterocyte, it is either degraded in the cytoplasm by haem oxygenase into ferrous iron or it may be exported intact via two known haem export proteins that are found in the enterocyte [17, 18]. Figure 1 depicts the proteins involved in the intestinal absorption of both non-haem and haem iron.

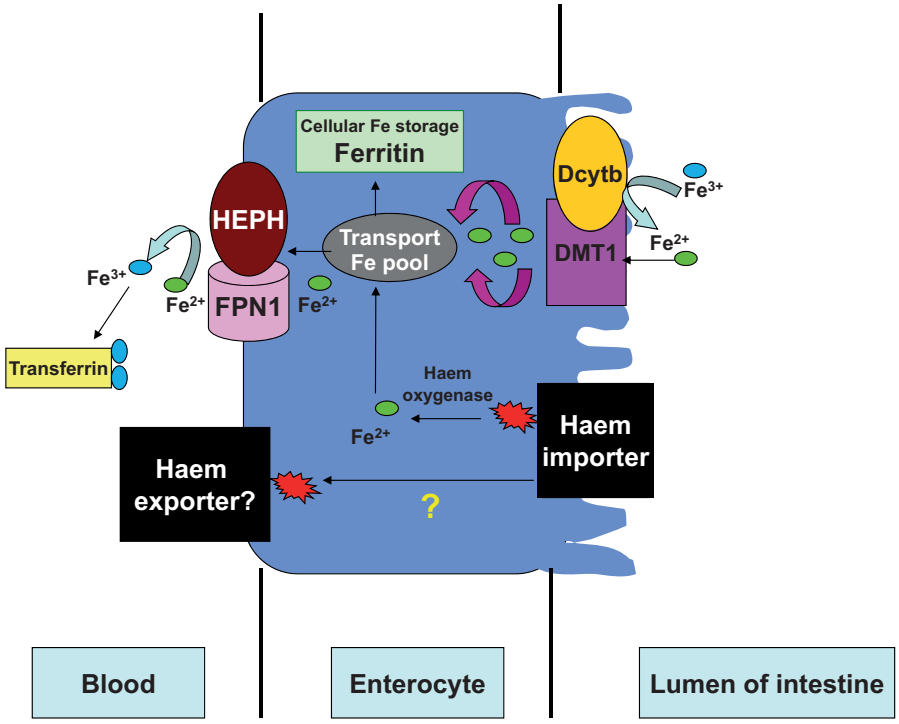

FIG. 1. Proteins involved in intestinal iron absorption.

When iron is consumed from dietary sources, the food ingested often contains a mixture of both haem and non-haem iron and both mechanisms of iron absorption are utilized to absorb the iron (Fig. 2). Once the diet mixes in the intestinal lumen, multiple dietary interactions can occur as a result of the enhancing and inhibiting components of the diet. Non-haem iron does not appear to substantially impact absorption of haem iron but studies have shown that animal tissue foods (meat, poultry or fish) will improve absorption of a non-haem iron source when ingested in the same meal [19, 20].

3.3. WHOLE BODY IRON PARTITIONING

Early studies of iron physiology demonstrated that the fraction of non-haem iron that is absorbed will decrease as the load of iron that is presented to the gut increases. This is illustrated by Bothwell and Finch [21]. For example, when 100 mg of iron is ingested, approximately 10% is absorbed, resulting in net iron absorption of 10 mg (100 mg × 0.1). When the load of iron presented to the gut is

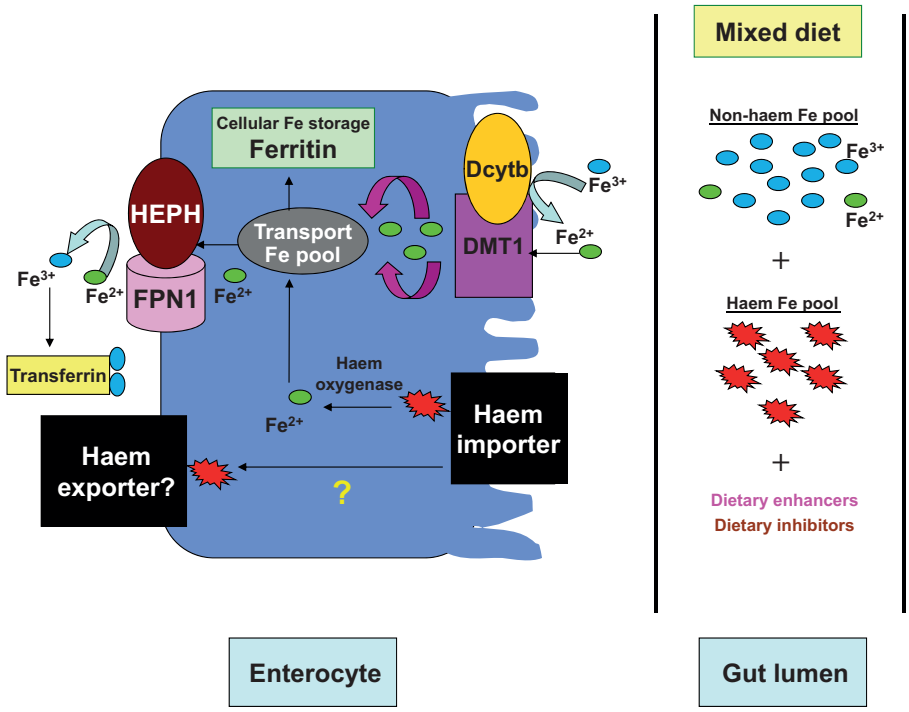

FIG. 2. Absorption of iron from a mixed diet involves both haem and non-haem pathways.

reduced, a far greater fraction of the iron will be absorbed but the net amount of iron absorbed will be considerably lower. For example, when 5 mg of iron is administered, approximately 1 mg of iron (20%) is absorbed. Thus, while absorption efficiency doubles, the net amount of iron absorbed is reduced to one tenth of that obtained from the 100 mg iron load. It is important to note that the examples above are based on iron absorbed in fasted subjects. The fraction of iron absorbed in the fasted state is typically much higher than that which occurs when iron is ingested as a component of a mixed meal that contains other inhibitory or enhancing components, and the amount of iron, or iron load, has substantially less influence on iron absorption when tested with food rather than in a fasting state [22].

Once iron exits the enterocyte, it is transported by circulating transferrin. The transferrin molecule can bind up to two atoms of iron and functions to keep iron in a non-reactive state as it is delivered to tissues that require iron. Iron dependent tissues express transferrin receptor on their cell membrane which binds to the iron-laden transferrin and allows it to be internalized and released for intracellular use or further metabolism. The majority of iron entering the

circulation is internalized into erythroid cells and is incorporated into haemoglobin. Within each RBC, there are approximately 250 000 molecules of haemoglobin — each can bind four atoms of iron. Thus, each RBC contains approximately 1 million atoms of iron. Of the iron that is absorbed into the body, roughly 80% is utilized by the RBC to facilitate oxygen transport. The remaining absorbed iron that is not utilized for RBC formation or found in myoglobin can be stored as ferritin or haemosiderin (an aggregate of iron). Iron is also an essential component of cytochromes and is a cofactor for many enzymes including those used for DNA synthesis [23].

Early studies using radioactive isotopes found that it takes 10–12 d for most of the absorbed iron to be incorporated into RBCs in healthy adult subjects [24, 25]. The timing of this process is integral to the design of iron bioavailability studies that utilize the erythrocyte incorporation method. The timing of this process may vary in infants. A study by Fomon et al. suggested that it may take nearly 28 d for all absorbed iron to become incorporated into RBCs in 56 and 168 day old infants [26].

As free iron is reactive, too much iron in the body can promote the generation of superoxide anions and hydroxyl radicals that can damage DNA, lipids and proteins. For this reason, most iron in the plasma is bound to transferrin and only very small amounts of iron circulate free as non-transferrin bound iron. Body iron stores in a typical adult male and female [21, 27, 28] are shown in Table 2.

RBCs have an average life span of 120 d. Once iron is incorporated into the RBC, the enrichment is typically stable over this 120 d period. As the RBC ages, the senescent RBCs are eventually ingested by macrophages. The majority of this RBC degradation occurs in the red pulp of the spleen. The liver also contributes, to a much smaller extent, to this process. The iron that is released from the catabolized haem is recycled to the bone marrow where it is again incorporated

TABLE 2. AVERAGE BODY IRON COMPARTMENTS IN ADULT MALES AND FEMALES

	Males	Females
Body stores (ferritin and haemosiderin) (mg)	1000	300
RBC mass (mg)	2300	1680
Myoglobin (mg)	320	205
Haem and non-haem enzymes (mg)	160	128

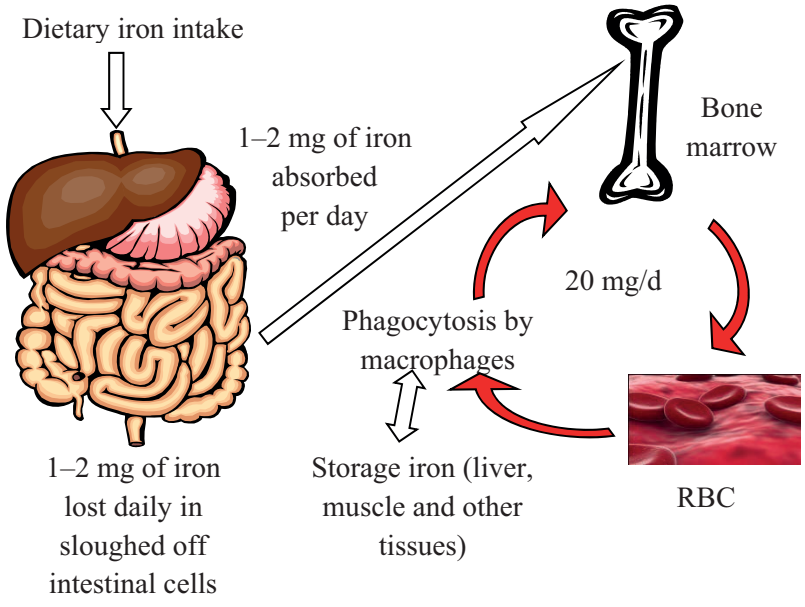

FIG. 3. Diagram of iron partitioning in the body (modified from Ref. [28]).

into RBCs or is stored in the body in the liver or bone marrow as ferritin or haemosiderin. Typical rates of iron absorption and partitioning in the body are detailed in Fig. 3.

Hepcidin is a small peptide hormone that is produced in the liver and functions to regulate intestinal iron absorption via its impact on the cellular iron export protein, FPN [29]. When hepcidin concentrations are elevated, the hepcidin protein binds to FPN causing it to be internalized, phosphorylated, ubiquitinated and then degraded [30, 31]. The end result of the hepcidin/FPN interaction is that intracellular iron is trapped and cannot be released from the enterocyte or other body tissues. It is increasingly recognized that many inflammatory processes cause a persistent elevation in hepcidin that serves to reduce iron absorption and iron mobilization from stores. This pathway is relevant to the anaemia of chronic inflammation and the known associations that are frequently observed between chronic infections and anaemia in the developing world [32].

The physiology of iron detailed above has implications for studies of iron bioavailability in normal healthy individuals. The majority of absorbed iron becomes incorporated into RBCs. For this reason, enrichment of orally administered stable iron isotopes in RBCs is commonly used as an index of iron absorption. The form of iron (haem versus non-haem) as well as the amount of

iron administered and other dietary factors of the test food and/or test meal must be considered when designing iron bioavailability studies.

3.4. SUMMARY

Humans have no excretory pathways that allow excess iron to be excreted from the body. The majority of absorbed iron is retained in the body to support the nearly 20 mg of iron being recycled daily in support of erythropoiesis [33]. Iron homeostasis must be very tightly controlled at the level of the intestine to increase or decrease entry of iron into the body in response to body iron stores and demands. In spite of numerous advances that have detailed the processes involved in intestinal absorption of iron, many questions on bioavailability of non-haem and haem iron remain.

These unique aspects of iron physiology inform the design of stable iron isotope bioavailability studies, the biological samples to use for assessment of isotopic enrichment and the timing of sample collection.

4. IRON BIOAVAILABILITY AND IRON ISOTOPES

4.1. DEFINITION OF IRON BIOAVAILABILITY

Bioavailability denotes the degree to which a particular nutrient can be absorbed and utilized from the dietary source in question [34]. Iron in the diet must be absorbed before the biological effects of this trace element are evident. As most absorbed iron becomes incorporated into RBCs, the amount of absorbed iron that becomes incorporated into RBC haemoglobin is often used as a measure of iron absorption.

Iron bioavailability can be influenced by:

— Characteristics of the test food itself (presence of inhibitors and enhancers);
— Composition of any other liquids or solids consumed along with the test food;
— Characteristics of the individuals' health status and physiology;
— The iron compound or form of iron administered.

Many variables are already known to impact iron bioavailability and these should be controlled for when designing a bioavailability study. These include the test conditions of the subject when studied (fed or fasted) and whether or not other components are present at the time the food is ingested (i.e. whether the food is tested when given as a mixed meal or if given along with other enhancing or inhibitory compounds). Components of the diet that enhance iron absorption include ascorbic acid and animal tissue (meat, poultry or fish). Dietary inhibitors of iron absorption include calcium, phytate, tannins and other polyphenols [35]. If bioavailability of an iron fortificant is being tested, this may be impacted by the formulation of the fortified food, the iron compound used, the way it is administered (capsule, powder, liquid, etc.) and the net iron load administered. When designing bioavailability studies, it is essential that as many of these variables as possible are controlled for in order to minimize variability and fully interpret the results generated.

In addition to close monitoring of the composition of the diet, careful selection of the study population is necessary to control for subject related variables that may also influence iron bioavailability. These variables include the age range, iron status, sex, physiological state, presence of inflammation or infections, and anthropometrics of the study population of interest. Other inter-individual characteristics may impact bioavailability but cannot be easily assessed or controlled for, including efficiency of digestion, gut transit time, the mucosal mass of key iron transport proteins and the presence of undiagnosed diseases, disorders or inflammatory processes.

4.2. IRON ISOTOPIC LABELS

Iron isotopes are frequently used as labels (sometimes referred to as tracers — in this publication, the term ‘label’ will be used) in iron bioavailability studies. The criteria for a useful label include: (i) that the label should be metabolized in the same way as the iron in the food or supplement it is being used to label and (ii) that the amount of label given should not interfere with the process that it is being used to examine. Iron isotopes can either be radioisotopes (i.e. they emit ionizing radiation) or stable isotopes (i.e. naturally occurring, non-radioactive isotopes that occur in fixed amounts in nature and in the foods that we eat). Isotopes of the same element have a similar number of protons (same atomic number) but differ from one another in their atomic mass due to variable numbers of neutrons.

4.2.1. Radioisotopes

Nearly all of the early work characterizing body iron pools and iron bioavailability was undertaken using iron radioisotopes [36], specifically ^{55}Fe and ^{59}Fe (Table 3).

Radioactive isotopes have some advantages in human studies in that they require a very small, essentially 'mass free', amount of iron label and the instrumentation needed for measurement of iron radioisotopes in biological samples is found in many research facilities. An additional advantage of the gamma emitting ^{59}Fe radioactive isotope is that whole body counting can be used to measure the fraction of iron isotope that is retained in the body after ingestion. Disadvantages to the use of radioisotopes include the fact that they have finite half-lives. This may introduce time limitations for shipping isotopes to isolated settings, storing isotopes before the study is implemented, and collecting and analysing the biological samples post-dosing. It is also becoming increasingly difficult to dispose of radioactive waste and many facilities do not have the special licences that are needed to administer radioactive isotopes to human subjects.

Typical radiation exposures from radioactive iron isotopes can be put into context by comparing the dose received to the mean yearly natural radiation exposure in the USA and the United Kingdom, which is expressed as committed effective dose equivalents (CEDEs). Typical doses of ^{55}Fe and ^{59}Fe used in an absorption study would represent a 5.4% increase in CEDEs for ^{59}Fe (19.7 d exposure) and 0.96% (3.5 d exposure) for ^{55}Fe [37]. There continue to be many safe applications of this approach [38–43] and several reviews on the use of radioisotopes in human nutrition have been published [37, 44]. As radioactive isotopes expose the subject to ionizing radiation, their current use is restricted primarily to non-pregnant, adult populations. These are often the population groups that are the least vulnerable to iron deficiency and stable iron isotopes are, therefore, needed when studying pregnant women, infants and children. For this reason, the remainder of this publication focuses only on stable iron isotope methodology, which can be applied in all population groups of interest.

TABLE 3. RADIOACTIVE IRON ISOTOPES USED IN HUMAN STUDIES

Isotope	Half-life	Type of radiation emitted
^{55}Fe	2.94 years	Low energy X rays
^{59}Fe	45 days	Beta (β) and gamma (γ) emissions

4.2.2. Stable isotopes

Stable iron isotopes do not emit radiation and are present in fixed amounts in the environment and in the foods that we eat, i.e. the naturally occurring abundance. The four stable isotopes of iron, the atomic mass and the natural abundance of each are detailed below (Table 4).

Units for equations are presented in milligrams because clinical haematological measures of iron status and dietary intakes are typically presented in this unit. To convert milligrams of iron into millimoles, the milligram quantity is divided by the atomic weight of iron: ($W_{\text{natural Fe}}$ = 55.845 mg/mmol).

$$\text{Fe (mmol)} = \frac{\text{Fe (mg)}}{W_{\text{natural Fe}}\ \text{(mg/mmol)}} = \frac{\text{Fe (mg)}}{55.845\ \text{(mg/mmol)}} \quad (1)$$

Administration of stable iron isotopes does not have any known adverse risks at the doses typically used in human studies. Since they are distinguished by mass differences, all three minor abundance stable iron isotopes can be given to the same subject to compare bioavailability of various foods or to allow for simultaneous administration of oral and intravenous isotopes. Multiple stable isotopes of different minerals can also be given to the same individual to examine interactions of iron with other nutrients such as calcium [47] or zinc [48]. Stable iron isotopes were first used in human studies in the 1960s [49] but their use in human nutrition studies was infrequent until the 1980s. Subsequently, research was undertaken to validate that radioisotope iron absorption data gave comparable data to those measured with stable isotopes in young and elderly men [50], and in healthy women [51]. Most stable iron isotope studies administer only

TABLE 4. ATOMIC MASS AND NATURAL ABUNDANCE OF STABLE IRON ISOTOPES (*based on Refs [45, 46]*)

Isotope	Atomic mass (g/mol or mg/mmol)	Naturally occurring abundance (at.%)
^{54}Fe	53.940	5.845
^{56}Fe	55.935	91.754
^{57}Fe	56.935	2.1191
^{58}Fe	57.933	0.2819

the two least abundant forms of iron (^{57}Fe and ^{58}Fe) and use the two remaining iron isotopes (^{54}Fe and ^{56}Fe) for internal calibration measurements as discussed later.

5. METHODS TO ASSESS IRON BIOAVAILABILITY

Basically, the methodology to assess iron bioavailability involves the administration of single or multiple oral stable iron isotope(s). Iron absorption is then estimated using one of three approaches:

(a) A metabolic balance study can be undertaken to recover the amount of oral stable iron isotope(s) excreted in faeces (faecal recovery method).
(b) A plasma sample can be obtained several hours post-dosing to assess plasma appearance kinetics (plasma appearance method).
(c) A blood sample can be collected 2 weeks post-dosing to assess the amount of stable iron isotope(s) incorporated into RBCs (erythrocyte iron incorporation method).

The first two approaches will be detailed only briefly, as they are less sensitive methods and are infrequently utilized at present. The majority of studies currently rely on the erythrocyte iron incorporation method which is described below in depth.

5.1. FAECAL ISOTOPE RECOVERY

Differences between the amount of oral stable isotope ingested and the amount lost from the body in faeces can be used to estimate iron absorption. Complete faecal collections are typically obtained for up to 7–10 d post-oral dosing, assuming that the majority of non-absorbed isotope in the gastrointestinal tract will have been passed over this interval of time [21]. Total dietary intake on the day of the absorption/bioavailability study is also monitored either with weighed diets or complete collections of duplicate meals. The equations used are presented below:

$$\text{net label absorbed (mg)} = \text{ingested Fe label (mg)} - \text{recovered Fe label (mg)} \quad (2)$$

$$\text{fractional Fe absorbed} = \frac{\text{net label absorbed (mg)}}{\text{ingested Fe label (mg)}} \quad (3)$$

$$\text{per cent Fe absorbed} = \text{fractional Fe absorbed} \times 100 \quad (4)$$

These measurements are then commonly applied to estimate the non-haem iron absorbed from the diet that was labelled by the stable isotope. This assumes that the fractional absorption is the same for the added stable isotope iron and the non-haem iron (see the discussion of extrinsic labelling in Section 6.6).

$$\text{diet non-haem Fe absorbed (mg)} = \text{diet non-haem Fe content (mg)} \times \text{fractional non-haem Fe absorbed} \quad (5)$$

This approach follows the same premise as a chemical balance method but is more accurate because the amount of stable iron isotope present in faeces can be directly traced to the test meal/test diet and can be distinguished from endogenous iron lost into the stool from sloughing off of intestinal cells or from endogenous faecal secretion (not an issue for iron but very important for other minerals and trace elements, such as calcium and zinc, where daily losses via this route are appreciable). An advantage to the metabolic balance approach is that it is non-invasive as it does not require collection of blood. To date, faecal recovery of orally administered iron isotopes has primarily been used in early studies assessing iron absorption in infants and adults [52–54]. The faecal recovery approach has also been used in comparisons with the erythrocyte iron incorporation method in infants [55].

The method has many disadvantages, and is imprecise and time consuming for both the subject and investigator. Subject compliance is essential as even small losses of excreted isotope that are not recovered will lead to an overestimation of the amount of iron absorbed. This is important as dietary iron intake averages only milligrams per day and the fraction of iron absorbed from the gut is typically low. In addition, if the stool collection containers contain iron or iron is introduced into the collection containers from the environment, this will reduce the enrichment of the isotope recovered and lead to an overestimation of net absorption. Faecal markers, such as dyes, radio-opaque pellets and rare metals (such as dysprosium), can be used to help correct for transit time differences. However, the faecal recovery approach is time consuming, requires a significant degree of subject compliance and places a significant burden on both the subject

and research investigator, and, therefore, this approach is rarely used at this time to assess iron bioavailability.

5.2. PLASMA ISOTOPE APPEARANCE

Plasma appearance of an orally administered iron isotope was used in early studies to assess plasma iron turnover by collecting multiple plasma samples at regular intervals post-dosing [21]. The method was suited to studies using radioactive iron isotopes because the small mass of iron in plasma was not a constraint to analytical measurement of plasma iron isotope clearance. As plasma iron averages only 1 μg/mL (0.0179 μmol/mL) and iron absorption is typically low, the dose of stable iron that would be needed to appreciably enrich plasma is generally not feasible given the design of most bioavailability studies.

For example, Whittaker et al. published data on plasma appearance of a stable iron isotope as a measure of iron absorption. In this study, a 5 mg oral dose of ^{54}Fe was administered to pregnant women and blood was collected at regular intervals for a 6 h period post-dosing [56]. Enrichment of the orally administered stable isotope in whole blood was compared to plasma enrichment obtained from a 200 μg dose of ^{57}Fe given intravenously as ferrous sulphate. Measurements were made using an early quadrupole ICPMS method with correction for polyatomic interferences [56]. More recently, Zimmermann et al. followed stable iron isotope appearance curves in whole blood obtained for 6 h post-dosing to assess changes in plasma hepcidin in response to iron loading [57]. Few studies at present use the plasma appearance approach as this method has been largely replaced by the erythrocyte iron incorporation method.

5.3. ERYTHROCYTE IRON INCORPORATION

Once absorbed, the majority of iron (often assumed to be approximately 80%) becomes incorporated into RBCs within 10–12 d. Once incorporated, enrichment will stay stable over the lifespan of the RBC (approximately 120 d in adults) and can, thus, be used as a proxy for iron absorption after either a single oral, multiple oral or oral and intravenous iron isotopes are given (Fig. 4).

Several assumptions are made when utilizing this approach, many of which were validated in early studies using radioactive isotopes undertaken in the 1940s [58, 59]. The following assumptions and requirements are used for the erythrocyte iron incorporation method:

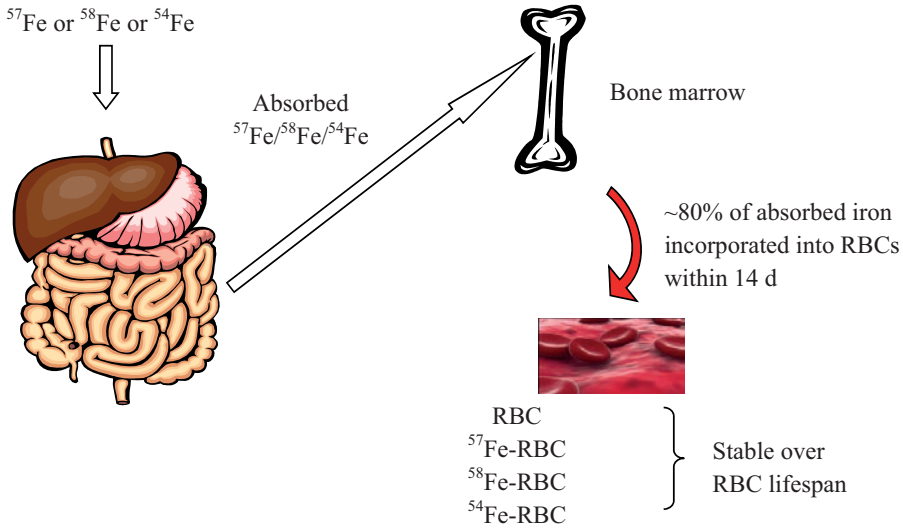

FIG. 4. Erythrocyte iron incorporation.

— Assumption that a fixed fraction of absorbed iron is incorporated into RBCs;
— Assumption that haemoglobin in RBCs contains a constant fraction of iron;
— Assumption that once absorbed iron has been incorporated into RBCs, it does not exchange with iron in plasma over the lifespan of the RBC;
— Requirement for an estimate of blood volume that is reliable for the study population of interest.

Key factors to consider when assessing iron bioavailability using stable isotope approaches are introduced below and are highlighted throughout the text:

— Characteristics of study population: sex, age range, health and iron status, physiological state.
— Study design: composition of test meals and number of administrations, use of reference dose.
— Administration of isotopes: in the fed or fasted state.
— Form of iron to be administered: sulphate, citrate, fumarate or other form.

— Number of isotopes to be given and the administration route: single isotope (oral administration), dual isotopes (oral administration), dual isotopes (oral and intravenous administration).
— Dose of stable iron isotope per administration.

6. STABLE ISOTOPE SELECTION AND DOSING

Regardless of the bioavailability approach selected, a dose of stable iron isotope will need to be selected that is sufficient for the study approach to be followed. Several points should be kept in mind during the purchase and preparation of the stable iron isotopes.

Dosing issues are dependent on the anticipated percentage of iron absorption, body pool size (RBC mass), accuracy and sensitivity of the mass spectrometer (limit of detection (LOD) and limit of quantification (LOQ)) as detailed below.

The LOD provides a measure of the lowest quantity of a substance that can be distinguished from the absence of that substance. This will determine whether or not the iron label can be detected when present in a biological sample of interest. The LOD is typically set at three times the relative standard deviation (RSD) of the mean ratio measurement (the average of a repeated number of isotope ratio measurements). In stable iron isotope studies, isotope ratio measurements are typically expressed in units of per cent enrichment or Δ% excess which is calculated as in Eq. (6):

$$\Delta\%\ \text{excess} = \frac{R_s - R_{\text{baseline Fe}}}{R_{\text{baseline Fe}}} \times 100 \tag{6}$$

where

R_s is the stable isotope label to reference isotope ratio in the sample;

and $R_{\text{baseline Fe}}$ is the stable isotope label to reference isotope ratio in the baseline sample.

In iron absorption studies, Δ% excess is used to express measurements of enrichment relative to baseline. Unless there was a previous isotope administration, the baseline measurement should reflect the natural abundance ratio. As an

example, to calculate Δ% excess when using measurements of ^{57}Fe:^{56}Fe ratios at baseline and after enrichment following administration of ^{57}Fe, using Eq. (6):

$$\Delta\% \text{ excess of } {}^{57}\text{Fe}:{}^{56}\text{Fe} = \frac{{}^{57}\text{Fe}:{}^{56}\text{Fe enriched} - {}^{57}\text{Fe}:{}^{56}\text{Fe baseline}}{{}^{57}\text{Fe}:{}^{56}\text{Fe baseline}} \times 100$$

The LOQ determines how much stable isotope label is needed to obtain a reliable measure of the amount or enrichment of label present or the limit at which one can reasonably tell the difference between two different values. The LOQ is typically set as ten times the RSD of the mean ratio measurement [60]. When using TIMS, the mean ratio measurement is often based on an average of 30 individual measurements.

When applying these concepts to study design, the lower the RSD of the mass spectrometer to be used, the lower the dose of stable isotope label that is required to elicit a measurable enrichment in the biological body fluid or tissue of interest. From a study design standpoint, lower doses decrease the risk of perturbing normal iron homeostasis, the risk of perturbing the normal ratio of iron to other components of the food matrix that may affect bioavailability, and the cost of the isotope required to complete the research.

As a general rule of thumb, although ^{58}Fe is more expensive when compared with ^{57}Fe or ^{54}Fe, a much lower dose of ^{58}Fe isotope is required to sufficiently enrich body pools because it is present at more than tenfold lower concentrations in the body. Lower iron doses are often essential, especially in certain groups such as exclusively breastfed infants, as human milk contains only 270 μg iron/L [61], and typical intakes from a single feed of human milk may be less than 30 μg of iron. For adults, the intake of iron in a single meal is considerably higher. For example, estimates from US adult men and women report typical iron intakes ranging from 12–18 mg/d [62]. The need to use a low dose of stable iron isotope must be counterbalanced against the amount required based on the sensitivity, precision and accuracy of the mass spectrometer.

Whenever possible, it is helpful to examine published data on doses used in previous studies in similar subjects to obtain information on the enrichment observed from the average dose of isotope given. If this information is available, it can be used to estimate dosing needs for a given target enrichment desired using the equations detailed below.

Example:

A previously published study administered 0.6 mg of ^{58}Fe orally and had an average excess of ^{58}Fe of 8% in RBCs collected 2 weeks post-dosing.

For a new study, the investigator wants to adjust the oral dose for a similar population. The RSD of the mass spectrometer for ^{58}Fe is 0.1%; therefore, the LOQ is 1% (i.e. ten times higher than the RSD of 0.1%).

While the LOQ is 1%, the investigator chooses to select a dose to achieve an average final enrichment of 4% which will allow for more variability in absorption among the new subject population.

Using the data above and a target enrichment of 4 Δ% excess of the iron label in the final sampling compartment, the new dose can be approximated as follows:

$$\text{new dose (mg)} = \frac{\text{new desired target } \Delta\% \text{ excess} \times \text{previous dose (mg)}}{\text{previous } \Delta\% \text{ excess in same sampling pool}} \quad (7)$$

Solving with the data in the example:

$$\text{new dose (mg)} = \frac{4\ \Delta\% \text{ excess} \times 0.6 \text{ mg } ^{58}\text{Fe}}{8\ \Delta\% \text{ excess in same sampling pool}} = 0.3 \text{ mg } ^{58}\text{Fe}$$

Assuming that one has enrichment data for one isotope but wants to change the stable isotope utilized in a subsequent study, Eq. (8) can be used.

Example:

The calculation above indicated that 0.3 mg of ^{58}Fe was required but now the investigator wants to administer ^{57}Fe to achieve the same enrichment post-dosing:

— Natural abundance values of each isotope are used.
— Natural abundance of ^{57}Fe = 2.1191%.
— Natural abundance of ^{58}Fe = 0.2819%.

To determine the dose of the new isotope required to give the same enrichment in the body pool of interest, the dose (in milligrams) of the previous isotope is used and adjusted for the isotopic differences in atomic mass and natural abundance as follows:

$$^{57}\text{Fe dose (mg)} = {}^{58}\text{Fe dose (mg)} \times \frac{m_{^{57}\text{Fe}}}{m_{^{58}\text{Fe}}} \times \frac{\text{NA}_{^{57}\text{Fe}}}{\text{NA}_{^{58}\text{Fe}}} \quad (8)$$

where

m is the atomic mass of the isotope indicated in subscript (Table 4);

and NA is the natural abundance of the isotope indicated in subscript (Table 4).

To solve the problem in the above example:

$$^{57}\text{Fe dose (mg)} = 0.3 \times \frac{56.935}{57.933} \times \frac{2.1191}{0.2819} = 2.216$$

This calculation indicates that 2.216 mg of ^{57}Fe will achieve the same enrichment as that obtained with 0.3 mg of ^{58}Fe.

If the dose of iron isotope required is large relative to the typical iron content of the test food, the iron label given can be split between multiple test meals. The advantage of this approach is to ensure that (i) the total amount of stable isotope label ingested is sufficient to adequately enrich the biological samples of interest (such as RBCs) and (ii) the dose of stable iron isotope given per test meal does not markedly impact the typical load of dietary iron ingested.

6.1. ISOTOPE PURCHASE

The amount of stable isotope label needed can be estimated after determining the number of subjects to be dosed and the dose per subject. Typically, ordering 20–30% extra will provide sufficient amounts to both account for sterility testing (if needed) as well as to include a few additional subjects to allow for subject dropout. It is preferable to purchase all stable isotope(s) needed for the study in one batch as this ensures that the same enrichment of the label(s) is used throughout the study which simplifies subsequent calculations. It also ensures that the amount of total iron administered per subject is consistent across the study. An additional benefit of obtaining all necessary label(s) in one batch is that prices are often based on the total amount of stable isotope purchased and it also minimizes pharmaceutical costs if external companies are involved with the preparation and testing of the doses. An example of the estimation used in ordering the appropriate amount of stable iron isotope when preparing doses is presented below.

It is assumed that the study design is to dose 20 women with 5 mg of ^{57}Fe orally: 5 mg ^{57}Fe × 20 subjects = 100 mg of ^{57}Fe is needed.

To add 30% extra: 100 mg ^{57}Fe × 1.3 = 130 mg of ^{57}Fe is needed.

To account for isotope enrichment (e.g. assume ^{57}Fe is 94% enriched): 130 mg ^{57}Fe/0.94 = 138 mg ^{57}Fe enriched material is required to provide 130 mg of ^{57}Fe.

Many isotope vendors will have several choices of enrichment available for each isotope and stable iron isotopes can be purchased as the metal or oxide. When multiple enrichments are available, it is generally preferable to purchase the highest enrichment available in order to minimize the total amount of iron required for either oral or intravenous dosing. This is particularly important to help ensure that the dose administered does not perturb normal physiology or alter the typical intake of iron from any given meal. The form of iron purchased (oxide or metal) should be taken into consideration when assessing the final form of stable isotope label to administer to minimize the work needed to convert the isotope into the form given to the study subjects and the possibility of contamination that might be introduced during this process.

Isotope costs vary in relation to natural abundance with cost typically increasing as the natural abundance of the stable isotope decreases. For example, as of 2011, ^{57}Fe was priced at approximately $13–23/mg of elemental material that was 94–96% enriched. Due to the lower natural abundance of ^{58}Fe, this isotope was more expensive and ranged in price from $43–53/mg of material (as the oxide or metal) that was 93–95% enriched.

6.2. PREPARATION OF STABLE ISOTOPE LABELS

The amount of isotope purchased should be weighed to validate the starting amount of isotope before preparing the label(s) for oral or intravenous use. The form of iron to be administered should mimic the form of iron typically ingested from dietary or supplemental sources. If comparing relative bioavailability from two sources, the total amount of iron load from each test meal should be kept equal between test substances in order to avoid differences in absorption caused by the mass of iron presented to the gut.

A method to convert iron metal into ferrous sulphate solutions has been published [63]. To improve shelf life and prevent oxidation, stable iron isotope labels are often stored under a nitrogen atmosphere or in containers that are flushed with argon before being sealed. When various forms of supplemental or fortificant iron are to be evaluated, these forms are typically best prepared by the pharmaceutical company or industry that manufactures these for commercial

FIG. 5. Packaging all dosing supplies in single use units facilitates field studies.

preparations to ensure that the stable iron isotope labels are as similar as possible to the compound being evaluated.

All of the chemicals that are used in the preparation of oral and intravenous doses should be free of exogenous iron. Glassware used to prepare the labels as well as vials used to store the final isotope solutions should be acid washed, rinsed thoroughly in deionized water and, for intravenous doses, be autoclaved prior to use. If exogenous iron is present in any of the chemicals or glassware during preparation, this will reduce the enrichment of the stable isotope label administered and can introduce an error in the subsequent calculations.

Based on the study design, doses can either be packaged as single use vials, so that once opened they can be immediately utilized or stored in one larger batch sufficient for dosing multiple study volunteers (Fig. 5). Each storage container should be clearly labelled with the isotope and total concentration of stable isotope per millilitre and stored in a secure setting at the appropriate temperature.

Whenever possible, doses should be stored in a hospital pharmacy and dispensed by trained pharmaceutical staff prior to each clinical study. If this is not possible, the best sanitary practices must be followed for the oral doses and sterility techniques for intravenous doses to protect patient safety. Packaging all ancillary supplies needed for dosing helps to standardize methods and facilitates implementation of studies in field settings.

6.3. ACCURATELY MEASURING THE DOSE ADMINISTERED

It is absolutely essential to ensure that an accurate measure of the total amount of isotope administered is obtained because all subsequent calculations are based on this measure. Should the dose of stable isotope label given not be accurately quantified, all subsequent data generated will be unreliable.

To accurately quantify the dose, the isotope solution is drawn up into a sterile syringe and the weight of the capped dosing syringe is recorded using a sensitive digital scale (optimally weighed to the nearest 0.001 g) (Fig. 6). Once the dose is administered, the empty capped syringe is weighed. The difference in weight can then be multiplied by the concentration of isotope per milligram assuming that the isotope solution weighs 1 g/mL. This amount is recorded as the amount of stable isotope given to the subject. When the label is administered as a dry powder, for example as ferrous fumarate, individual doses are prepared and weighed on a sensitive digital scale (optimally weighed to the nearest 0.001 g) before being added to the test meal (Fig. 7). The empty dose container is re-weighed and the administered dose calculated.

When the iron isotope is added to a food source, the food should also be pre- and post-weighed to ensure that all food is consumed. Complete food consumption can be facilitated by using spatulas and rinsing with water (Fig. 8). It is preferable to choose a test meal and amount that can be easily consumed by the age group being studied rather than to attempt to account for the fraction of the dose that was not ingested. Addition of the isotope to only the liquid portion of the meal is not recommended as this may increase the isotope absorption relative to mixing the isotope into the solid foods [64].

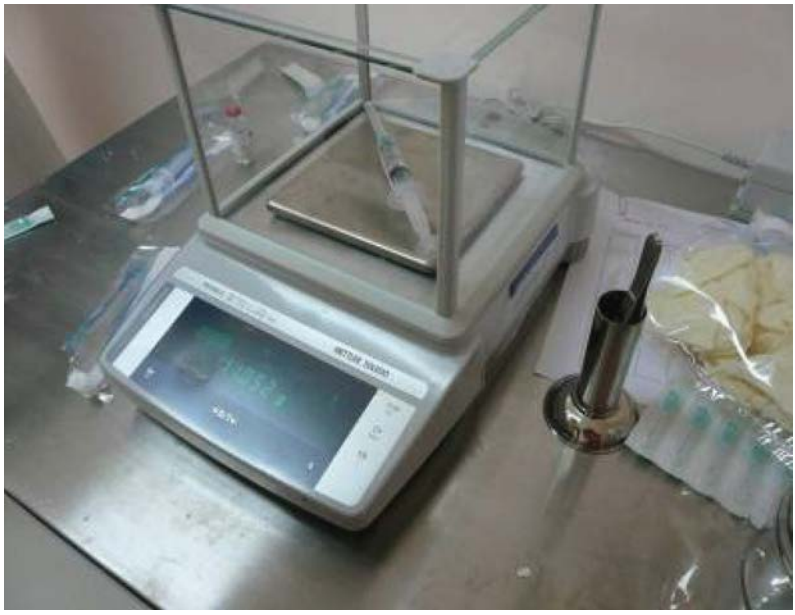

FIG. 6. Syringes containing stable isotope doses are pre- and post-weighed to determine the dose administered.

FIG. 7. Adding a weighed quantity of isotopic solution extrinsically to the test meal.

FIG. 8. All containers containing stable isotope doses should be rinsed and the rinse consumed.

6.4. STERILITY TESTING

Before preparing the stable iron isotope labels to be used in a research study, the investigator must first become acquainted with the local institutional rules and regulations for human research as these vary from institution to institution. In general, once the stable isotope doses for oral administration have been prepared and filtered through a 0.3 μm filter, they are often then sent out to be certified for sterility. This definitely applies to doses for intravenous administration. Many external laboratory facilities are available for this certification that typically takes 7–10 d.

6.5. CONFIRMING THE IRON CONTENT AND ENRICHMENT OF THE STABLE ISOTOPE LABELS

An enrichment sheet detailing the isotopic composition will be shipped along with the purchased enriched stable isotope. While this information is useful in planning the dose preparation, good practice requires that the isotopic composition of the final labelled material is verified by measuring the isotopic ratios and the total iron content. Such verification confirms the data provided by the isotope vendor and ascertains that no exogenous iron contamination has been introduced to dilute the isotope enrichment during the preparation of the doses. Verification of the dose and enrichment is essential to prevent errors that can jeopardize or ruin a study. Such errors have occurred when the purchased stable isotope was not enriched as claimed by the supplier, or when the iron content of the dose was inadvertently increased by iron contamination of glassware during dose preparation or sterility testing. To avoid such errors, it is extremely important to conduct a final verification of the dose enrichment and iron content.

An example of the enrichment information provided by the supplier is shown in Table 5. This information should be verified by measuring the isotopic ratios.

The total iron content of the dose should also be verified. Determination of the total iron content can be made by (i) applying mass corrections to the elemental concentrations obtained by either atomic absorption spectrophotometry (AAS) or inductively coupled plasma-atomic emission spectrometry (ICP-AES); or (ii) reverse isotopic dilution. Both have been described by Patterson et al. [60] and the first method will be further described here.

The AAS or ICP-AES instruments report weight concentrations in milligrams per millilitre based on the atomic weight of the naturally occurring element used in the standards for these instruments. However, this is not the average atomic weight of the iron in the enriched isotope solution. Since the light absorbance or emission readings of these AAS or ICP-AES instruments,

TABLE 5. EXAMPLE OF INFORMATION SENT WITH STABLE IRON ISOTOPES WHEN PURCHASED

Isotope	Naturally occurring abundance (at.%)	Enrichment of purchased ^{57}Fe (at.%)	Enrichment of purchased ^{58}Fe (at.%)
^{54}Fe	5.845	0.004	<0.010
^{56}Fe	91.754	2.996	0.129
^{57}Fe	2.1191	94.00	1.861
^{58}Fe	0.2819	3.000	98.00

respectively, are proportional to the number of atoms (millimolar concentration), the weight based concentrations reported by the AAS or ICP-AES can be corrected using the atomic weight of the isotope solution to determine the iron concentration of the enriched stable isotope in milligrams per millilitre. The calculated correction uses information on the enrichment data of the isotope label (provided by the supplier as in Table 5), the atomic weight of natural iron and the atomic mass of the iron isotopes.

For example, it is assumed that the goal is to prepare a solution of ^{57}Fe label with a concentration of 10 mg ^{57}Fe/mL, based on the ^{57}Fe listed in Table 5. As stable iron isotopes are not available at 100% enrichment, the total iron content of the solution will be higher than the stable isotope label iron concentration per unit volume. Once the label has been prepared and the fractional abundance of each isotope has been validated, a mass corrected element concentration of enriched isotope per unit is obtained.

Example: Calculation of the mass corrected concentrations of total iron and of ^{57}Fe isotope in the label

The following example demonstrates the calculation of the mass corrected concentrations of total iron and of ^{57}Fe isotope in the label in four steps (Eqs (9)–(11)). The first step is to calculate the atomic weight of the purchased ^{57}Fe-enriched iron sample, based on the supplier information:

$$W_S = \frac{\left[\left(m_{54_{Fe}} \times E_{54_{Fe}}\right)+\left(m_{56_{Fe}} \times E_{56_{Fe}}\right)+\left(m_{57_{Fe}} \times E_{57_{Fe}}\right)+\left(m_{58_{Fe}} \times E_{58_{Fe}}\right)\right]}{100} \quad (9)$$

where

W_S is the average atomic weight (mg/mmol) of the sample containing multiple isotopes;

m is the atomic mass (mg/mmol) of the isotope indicated in the subscript (from Table 4);

and E is the enrichment level (at.%) of the isotope in the subscript.

This example will use the information for the purchased highly enriched ^{57}Fe detailed in Table 5.

$$W_S = \frac{\left[\left(53.940\times 0.004\right)+\left(55.935\times 2.996\right)+\left(56.935\times 94.00\right)+\left(57.933\times 3.000\right)\right]}{100}$$
$$= 56.935 \text{ mg/mmol}$$

The average atomic weight for this particular highly enriched sample is essentially the same as for ^{57}Fe but this may not be the case if the sample is less highly enriched. This average atomic weight will be applied to determine the total iron concentration of the sample, but first the measured concentrations must be converted to millimoles per millilitre.

Although trace element concentrations are detected by AAS or ICP-AES in proportion to their molar concentrations, the instrumentation has reported the results in weight concentrations, using the average atomic weight of iron ($W_{\text{natural Fe}} = 55.845$ mg/mmol). Thus, the millimolar concentration detected can be determined by applying Eq. (1) to the instrument reported weight concentration (10.424 mg/mL in this example):

$$\text{uncorrected Fe concentration (mmol/mL)} = \frac{\text{uncorrected Fe concentration (mg/mL)}}{W_{\text{natural Fe}}\,(\text{mg/mmol})}$$
$$= \frac{10.424\ (\text{mg/mL})}{55.845\ (\text{mg/mmol})} = 0.1867\ \left(\text{mmol Fe/mL}\right)$$

The mass corrected total iron concentration can be determined by applying the average atomic weight, W_S, calculated above for this highly enriched isotope preparation, to the detected millimolar iron concentration as follows:

mass corrected Fe concentration (mg/mL)

= Fe concentration (mmol/mL) × W_s

= 0.1867 (mmol Fe/mL) × 56.935 (mg/mmol) = 10.63 (mg Fe/mL) (10)

The concentration of ^{57}Fe of the dose can be calculated (Eq. (11)) using the total detected millimolar concentration (calculated above), the enrichment reported by the supplier (94% from Table 5) and the atomic mass of the isotope (56.935 mg/mmol from Table 4):

$$^{57}\text{Fe isotope}(\text{mg/mL}) = \text{Fe concentration}(\text{mmol/mL}) \times E_{^{57}\text{Fe}} \times m_{^{57}\text{Fe}} \quad (11)$$

For this example:

$$^{57}\text{Fe isotope}\left(\text{mg/mL}\right) = \frac{0.1867\ (\text{mmol Fe})}{(\text{mL})} \times \frac{94\ (\text{mmol}\,^{57}\text{Fe})}{100\ (\text{mmol Fe})} \times \frac{56.935\ (\text{mg}\,^{57}\text{Fe})}{(\text{mmol}\,^{57}\text{Fe})}$$

$$= 9.99\ \text{mg}\,^{57}\text{Fe/mL}$$

After the isotope solutions have been prepared and the calculation above has been completed, it is helpful to label the doses with the stable isotope concentration per millilitre. In the example above, if the intended dose was 5 mg of ^{57}Fe, then 0.5 mL of the dosing stock solution would be needed based on the calculated ^{57}Fe concentration of 9.99 mg/ml. The actual iron content of this dose would be higher (5.32 mg/ml) because the ^{57}Fe label is not 100% enriched. Careful labelling will help avoid dosing errors.

6.6. EXTRINSIC VERSUS INTRINSIC LABELLING OF TEST MEALS

Isotopes can be added to the test meal either extrinsically or intrinsically:

— In extrinsic labelling, the stable isotope label is added directly onto or mixed with the test meal being studied. It is not incorporated into the food as would be the native iron found in the food. As it may not be located in the same compartments of the plant or bound to the same substances that it would be naturally, there is a risk that the bioavailability of the extrinsic label will not mimic the bioavailability of the iron found naturally in the test substance.

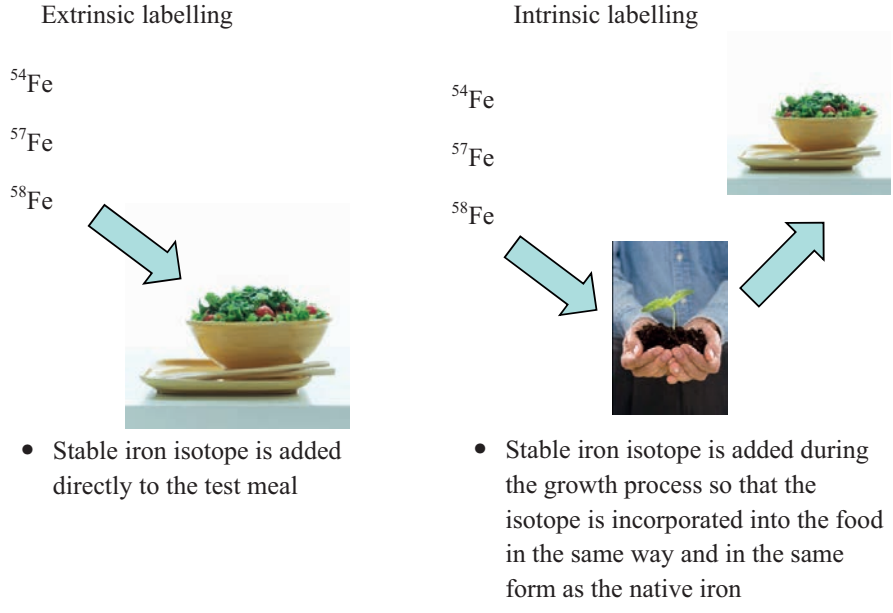

FIG. 9. Extrinsic versus intrinsic labelling.

— In intrinsic labelling, the stable iron isotope is incorporated into the food during the growth process to produce a labelled food which resembles the native food to the greatest extent possible. For a plant source, this would involve growing the food with an enriched source of iron (typically hydroponically) and then harvesting the food and determining the enrichment of the final product obtained. The advantage of this approach is that the label is then located in the same biological compartments and in the same form as the naturally occurring element (Fig. 9).

To prepare intrinsically labelled foods can be difficult and expensive, particularly if the plant source in question does not incorporate much iron and/or the edible portion of the plant is low in iron. Similar challenges occur when labelling animal food sources such as milk. For example, animal studies administering very large doses of intravenous stable iron isotope to lactating baboons have not been able to produce labelled milk [65]. Administration of radioactive iron isotopes to lactating sows has also failed to result in appreciable transfer of intramuscularly administered iron isotope to milk [66]. It appears that the pool of iron transferred to milk does not turn over rapidly or readily exchange with other body pools of iron. In instances such as this, bioavailability studies can only be undertaken by adding iron extrinsically to milk, for example to human

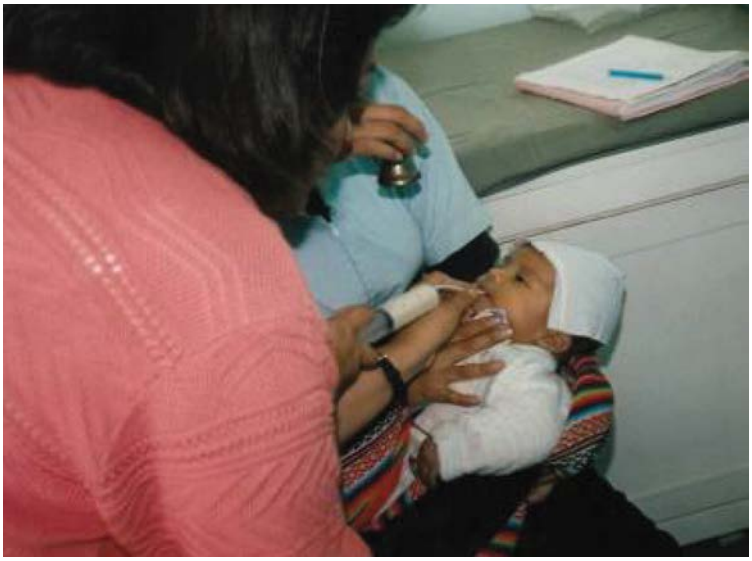

FIG. 10. Study of iron bioavailability in a Peruvian infant using extrinsically labelled human milk.

milk and then feeding the labelled milk to the infant. The extrinsic labelling technique has been used to assess iron absorption from human milk [67, 68]. The human milk can either be expressed and the iron isotope added to one or multiple feeds, or the stable iron isotope label can be administered directly into the mouth of the infant in the middle of a feed (Fig. 10). Extrinsically labelled human milk has also been used to assess the impact of lactoferrin on iron absorption in 2–10 month old infants, demonstrating that removal of lactoferrin leads to a significant increase in the absorption of iron from human milk [69].

Labelling a pharmaceutical product involves having enriched iron isotope replace the iron typically used during manufacturing. The process often involves complicated equipment and highly regulated technical methods. Extrinsic labelling cannot be used to study the bioavailability of commercial fortification forms of iron such as electrolytic iron and other elemental iron powders prepared for food fortification, Fe–amino acid chelates or NaFeEDTA. If the form of iron can be accurately reproduced in small quantities, it is generally preferable to have the manufacturer of the test compound make the enriched product to ensure that the isotope is in a form identical to that of the substance it is being used to trace.

For some foods, there are available data on relative differences in iron absorption from both intrinsically and extrinsically labelled food sources [64, 66, 70–76]. Based on this research, several investigators concluded that practically all the non-haem iron contained in a single meal contributes to a fully

exchangeable common pool of non-haem iron in the intestinal lumen, and that this generally validates the use of extrinsic labelling for studies of iron absorption from foods [64, 73–76].

7. ERYTHROCYTE IRON INCORPORATION: ORAL ADMINISTRATION OF LABELS

Many studies of erythrocyte iron incorporation utilize a paired study design and administer two oral iron isotopes (double oral isotope approach) to compare the relative bioavailability between two test meals (foods or fortificants) or between one test meal and a reference dose. The reference dose utilized is typically an aqueous dose of ferrous sulphate given along with ascorbic acid to ensure maximum absorption. If the dose of isotope needed for adequate enrichment is large, or if the amount of iron typically consumed is low, the oral isotope dose can be administered over several meals or over multiple days. Two weeks after the last label is ingested, a blood sample is collected to assess the relative amount of each isotope in RBCs. Most studies using this approach utilize the two lowest abundance iron isotopes (^{58}Fe and ^{57}Fe).

Most importantly, the paired study design allows each person to serve as their own control as iron status influences iron absorption significantly. In addition, with this approach estimations of blood volume and the fraction of absorbed iron that is incorporated into RBCs (discussed below) become less important as the information of interest is the relative difference in bioavailability between the test meals.

A step by step approach of analysis of the isotopic data collected from this type of study is detailed below. The first calculation estimates the size of the circulating iron pool.

7.1. DETERMINATION OF THE CIRCULATING IRON BODY POOL

$$\text{circulating Fe}\left(\text{mg}\right) = \text{Hb (g/L)} \times \text{BV}\left(\text{mL}\right) \times \frac{3.47\ \text{(mg Fe)}}{\text{g Hb}} \times \frac{1\ \text{(L)}}{1000\ \text{(mL)}} \quad (12)$$

where

Hb (g/L) is the haemoglobin concentration measured in whole blood collected from the subject on the first day of the study;

and BV is the blood volume, commonly determined from height and weight as described below for specific age and gender groups.

The subject's weight should be obtained on the first day of the study with a sensitive scale (to the nearest 0.1 kg). The amount of iron present in haemoglobin is based on an estimate as each gram of haemoglobin contains on average 3.47 mg of iron. Multiplication by 1/1000 converts the units for haemoglobin from grams per litre to grams per millilitre, to express all volumes in millilitres.

As seen above, estimation of the circulating iron pool requires an estimate of blood volume which is influenced by age, sex, body size, body composition and physiological state. Blood volume can either be directly measured or estimated based on weight and/or height with established formulas. Direct assessment approaches will be mentioned briefly but for the majority of bioavailability research applications, estimated measures of blood volume are generally utilized.

7.2. DETERMINATION OF BLOOD VOLUME

7.2.1. Direct measures

Techniques for assessing blood volume involve intravenous dosing with either a radioisotope (tagged to either RBCs or albumin) or an intravenously administered dye (Evans blue or indocyanine green) [77]. Chromium in the +6 oxidation state binds irreversibly to RBCs and of the available methods, ^{51}Cr–RBC volume (^{51}Cr–RBC) is considered to be the gold standard [77, 78]. While ^{51}Cr is a radioactive isotope, ^{53}Cr is stable and has also been used intravenously in pregnant women to obtain measures of blood volume [79, 80]. Human serum albumin (HSA) can be tagged with iodine isotopes (^{125}I or ^{131}I) to determine blood volume as well [81]. Recently, a semi-automated system which labels HSA with ^{131}I (the ^{131}I–HSA method) has been approved by the US Food and Drug Administration (FDA) [82]. Evans blue dye can be used to measure plasma volume and, subsequently, blood volume by spectrophotometry [83]. Although this method has the advantage of not involving radiation, Evans blue is a carcinogen and is no longer approved for use in the USA [77]. Indocyanine green is another dye method to assess plasma volume and there is no evidence of toxicity; however, it has not been FDA approved.

In many instances, the approaches that directly measure blood volume will not be possible due to the cost and limited access to the resources needed to undertake these studies. However, when comparing the bioavailability of two test meals, the relative difference is the information of interest and estimates of blood volume are generally sufficient. A number of robust equations exist to estimate blood volume based on combinations of body weight, length, surface area, lean body mass and age.

7.2.2. Indirect measures

7.2.2.1. Infants

Reliable blood volume averages in neonates are challenging due to variability in birth size, gestational age at birth and differences that also occur as a function of variable cord clamping times [84–86]. Common estimates used in iron studies assume a blood volume of 80 mL/kg during the first year of life [87, 88].

7.2.2.2. Children

Previous iron bioavailability studies in children have assumed set values of blood volume in children based on body weight and gender as 75 mL/kg in boys and 66 mL/kg in girls [89]. Alternatively, prediction equations have been published for infants and children between the ages of 0 and 14 years as a function of weight, height and body surface area for males and females [90]. Linderkamp et al. found that the strongest prediction equations for girls and boys were based on a combination of the child's weight and height [90] (Eqs (13, 14)).

Estimation of blood volume for males aged 2–14 years and females aged 2–6 years [90]:

$$\log BV = [0.6459 \times \log (\text{weight})] + [0.002743 \times \text{height}] + 2.0324 \qquad (13)$$

where

BV is the blood volume in millilitres;
height is in centimetres;

and weight is in kilograms.

Estimation of blood volume for females aged 7–14 years [90]:

$$\log BV = [0.06412 \times \log (\text{weight})] + [0.001270 \times \text{height}] + 2.2169 \qquad (14)$$

7.2.2.3. Healthy adults

Studies in healthy, non-pregnant adults have often assumed a fixed blood volume of approximately 65 mL/kg based on weight alone, or normative equations can be used that are based on both height and weight and other measures of body composition [91, 92].

Estimation of adult blood volume for women (derived from women 144–179 cm tall, weighing 45–73 kg) [91]:

$$BV = (16.52 \times \text{height}) + (38.46 \times \text{weight}) - 1369 \quad (15)$$

Estimation of adult blood volume for men [92]:

$$BV = (28.5 \times \text{height}) + (31.6 \times \text{weight}) - 2820 \quad (16)$$

7.2.2.4. Pregnant women

Blood volume estimates are challenging to predict as pregnancy is a dynamic physiological state and plasma volume and red cell mass change throughout gestation (about a 50% and 30% increase, respectively). Blood volume during pregnancy has previously been estimated to be approximately 70 mL/kg [93]. The importance of accurate estimates of blood volume has been highlighted by Whittaker et al. [94]. For example, if blood volume estimates were inaccurate by 10 mL/kg, estimates of iron incorporation would be inaccurate by 12%. Medical conditions during pregnancy, such as pre-eclampsia may also impact blood volume estimates and need to be taken into account [80].

7.3. DETERMINATION OF ISOTOPE INCORPORATED INTO RED BLOOD CELLS

Once blood volume has been estimated or measured, the next step involves measurement of the net amount of stable isotope that has been incorporated into the RBCs. After circulating iron has been estimated from Eq. (12), the total amount of stable isotope incorporated into RBCs can be estimated. Equations for this process have been published [26], and are detailed below in Eq. (17).

Amount of iron isotope incorporated into RBCs:

$$^{57}\text{Fe}_{\text{inc}} = \frac{R^t - R^0}{R^0} \times \frac{\text{circulating Fe (mg)}}{W_{\text{natural Fe}}} \times \frac{\text{NA}_{^{57}\text{Fe}}}{100} \times m_{^{57}\text{Fe}} \quad (17)$$

where

$^{57}Fe_{inc}$ is the RBC iron isotope incorporation in milligrams;
R^t is the ^{57}Fe:^{56}Fe ratio at time t after dosing;
R^0 is the ^{57}Fe:^{56}Fe ratio at baseline;
$W_{natural\ Fe}$ is the average atomic weight of Fe = 55.845 mg/mmol;
NA_{57Fe} is the natural abundance of ^{57}Fe = 2.1191at.% (Table 4);

and m_{57Fe} is the atomic mass of ^{57}Fe = 56.935 mg/mmol (Table 4).

A similar calculation would apply for administered ^{58}Fe, using the associated ratios, natural abundance (NA_{58Fe} = 0.2819at.%) and atomic mass (m_{58Fe} = 57.933).

$$^{58}Fe_{inc} = \frac{R^t - R^0}{R^0} \times \frac{\text{circulating Fe (mg)}}{W_{natural\ Fe}} \times \frac{NA_{58Fe}}{100} \times m_{58Fe}$$

where

$^{58}Fe_{inc}$ is the RBC iron isotope incorporation in milligrams;
R^t is the ^{58}Fe:^{56}Fe ratio at time t after dosing;
R^0 is the ^{58}Fe:^{56}Fe ratio at baseline;
$W_{natural\ Fe}$ is the average atomic weight of Fe = 55.845 mg/mmol;
NA_{58Fe} is the natural abundance of ^{58}Fe = 0.2819at.% (Table 4);

and m_{58Fe} is the atomic mass of ^{58}Fe = 57.933 mg/mmol (Table 4).

7.4. ESTIMATION OF IRON ABSORPTION, BASED ON RED BLOOD CELL INCORPORATION

A large proportion, but not all, of the absorbed iron is typically incorporated into RBCs in healthy adults and children. An estimate of RBC incorporation is necessary to account for the fraction of iron that was absorbed but not incorporated into RBCs. Mean RBC incorporation has been reported as ~80–85% in 8 healthy US men [95], ~60% in 31 healthy US men [96], ~80% in 36 US women with a mean serum ferritin of about 20 µg/L [97], ~93% in 25 healthy Thai women [98], ~80% in 30 healthy British men [99], and ~76% and 73%, respectively, in 28 iron deficient and 8 iron sufficient Canadian infants [100]. A commonly used assumption of 80% RBC incorporation can help facilitate

comparisons between iron bioavailability studies. This assumption is used here in the calculation of absorption.

Calculation of per cent ^{57}Fe absorption:

$$^{57}Fe_{absorbed}(\%) = \frac{^{57}Fe_{inc}}{^{57}Fe_{dose}} \times \frac{100}{80} \times 100 \quad (18)$$

where $^{57}Fe_{inc}$ is from Eq. (17);

and the assumed incorporation of the absorbed isotope into erythrocytes is 80%.

The calculation for per cent ^{58}Fe absorbed is similar but first an adjustment is made for cross-contamination.

7.5. CORRECTIONS FOR CROSS-CONTAMINATION FROM MULTIPLE LABELS

When two stable iron isotope labels are administered, an additional challenge becomes evident because the ^{58}Fe and ^{57}Fe enriched stable iron isotopes that are commercially available are not 100% enriched. Iron stable isotopes can typically be purchased in enrichments over 90% but the ^{57}Fe purchased also contains a small amount of ^{58}Fe, and the ^{58}Fe contains a small amount of ^{57}Fe as shown in the example of the isotopic distribution for iron isotopes provided in Table 5.

As already discussed, a much larger mass of ^{57}Fe is needed to get the same enrichment of RBCs as that which would be needed from ^{58}Fe. The cross-contamination of isotopes that occurs when both isotopes are used generally leads to a larger measurement error for the ^{58}Fe data because the larger dose of ^{57}Fe given contributes a larger fraction of ^{58}Fe relative to the ^{58}Fe label administered. Consequently, it is common to only adjust for the cross-contamination of ^{58}Fe from the ^{57}Fe dose. The calculated adjustment, as described by Kastenmayer et al. [63], is presented here, and alternative approaches have been published by Walczyk [101] and by Toffolo et al. [102], with the latter offering software programs available on request[1].

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The adjustment for cross-contamination is described in three steps (Eqs (19)–(21)). First, calculation of the amount of ^{58}Fe coming from the ^{57}Fe label and incorporated into erythrocytes:

$$^{58}Fe^{**}_{inc} = {}^{58}Fe^{*} \times \frac{^{57}Fe_{absorbed}\,(\%)}{100} \times \frac{80}{100} \tag{19}$$

where

$^{58}Fe^{**}_{inc}$ is the ^{58}Fe incorporated (mg) into erythrocytes from the ^{57}Fe label;
$^{58}Fe^{*}$ is the ^{58}Fe administered with the ^{57}Fe label (mg);
the assumed RBC incorporation of the absorbed isotope is 80%;

and an identical absorption is assumed for the ^{58}Fe and the ^{57}Fe present in the ^{57}Fe label.

Second, correction of the amount of incorporated ^{58}Fe by subtracting the portion which came from the ^{57}Fe label (calculated in Eq. (19)) from the total amount of incorporated ^{58}Fe:

$$^{58}Fe_{inc/corr} = \text{total}\,{}^{58}Fe_{inc} - {}^{58}Fe^{**}_{inc} \tag{20}$$

Finally, calculation of per cent ^{58}Fe absorption:

$$^{58}Fe_{absorbed}\,(\%) = \frac{^{58}Fe_{inc/corr}}{^{58}Fe_{dose}} \times \frac{100}{80} \times 100 \tag{21}$$

8. ERYTHROCYTE IRON INCORPORATION: INTRAVENOUS ADMINISTRATION OF LABELS

8.1. WHEN IS INTRAVENOUS ADMINISTRATION HELPFUL?

For some physiological states, the incorporation rate of absorbed iron into RBCs may be highly variable. If so, using the above mentioned assumption of an 80% incorporation rate would limit the interpretation of study findings. RBC iron incorporation has been reported to be inversely related to body iron status [96, 103],

and to decrease with iron supplementation of healthy women [104]. Such differences in RBC iron incorporation between individuals will not generally affect studies of bioavailability of iron from food sources and/or iron fortificants where individuals serve as their own controls. However, in some studies, it may be beneficial to measure RBC iron incorporation to directly adjust for the amount of orally administered isotope that is incorporated into RBCs. Several examples where direct measurement of this value may be helpful are detailed below.

8.1.1. Pregnant women

Pregnancy is a highly dynamic state and many variables could potentially alter both the ability of pregnant women to appropriately expand their blood volume during pregnancy and the utilization of absorbed iron for maternal RBC formation. If the primary research question is not focused on relative differences in bioavailability of two foods in a pregnant cohort with well defined iron and health status, an intravenous isotope must be given to fully explore iron dynamics at this life stage. This was found to be necessary in a study of pregnant Peruvian women addressing the impact of prenatal iron supplementation on iron absorption [93]. In this study, RBC iron incorporation was highly variable among these pregnant women and was influenced by iron supplementation ranging from a mean of 92% (in women not supplemented with iron) to a mean of 76% (in women receiving prenatal iron supplements). If an 80% estimate of RBC iron incorporation had been used, this would have altered the interpretation of the study findings in this cohort [93].

8.1.2. Premature infants

Studies using stable iron isotopes have noted that far less than 80% of absorbed iron appears to be incorporated into RBCs in premature infants [105, 106]. In these instances, additional information and more accurate data may be obtained if erythrocyte iron incorporation is directly measured.

8.1.3. Individuals with diseases that impact iron homeostasis

Many diseases, inflammatory states and infections could potentially impact RBC catabolism, macrophage iron recycling or blood volume estimations [98, 107]. In these situations, it may also be beneficial to directly measure RBC iron incorporation instead of using assumptions for this measure.

8.2. MEASUREMENT OF ISOTOPE INCORPORATED INTO RED BLOOD CELLS

The fraction of intravenous iron isotope that is incorporated into RBCs can be directly measured if an intravenous dose of iron is administered. Ferrous citrate is often utilized for these studies as it appears to be less anaphylactic (i.e. less likely to lead to allergic reactions that can be life threatening) compared to several other forms of intravenous iron that are available. In all instances, the subject should be under medical supervision and be closely monitored during any intravenous infusion of iron.

In studies where an intravenous dose is administered, a second label is given orally and the data can then be adjusted for the actual fraction of iron that becomes incorporated into RBCs. This approach assumes that the metabolism of the intravenous iron (in whatever form it is administered) is similar to the metabolism of the oral label once absorbed. While the approach may have limitations, it does allow perturbations in RBC iron incorporation to be identified which otherwise would not be measurable.

The calculations used with oral and intravenous labels are very similar to those detailed above for administration of two oral labels. The size of the circulating iron pool (Eq. (12)) is applied to determine the amount of the intravenous isotope incorporated into RBCs (Eq. (17)). This is then expressed as a percentage of the intravenous isotope dose administered and is assumed to represent the percentage of RBC incorporation of the absorbed oral label. The measured incorporation rate into RBCs is applied (instead of an assumed 80%) in the subsequent calculation of percentage of iron absorption.

8.3. SPECIAL CONCERNS WHEN USING INTRAVENOUS ADMINISTRATIONS OF IRON ISOTOPES

Most bioavailability studies do not require the use of intravenous iron isotopes but it is important to note that whenever intravenous iron isotopes are utilized, additional safety precautions are required because intravenous iron can be anaphylactic.

The majority of the safety literature on intravenous iron dosing has been compiled from clinical uses of intravenous iron for the management of the anaemia resulting from kidney disease. At present, the FDA has approved three forms of intravenous iron for use in the USA: iron dextran, sodium ferric gluconate complex in sucrose and iron sucrose [108]. Reviews are available on the risk of hypersensitivity or death caused by these intravenous iron preparations [108, 109]. Reported rates of all adverse events from the use of iron dextran,

ferric gluconate and iron sucrose are approximately 29.2, 10.5 and 4.2 reports per million 100 mg dose equivalents for the forms above, respectively [108]. When presented as fatal events, these numbers are 1.4, 0.6 and 0.0 reports per million 100 mg dose equivalents for iron dextran, ferric gluconate and iron sucrose, respectively [108, 109].

The possibility, albeit very rare, of anaphylactic events necessitates that appropriate medical resources be on hand when administering intravenous iron isotopes. While the doses used to clinically treat anaemia are substantially greater than the doses typically given intravenously for research studies, the anaphylactic potential of the compound remains evident also at smaller doses. To minimize possible anaphylactic events, the majority of studies administering intravenous stable iron isotopes have utilized intravenous ferrous citrate infusions, have given only small doses (<0.6 mg) and have infused these amounts slowly under medical supervision [93, 98, 105, 106, 110]. All anaphylactic precautions and medical treatment resources should be on hand when infusing intravenous stable iron isotopes. As in all human research, research plans must be approved by the institutional human research review committees.

Doses prepared for intravenous administration should be tested for pyrogens in addition to the sterility testing. Pyrogens are substances that are capable of producing a fever. External laboratories or clinical laboratories in hospital settings are typically utilized for this type of testing.

9. REDUCING INTER-SUBJECT VARIABILITY

9.1. NORMALIZATION TO A REFERENCE DOSE

As iron absorption is heavily influenced by the iron status of the individual, iron absorption data are variable between subjects. One way to control for inter-subject variability is to provide one stable isotope as a reference dose in addition to another stable iron isotope given along with the test meal or iron compound. The reference dose should be selected to represent iron absorption under optimal conditions and is typically given to subjects when they are in a fasted state. The reference dose is frequently 3 mg of iron as ferrous sulphate mixed with ascorbic acid at a 2:1 ascorbate:iron molar ratio [111]. To allow for comparisons between study subjects, the reference dose absorption data are standardized to a fixed reference value (typically 40%) and the fraction of the test meal absorbed relative to the reference dose is then calculated as follows.

Normalizing iron absorption data to a reference dose:

$$\text{absorption}_N = \text{absorption}_O \times \frac{40}{\text{absorption}_{Ref}} \quad (22)$$

where

absorption is expressed as a percentage of the ingested dose;

and subscripts N, O and Ref indicate normalized, observed and reference dose, respectively.

Example: Normalizing data

As an example, the measured iron absorption from a test meal was 5% while the absorption of the reference dose of iron was 11%. Using the procedure above, the reference dose absorption is normalized to 40% and this ratio is then used to adjust the test meal data as follows:

$$\text{absorption}_N = 5 \times \frac{40}{11} = 18.2\%$$

Applying this normalization process to the data generated from each subject provides a means of correction for differences between subjects. This approach has been utilized in some studies of iron absorption [112, 113].

9.2. NORMALIZATION TO IRON STATUS

An alternative approach to adjust for the impact of iron status on iron absorption from test meals has been developed by Cook et al. based on studies in adults. Equation (23) is based on the inverse relationship between serum ferritin and iron absorption [114], and can be used to adjust iron absorption data to a reference serum ferritin concentration (typically to reference values of 23 or 40 µg/L) as follows:

Normalizing absorption data to a reference serum ferritin concentration:

$$\log\left(\text{absorption}_N\right)=\log\left(\text{absorption}_O\right)+\log\left(\text{ferritin}_O\right)-\log\left(\text{ferritin}_R\right) \quad (23)$$

where

absorption_N is normalized dietary iron absorption (%);
absorption_O is observed dietary iron absorption (%);
ferritin_O is observed serum ferritin concentration (μg/L);

and ferritin_R is reference serum ferritin concentration (μg/L).

This normalization of absorption results to a common iron status is useful for comparing iron bioavailability between studies involving different subjects [111, 115]. As demonstrated in adults, there is a linear relationship between log iron absorption and log serum ferritin. The slope of the line has been found to be constant across a range of serum ferritin concentrations from deficient to adequate (<10 to 100 μg ferritin/L); only the intercept of the line varies with the iron bioavailability of the test meal [111]. Thus, the measurement of relative iron bioavailability between different iron sources (expressed as a ratio) is independent of iron status.

Further comparison between studies is possible using the relationship that subjects with a serum ferritin of 23 μg/L absorb 40% of a reference dose of iron [116].

The normalization of iron absorption using serum ferritin has the limitation that ferritin is also an acute phase protein, i.e. the concentration of ferritin can increase as a result of inflammation or infection. Individuals with concurrent infections may, therefore, appear iron sufficient due to their artificially high serum ferritin value.

10. INSTRUMENTATION AND TECHNIQUES USED FOR MEASUREMENTS

The two primary analytical methods that are currently utilized for analysis of stable iron isotopes are TIMS and ICPMS. The principles of each approach

along with their advantages and disadvantages are detailed below. Regardless of which analytical method is utilized, the LOD and the LOQ should be kept in mind when selecting the doses of isotope(s) to administer.

Key components of both ICPMS and TIMS instruments include: (i) a sample inlet which provides a means of introducing or loading the sample into the mass spectrometer; (ii) a source capable of ionizing and focusing the sample to produce gas phase ions; (iii) a mass analyser capable of separating the ions generated according to their mass to charge (m/z) ratio; (iv) an ion detector which will detect the amount of each stable iron isotope of interest; and (v) a data system to process and collect the data generated. Both ICPMS and TIMS have individual strengths and weaknesses as detailed below along with a brief description of each approach.

10.1. THERMAL IONIZATION MASS SPECTROMETRY

Thermal ionization differs from ICPMS in the way the samples are ionized. In TIMS, the iron is first chemically purified and isolated from the biological fluid or sample of interest. For iron, this first step generally requires acid digestion of the sample (typically faeces or whole blood). Once the sample is digested, it is reconstituted in dilute HCl and the iron is then purified. The most common means of separating iron from other elements is anion exchange chromatography in HCl medium. Using this approach, the retention of iron on the anion exchange resin increases with HCl molarity but Cr, Ni and other elements are not retained. This allows the sample to be purified prior to loading on a rhenium filament. Examples of a sample digestion procedure and methods for anion ion exchange chromatography are provided in Appendices I and II.

Extracted iron is reconstituted in dilute ultrapure HNO_3 before being loaded onto ultrapure metal filaments. Filaments for iron analyses are typically comprised of rhenium as this metal allows for optimal ionization. Rhenium filaments used for iron measures are zone refined and ultrapure (99.99% pure).

To enhance ionization efficiency, 3–4 μL of silica gel slurry is first heated onto the filament. Next, approximately 5–8 μL of the extracted iron is then added and dried onto the filament followed by 3–4 μL of phosphoric acid (0.7M). Filament loading systems are available that allow multiple samples to be loaded and heated concurrently. Filaments are then loaded onto a sample carousel (or wheel) and inserted into the source housing of the mass spectrometer. Most TIMS sample wheels can accommodate from 13 to 21 samples at a time. The more samples that can be introduced into the source, the more efficient the process becomes due to less time being wasted as the vacuum is re-established between sample magazines. In the new TIMS instrumentation, the establishment of the

vacuum is rapid and takes approximately 30 min to reach 10^{-7} torr (1.33×10^{-5} Pa). This process can be considerably longer in older instruments. To ionize the sample, filaments are heated to 1100–1300°C under vacuum. An external pyrometer can be used to control the process and account for filament to filament variability. The positive ions generated are first focused through an electric field in the source and then mass-separated using either a quadrupole or magnetic sector mass analyser. The differences in these two types of mass analysers are detailed below.

10.1.1. Quadrupole mass analyser

A quadrupole mass analyser (Fig. 11) is comprised of four rods approximately 1 cm in diameter and 20 cm in length. By adjusting the combination of voltages and radiofrequencies applied to the rods, specific m/z ions are selected and focused along the length of the rods to the detector. The cycling of the quadrupole voltages and radiofrequencies allows different masses of interest to be collected. The speed at which settings are altered on the rods determines the scan speed. With quadrupole instruments, only one specific m/z value can reach the detector at any given time. The other m/z ions do not have a stable trajectory and are ejected from the quadrupole. The limitation to this approach is that it suffers from spectral interferences and lower sensitivity and precision due to the inability to focus all ions concurrently.

Early TIMS instruments relied on a quadrupole for mass separation and achieved RSDs of approximately 1% for mineral isotopic measures [118, 119]. These were first released in the 1980s but have now been largely replaced by magnetic sector instrumentation due to the significant increase in precision and accuracy.

10.1.2. Magnetic sector mass analyser

In sector instruments, ions are focused with magnetic and electrical fields so that multiple dispersed ions can be collected concurrently. Charged particles

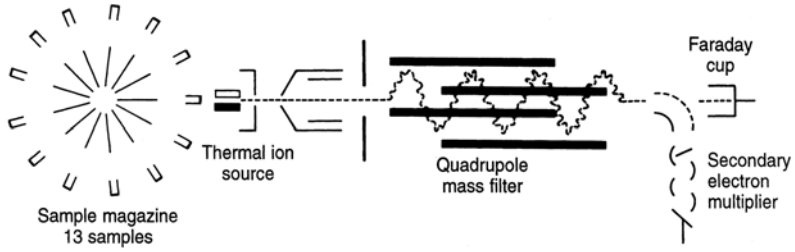


FIG. 11. Quadrupole mass analyser (reproduced from Ref. [117] with permission).

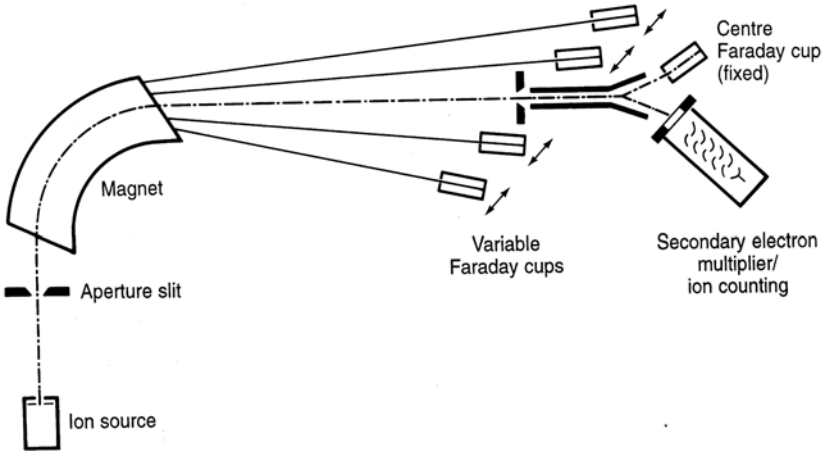


FIG. 12. Multicollector magnetic sector mass analyser (reproduced from Ref. [117] with permission).

that enter this field will exhibit a fixed curvature that is dependent on their mass, with heavier ions being deflected less than lighter ions. The advantages of this approach are that the LOD is typically a factor of at least tenfold lower than that obtained with the quadrupole instruments. Sector instruments can be either single collector (the ion beams are measured sequentially) or multicollector (multiple ion beams are measured simultaneously). In multicollector instruments (Fig. 12), movable detectors are configured such that they are centred to collect the ions of interest. The mass range of ions that can be collected varies based on the instrument, optics configuration and the number of collection cups.

Using magnetic sector instruments with multiple Faraday collectors enables all iron isotopes to be measured simultaneously. Using this approach, RSDs <0.001% can routinely be obtained for ^{57}Fe and <0.1% for ^{58}Fe. Iron suffers from isobaric interferences due to ^{58}Ni. The newer model magnetic sector instruments have internal software programs that allow for interference corrections to be automatically accounted for by normalizing the data to the expected ratio of ^{58}Ni:^{60}Ni.

An additional advantage of newer TIMS instrumentation is the availability of software with the capability of running automated heating programs using an attached pyrometer. This software allows filaments to be heated to a defined temperature or current before collecting isotopic data. These advances reduce the personnel time necessary to run these analyses.

Isotopic fractionation is an issue of concern with TIMS. This occurs because there is a mass-dependent fractionation of iron isotopes as iron is evaporated from the rhenium filament. Lighter isotopes will be evaporated from

the filament faster and reach the detectors faster than the heavier isotopes. To internally correct for this and to make the iron measures independent of total mass of iron present, a ratio is made between each administered iron isotope (typically ^{57}Fe and ^{58}Fe) to another non-administered isotope (^{56}Fe). Isotopic fractionation can then be controlled for by measuring the ratio of two isotopes that were not given (^{54}Fe:^{56}Fe) and correcting this ratio for the isotopic fractionation observed. This normalization factor can then be applied to all other iron ratios to correct for isotopic fractionation.

Isotopic fractionation is a concern with both ICPMS and TIMS. However, the impact of isotopic fractionation with TIMS occurs in the parts per thousands and tends to be stable over time. In comparison, with ICPMS, isotope fractionation (mass bias) is influenced by the settings used, is less stable over time and can be in the parts per hundred range [120, 121].

Iron analyses with TIMS have traditionally been undertaken by monitoring positive ions but these analyses can also be undertaken by monitoring FeF_4^- molecular ions using negative ion TIMS [101, 122–124].

10.2. INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY

In ICPMS, the sample introduction system includes a peristaltic pump, nebulizer and spray chamber. The droplets generated in the nebulizer and spray chamber then enter the ion source, which in ICPMS involves passing the sample through a plasma which converts the elemental atoms into ions. The plasma is most frequently generated by passing argon through concentric quartz tubes. The tubes are wrapped at one end with a radiofrequency coil. The radiofrequency generator in combination with the argon produces the plasma. The very high temperature of the plasma (approximately 6000°C) ionizes the introduced sample. The ions that are generated are focused through a series of lenses and then, subsequently, enter a mass filter which can be a quadrupole, magnetic sector or flight tube as detailed below. Focused ions of interest are detected (typically using Faraday cups) and quantified. As for TIMS, the entire system operates under a vacuum to ensure that the ions generated have as large as possible a mean free path (the distance they can travel without colliding with other particles, such as other gas molecules).

In general, advantages to ICPMS are that multiple minerals can be measured simultaneously, the method is rapid and once calibrated large numbers of samples can be analysed within a short period of time. Disadvantages of ICPMS are largely due to isobaric and spectral interferences, which tend to be bigger concerns for analytes such as iron that have an atomic mass of less than 80 amu. These interferences can be due to trace components or major

components of the sample combining with the argon, solvent or acid based species to produce polyatomic, isobaric, doubly charged and oxide based spectral interferences. Common interferences for iron are detailed in Table 6.

The characteristics of the plasma source generated can potentially introduce variability and measurement error because the stability of the ion signal is dependent on the plasma source. Isotopic fractionation is also a concern and may be controlled for by tight regulation of instrument conditions and by correcting variations to internal standards that are run at regular intervals along with the unknown samples. Numerous reviews of this method and its strengths and weaknesses have been published [101, 118, 121, 126, 128–130].

Several types of inductively coupled plasma (ICP) instrumentation are available that, as TIMS instrumentation, differ primarily based on the mass analyser used for separation of the isotopes. The primary mass analysers available include quadrupole, magnetic sector and time of flight (TOF) instruments. In TOF instrumentation, the velocity of the ion varies in relation to its m/z ratio, such that lighter ions will reach the end of the flight tube before heavier ions. To date, the use of this approach for isotope ratio work is not widespread.

Newer modifications to the design of quadrupole ICP mass spectrometers have been developed to improve performance and minimize interferences. Adaptations to the standard quadrupole include the addition of collision/reaction cells following the quadrupole. Other adaptations utilize chemical reaction cells to minimize plasma-based polyatomic species. In these systems, reaction cell quadrupoles provide an area for a reaction gas to be introduced prior to the quadrupole to assist in ejecting the precursor ions to prevent the formation of polyatomic interferences.

Single collector, high resolution ICPMS instruments typically achieve lower RSDs than the newer ICP sector instruments that are multicollector instruments [101]. To date, few data are available using these instruments for

TABLE 6. EXAMPLES OF INTERFERENCES WITH IRON ANALYSES USING ICPMS (*based on Refs [125–127]*)

m/z	Spectral interferences
^{54}Fe	$^{54}Cr^{+}$, $^{108}Pb^{++}$, $^{108}Cd^{++}$, $^{40}Ar^{14}N^{+}$, $^{38}ArO^{+}$, $^{36}ArO^{+}$, $^{37}ClOH^{+}$, $^{53}Cr^{1}H$
^{56}Fe	$^{112}Cd^{++}$, $^{112}Sn^{++}$, $^{55}Mn^{1}H$, $^{40}ArO^{+}$, $^{36}Ar^{14}N^{16}O^{+}$, $^{40}CaO^{+}$
^{57}Fe	$^{56}Fe^{1}H$, $^{114}Cd^{++}$, $^{114}Sn^{++}$, $^{40}ArOH^{+}$, $^{38}Ar^{18}OH^{+}$, $^{40}OH^{+}$, $^{40}Ca^{17}O^{+}$, $^{40}Ca^{16}O^{1}H$
^{58}Fe	^{58}Ni, $^{116}Cd^{++}$, $^{116}Sn^{++}$, $^{56}Fe^{1}H$, $^{40}Ar^{18}O^{+}$, $^{40}Ar^{17}OH^{+}$, $^{40}Ca^{18}O^{+}$, $^{42}Ca^{16}O^{+}$

human nutritional or biomedical applications. Multicollector detectors are particularly beneficial for high precision isotope ratio measures. These instruments can achieve a 10 ppm precision (0.001% RSD). Multicollector detectors will provide a precision comparable to TIMS but it is also likely that the sample preparation methods will increase to allow for precise quantitation and these may be as consuming as those required for TIMS measurements [101].

10.3. ADDITIONAL FACILITIES NECESSARY FOR STABLE ISOTOPE WORK

In addition to the required mass spectrometric instrumentation, stable iron isotope studies require additional infrastructure to process the samples prior to isotope analysis. To minimize environmental contamination, samples are often processed in a clean room facility. All reagents that are used to chemically isolate or process the samples must be ultrapure and free of exogenous iron. Sources of ultrapure water are needed to prepare solutions and to wash glassware. All glassware that is used in sample processing must be acid washed and rinsed in deionized water prior to use. As the ultrapure acids needed for these analyses are expensive, many laboratories utilize on-site acid distillation facilities to produce their own clean acids to minimize supply costs.

Biological samples (such as RBCs or faecal samples) often require additional processing prior to analysis. To minimize environmental contamination, reduce the amount of acid needed and speed the digestion process, microwave digestion systems can be used to process these biological samples. When isolating iron using anion exchange chromatography prior to TIMS analyses or when loading filaments for TIMS work, use of a chemical fume hood helps to minimize contamination. Other equipment used to support these types of studies includes highly precise digital scales, pipettes, laboratory ware and standards to check mass spectrometric precision and accuracy.

11. ETHICAL CONSIDERATIONS AND INSTITUTIONAL REVIEW OF HUMAN RESEARCH

All research in humans must first be approved by the local institutional human review board as well as by affiliated review boards at collaborating institutions before being implemented. All procedures used in the design,

implementation and consenting process should follow existing ethical and best practice guidelines.

Research subjects should have a clear understanding of the study design, procedures that will be followed, the amount of time the study will require of them, what benefits and/or risks might be associated with the research, and who 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.

In iron bioavailability studies, it is important to present a statement describing what a stable iron isotope is and that there are no known risks associated with their use. The discomfort and possible risk of infection that may occur from blood collections should be mentioned and informed consent must 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 consent. The age cut-offs and preferred means of obtaining assent often vary between research institutions due to local customs. Requirements for the consent/assent process need to be respected while following accepted guidelines. If the review committee has never been involved in reviewing stable isotope research, it may take slightly longer to provide the committees with the necessary information for them to fully evaluate the protocol. As such, it is suggested to begin this process or discuss this with the appropriate contact from the institutional review board during the planning stages to facilitate implementation of the research and minimize the time required for the approval process. All of the patient data collected are confidential and plans must be in place to protect the confidentiality of all identifying information. Examples of human research guidelines include:

— Council for International Organizations of Medical Sciences: International Ethical Guidelines for Biomedical Research Involving Human Subjects (http://www.cioms.ch/publications/guidelines/guidelines_nov_2002_blurb.htm).
— World Medical Association: Declaration of Helsinki. Ethical Principles for Medical Research Involving Human Subjects (http://www.wma.net/en/30publications/10policies/b3/index.html).

The consent form must be written or translated into the language(s) spoken by the individuals enrolled in the study and a witness to the consent process is often required. In some instances, the population group of interest may not be literate so that verbal informed consent scripts may be required and alternative means of verifying consent (thumbprint, etc.) must be put into place. Guidelines

for both preparing consent forms and obtaining informed consent are listed below:

— WHO: The Process of Obtaining Informed Consent (http://www.who.int/rpc/research_ethics/Process_seeking_IF_printing.pdf).
— WHO: Informed Consent Templates (http://www.who.int/rpc/research_ethics/informed_consent/en/index.html).

Full disclosure of all potential risks is mandatory. In iron bioavailability studies, these risks primarily include the pain, discomfort and possible risks of bruising and infection associated with the venipuncture used to collect blood for iron status and iron isotope ratio measures. If a stable iron isotope will be administered intravenously, more detailed information regarding the possible risks — including the risk of anaphylaxis — must be provided and appropriate precautions must be in place.

Remuneration for subject time and inconvenience is sometimes provided to individuals participating in bioavailability studies. The amount and type of compensation should be given at a level that provides compensation for the amount of personal effort required yet not be so high that it would coerce subjects into participating when they otherwise would not be willing. The type of remuneration given must also 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.

12. STATISTICAL CONSIDERATIONS

Consideration and attention must be given to the number of subjects to be studied at the design phase of the study. The sample size should be justified and selected based on the power desired and the minimal detectable difference that is thought to be clinically important or biologically meaningful to the research in question.

Existing relevant data is utilized whenever possible to estimate means and standard deviations anticipated from similar studies in a given sample size. Using these estimates, multiple resources are available on the Internet to assist with the determination of adequate sample sizes, minimal detectable differences and

power calculations. Examples of web sites with information on statistical power calculations include:

— Mathematical Sciences, The University of Iowa (http://www.stat.uiowa.edu/~rlenth/Power/);
— Biostatistics Center, Massachusetts General Hospital (http://hedwig.mgh.harvard.edu/sample_size/size.html);
— Department of Biostatistics, Vanderbilt University (http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize);
— Statpages.net web site (http://statpages.org/).

13. PUBLICATION OF DATA IN PEER-REVIEWED JOURNALS

When presenting data using this methodology, details of the study design and implementation are provided in the methods section of the publication:

— Source and enrichment of stable isotopes utilized in the study.
— All details of how the labels were prepared for dosing, what form they were administered in and whether they were administered or prepared with any ascorbic acid.
— An assurance that the labels were tested for sterility (and pyrogenicity if applicable) prior to use.
— Details on the validation of the total iron content and enrichment of doses utilized.
— Certification that the study was approved by the institutional review board and that informed consent was obtained from all participants.
— Details on the mass spectrometric approach utilized for isotope analysis including: model of mass spectrometer, typical RSD for isotopes of interest, analytical details on methods of mass spectrometric data collection and/or citation to source where these have been published previously.
— Details of the calculations used in determination of iron bioavailability and/or citation of a source where these have been published previously.
— Justification for the sample size selected in relation to primary study outcomes.
— The statistical methods used to evaluate the study data.

It should be noted that peer-reviewed journals increasingly require advanced registration at the US National Institutes of Health's clinical trials web site[2] before the study is conducted.

[2] http://www.clinicaltrials.gov/

Appendix I

IRON ISOLATION FROM BIOLOGICAL SAMPLES

Digesting RBCs prior to analysis by TIMS: performed in the fume hood.
Supplies:

— Ultrapure acids (HNO_3 and HCl);
— Acid washed 10 mL of borosilicate glass Erlenmeyer flasks or a 10 mL acid washed polytetrafluoroethylene (PTFE) vial;
— Pipette and acid washed or trace metal free pipette tips;
— Hotplate;
— Fume hood.

Digestion and drying down blood samples:

(1) A 10 mL PTFE vial (or 10 mL acid washed Erlenmeyer flask) is labelled for the samples to be digested in.
(2) 0.5 mL of blood is added to each corresponding container.
(3) 1 mL of stock 'non-diluted' ultrapure HNO_3 is added to the blood samples.
(4) If a stopping point is necessary, the samples can sit overnight (covered with parafilm) or one can proceed immediately to the next step.
(5) The samples are placed on a hotplate and heated at 38°C.
(6) The containers are checked every 15 min to monitor the digestion progress. Sample colour changes from brown to yellow are a sign that digestion is occurring.
(7) Once the sample is fully digested (clear yellow in appearance), the temperature is elevated to approximately 60–71°C to evaporate off the acid. The sample is monitored closely during this process.
(8) Samples are dried down adequately when all of the acid is evaporated. The residue will look hardened and yellow in appearance. When this occurs, it is removed from the hotplate. The sample is removed from the hotplate immediately after the liquid is evaporated to avoid burning the digested sample residue.
(9) The sample residue is resuspended in 2 mL of 6M ultrapure HCl; this is the final step prior to anion exchange chromatography. Samples will resuspend easiest if done immediately.

Note: Step 7 takes several hours to complete but the time may be reduced by leaving the samples in HNO_3 overnight (in step 4).

Appendix II

IRON ANION EXCHANGE CHROMATOGRAPHY

Supplies:

— Waterproof marker for labelling glassware;
— 10 mL borosilicate glass beakers (acid washed);
— Small plastic transfer pipettes;
— 0.16 cm porous (pore size 25 μm) PTFE sheet;
— 4 mm arch belt punch;
— 1.5 mL microcentrifuge tubes (acid washed);
— 200 μL pipette tips (acid washed);
— 1 mL pipette tips (acid washed);
— Pipettors;
— Ultrapure HCl (stock is approximately 10.2M);
— Ultrapure HNO_3 (will need 3% HNO_3);
— 6M HCl (ultrapure);
— 0.5M HCl (ultrapure);
— 3M HCl (ultrapure, for acid washing columns);
— 3% HCl (for acid washing plastic tips and tubes);
— 10% HNO_3 (for acid washing glassware);
— Deionized water (purified, 18.2 MΩ · cm);
— Anion exchange resin AG 1-X8 (100–200 mesh, chloride form);
— Wash bottles (for 6M HCl, water and resin slurry);
— 0.1% phenol red;
— Rack to hold columns and basin to collect flow-through.

(a) Columns

Columns are prepared by cutting the top off the bulb of the plastic transfer pipette; the frit is prepared by using the 4 mm arch belt punch to remove 4 mm circles from the PTFE sheet. The PTFE circle is then fit snuggly into the base of the long end of the prepared plastic transfer pipette (above). The frit must be snug to prevent flow-through of resin. The assembled columns are acid washed by soaking overnight in 3M HCl and rinsing seven times in deionized water.

(b) Preparation

A slurry of anion exchange resin is made: approximately 2.5 cm resin with 5 cm deionized water.

All glassware is acid washed by soaking overnight in 10% HNO_3 and then rinsed seven times in deionized water. All plasticware (tips and microcentrifuge tubes) is acid washed in 3% HCl and then rinsed seven times in deionized water.

(c) Making and testing acids

The molarity of the stock ultrapure HCl acid is tested (see suggested titration method below). Based on the measured molarity of ultrapure HCl, the volumes needed to prepare 6M and 0.5M HCl for use in the protocol are calculated.

(d) Making an acid of a specific molarity

The following formula is used:

$$C_1V_1 = C_2V_2$$

where

C_1 is the molarity of the target acid;
C_2 is the molarity of the stock acid;
V_1 is the volume of the target acid;

and V_2 is the volume of the stock acid necessary to reach the target molarity.

Example:

To make 500 mL of 6M HCl with 10.2M stock HCl, the formula is set up the following way, and then solved for 'V_2':

$$6\ (M) \times 0.500\ (L) = 10.2\ (M) \times V_2\ (L)$$

$$V_2 = 0.2941\ L = 294.1\ mL$$

Therefore, to make the solution, 294.1 mL of ultrapure stock HCl is added to 205.9 mL of deionized water

(e) Testing the molarity of ultrapure stock HCl

Ultrapure stock HCl is approximately 10–10.2M

(1) One drop (or 4 μL) of phenol red is added to a small beaker.
(2) 100 μL of stock ultrapure HCl is added to the beaker (to test the molarity of the stock solution).
(3) 100 μL of 1M NaOH is added to the beaker and it is swirled to mix.
(4) Eight additional 100 μL aliquots of 1M NaOH are added one at a time, ensuring that complete mixing occurs between each addition. At this point, the solution may partially change to pink but when well mixed it should still be clear. At the completion of steps 3 and 4, a total of 900 μL of 1M NaOH has been added to the titration.
(5) 1M NaOH is added in 20 μL aliquots one at a time, swirling the beaker to mix fully after each addition. A colour change should occur after approximately four to six aliquots of 20 μL have been added. If neutralization occurs (the colour changes to a pink/purple colour) after four aliquots (980 μL of 1M NaOH added to the beaker in total), the molarity of the stock acid is 9.8M, if after five aliquots (1000 μL of 1M NaOH added to the beaker in total), the molarity of the stock acid is 10M, etc.

(f) Testing the molarity of prepared acids

6M HCl

If 6M HCl was prepared correctly, a colour change at neutralization should occur when exactly 600 μL of 1M NaOH is added to 100 μl of 6M HCl. If titration leads to a colour change (pink/purple, and does not change back to clear after mixing the solution) before the entire 600 μL of 1M NaOH is added, then the molarity of the prepared HCl is too low for use in this procedure. Calculations as well as the molarity of the stock acid used should be carefully checked.

(1) One drop of phenol red is added to a small beaker.
(2) 100 μL of the prepared 6M HCl is added to the beaker (to test the molarity of the 6M solution).
(3) 100 μL of 1M NaOH is added to the beaker and the beaker swirled to mix.
(4) Four additional 100 μL aliquots of 1M NaOH are added one at a time, ensuring that complete mixing occurs between each addition. At this point, the solution may partially change to pink but when well mixed

should still be clear. At the completion of steps 3 and 4, a total of 500 µL of 1M NaOH has been added to the titration.

(5) Next, 1M NaOH is added in 20 µL aliquots one at a time, swirling the beaker to mix fully after each addition. A colour change should occur after five aliquots have been added. At the completion of steps 3 through 5, a total of 600 µL of 1M NaOH has been added to the titration and the colour should have changed at neutralization (the colour change must be between 6.0 and 6.2M).

0.5M HCl

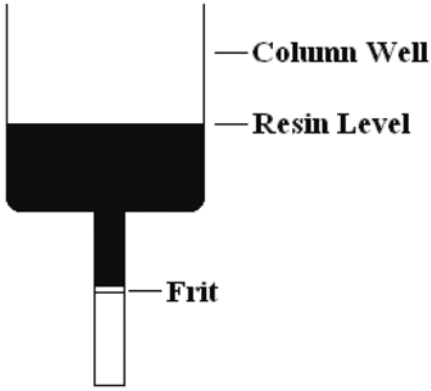
If 0.5M HCl was prepared correctly, a colour change at neutralization should occur when exactly 500 µL of 0.1M NaOH is added to 100 µl of 0.5M HCl. If titration leads to a colour change (pink/purple, and does not change back to clear after fully mixing the solution) before the entire 500 µL of 1M NaOH is added, then the molarity of the prepared HCl is too low for use in this procedure. Calculations as well as the molarity of the stock acid used should be carefully checked.

(1) One drop of phenol red is added to a small beaker.
(2) 100 µL of the prepared 0.5M HCl is added to the beaker (to test the molarity of the 0.5M solution).
(3) 100 µL of 0.1M NaOH is added to the beaker and the beaker swirled to mix.
(4) Three additional 100 µL aliquots of 0.1M NaOH are added one at a time, ensuring that complete mixing occurs between each addition. At this point, the solution may partially change to pink but when well mixed should still be clear. At the completion of steps 3 and 4, a total of 400 µL of 0.1M NaOH has been added to the titration.
(5) Next, 0.1M NaOH is added in 20 µL aliquots one at a time, swirling the beaker to mix fully after each addition. A colour change should occur after five aliquots have been added. At the completion of steps 3 through 5, a total of 500 µL of 0.1M NaOH has been added to the titration and the colour should have changed at neutralization (the colour change should be at exactly 0.5M).

(g) Protocol

Note: The column is kept moist at all times:

(1) The sample to be run is acquired (should already be digested in HNO_3, dried down and dissolved in 2 mL of 6M HCl). This may require stirring the sample with the pipette tip and/or heating; the sample is allowed to return to room temperature if heated to resuspend.
(2) Columns immediately removed from a 3M HCl beaker and rinsed with deionized water are chosen. Each column is checked for speed (drops should fall evenly) and the absence of bubbles above or below the frit.
(3) While there is still water in the column, resin/deionized water slurry is added. Note that:
   — The resin is thoroughly shaken before adding it.
   — No bubbles or air spaces should be visible in the resin.
   — After addition of the resin, the column should look similar to the following:



   — For samples containing greater amounts of iron, more resin may be added or longer columns can be used.
(4) The resin is rinsed twice with deionized water (the column well is filled twice, allowing the contents to drip through; approximately 4 mL in total).

Note: From this point on, no deionized water should enter the column.

(5) The resin is rinsed twice with 6M HCl (the column well is filled twice, allowing the contents to drip through; approximately 4 mL in total).

(6) The resin is rinsed a third time with 6M HCl (the column well is filled once, allowing the contents to drip through; approximately 2 mL in total). Step 7 follows on immediately; step 6 should be performed in batches, so that step 7 is commenced while there is still 6M HCl in the column.
(7) The load sample is dissolved in 6M HCl: Unless there is a very large amount of iron in the sample, the drops coming out at this point should not be yellow. Yellow drops indicate that the iron is not binding to the resin, possibly due to an error of using 0.5M HCl instead of 6M HCl. A new pipette tip is used for each sample.
(8) The column is rinsed with approximately 4 mL of 6M HCl (the column well is filled twice, allowing the contents to drip through).
(9) The column rack is transferred to paper towels. Pre-labelled beakers are placed below the corresponding column to collect the eluted sample.
(10) 0.5–1 mL of 0.5M HCl is added to the column well (0.1 mL for small volumes).
(11) Another 1–2 mL of 0.5M HCl is added to the column well (0.5 mL for small volumes).
(12) The sample is dried to remove HCl (the beaker is placed on a hotplate at 38°C until dry) and resuspended in 40 µL of 3% ultrapure HNO_3; it is transferred to a pre-labelled 1.5 mL microcentrifuge tube.

Appendix III

CHECKLIST FOR STUDY REQUIREMENTS

Item to complete	Why necessary	Completed
Obtain necessary funding for the study	Study design and scope must fit within research budget	Yes/No
Identify mass spectrometry laboratory that will analyse biological samples generated	Will determine which isotopes to administer Will provide information on what isotope doses are required based on instrument precision Will tell you what sample collection containers are needed and how these need to be prepared (acid washed, mineral free, etc.)	Yes/No
Purchase stable iron isotopes	Ensure that the required isotope is in stock and is purchased in the form best suited for conversion into the form to be fed	Yes/No
Obtain approval from the institutional review boards from all institutions involved in the study	Prepare documentation for human use approval Develop consent/assent forms needed Translate consent/assent forms as necessary Ensure that the intended study design, dose, collection methods for biological samples and study protocol are approved prior to implementing the study	Yes/No
Prepare isotope(s) for oral and/or intravenous administration	Convert stable iron isotopes (as metal or oxide) into the final form and dose needed Ensure that the dose needed is dispensed in the appropriate container (trace mineral free) for either individual doses or in a large container for multiple oral dosing Finish all sterility/pyrogenicity testing as required by the institutional review board Validate total iron content and final iron isotopic enrichment of doses to be used	Yes/No
Train all clinical and research personnel	Hold training sessions for all nursing, medical and field staff that will be involved in the study Ensure that all clinical materials are on-site and available (a generator if power supply needed, electronic scales, venipuncture supplies)	Yes/No

REFERENCES

[1] BOY, E., et al., Achievements, challenges, and promising new approaches in vitamin and mineral deficiency control, Nutr Rev. **67** Suppl. 1 (2009) S24–S30.

[2] WORLD HEALTH ORGANIZATION, Worldwide Prevalence of Anaemia 1993–2005 (De BENOIST, B., McLEA, E., EGLI, I., COGSWELl, M., Eds), WHO, Geneva (2008) 1–40.

[3] WORLD HEALTH ORGANIZATION, The World Health Report 2002, Reducing Risks, Promoting Healthy Life, WHO, Geneva (2002).

[4] BEARD, J.L., Why iron deficiency is important in infant development, J. Nutr. **138** (2008) 2534–2536.

[5] LOZOFF, B., et al., Long-lasting neural and behavioral effects of iron deficiency in infancy, Nutr. Rev. **64** (2006) S34–S43.

[6] WORLD HEALTH ORGANIZATION/CENTERS FOR DISEASE CONTROL AND PREVENTION, Assessing the Iron Status of Populations: Report of a Joint World Health Organization (WHO)/Centers for Disease Control and Prevention (CDC) Technical Consultation on the Assessment of Iron Status at the Population Level, WHO Press, Geneva (2004) 1–35.

[7] NICOLAS, G., et al., Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice, Proc. Natl. Acad. Sci. **98** (2001) 8780–8785.

[8] KRAUSE, A., et al., LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity, FEBS Lett. **480** (2000) 147–150.

[9] PARK, C.H., et al., Hepcidin, a urinary antimicrobial peptide synthesized in the liver, J. Biol. Chem. **276** (2001) 7806–7810.

[10] PIGEON, C., et al., A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload, J. Biol. Chem. **276** (2001) 7811–7819.

[11] GUNSHIN, H., et al., Cloning and characterization of a mammalian proton-coupled metal-ion transporter, Nature **388** (1997) 482–488.

[12] McKIE, A.T., et al., An iron-regulated ferric reductase associated with the absorption of dietary iron, Science **291** (2001) 1755–1759.

[13] DE, D., et al., Ferroxidase activity is required for the stability of cell surface ferroportin in cells expressing GPI-ceruloplasmin, EMBO J. **26** (2007) 2823–2831.

[14] WEST, A.R., OATES, P.S., Mechanisms of heme iron absorption: current questions and controversies, World J. Gastroenterol. **14** (2008) 4101–4110.

[15] SHAYEGHI, M., et al., Identification of an intestinal heme transporter, Cell **122** (2005) 789–801.

[16] QIU, A., et al., Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption, Cell **127** (2006) 917–928.

[17] ANDREWS, N.C., Understanding heme transport, N. Engl. J. Med. **353** (2005) 2508–2509.

[18] LATUNDE-DADA, G.O., et al., Recent advances in mammalian haem transport, Trends Biochem. Sci. **31** (2006) 182–188.

[19] LAYRISSE, M., et al., Iron fortification of food: its measurement by the extrinsic tag method, Blood **41** (1973) 333–352.

[20] COOK, J.D., MONSEN, E.R., Food iron absorption in human subjects. III. Comparison of the effect of animal proteins on nonheme iron absorption, Am. J. Clin. Nutr. **29** (1976) 859–867.
[21] BOTHWELL, T.H., FINCH, C.A., Iron Metabolism, Little Brown and Company, Boston (1962).
[22] BJORN-RASMUSSEN, E., et al., Absorption of 'fortification' iron. Bioavailability in man of different samples of reduced Fe, and prediction of the effects of Fe fortification, Br. J. Nutr. **37** (1977) 375–388.
[23] ANDREWS, N.C., Forging a field: the golden age of iron biology, Blood **112** (2008) 219–230.
[24] DUBACH, R., et al., Studies in iron transportation and metabolism; utilization of intravenously injected radioactive iron for hemoglobin synthesis, and an evaluation of the radioactive iron method for studying iron absorption, J. Lab Clin. Med. **31** (1946) 1201–1222.
[25] FINCH, C.A., GIBSON, J.G., Iron metabolism; utilization of intravenous radioactive iron, Blood **4** (1949) 905–927.
[26] FOMON, S.J., Time course of and effect of dietary iron level on iron incorporation into erythrocytes by infants, J. Nutr. **130** (2000) 541–545.
[27] FINCH, C.A., HUEBERS, H., Perspectives in iron metabolism, N. Engl. J. Med. **306** (1982) 1520–1528.
[28] LYNCH, S., "Iron metabolism", Nutritional Anemia (KRAEMER, K., ZIMMERMAN, M.B., Eds), Sight and Life Press, Basel (2007) 58–75.
[29] NEMETH, E., GANZ, T., Regulation of iron metabolism by hepcidin, Ann. Rev. Nutr. **26** (2006) 323–342.
[30] NEMETH, E., et al., Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization, Science **306** (2004) 2090–2093.
[31] DE, D., et al., The molecular mechanism of hepcidin-mediated ferroportin down-regulation, Mol. Biol. Cell. **18** (2007) 2569–2578.
[32] COLLINS, J.F., Hepcidin regulation of iron transport, J. Nutr. **138** (2008) 2284–2288.
[33] GANZ, T., Iron homeostasis: fitting the puzzle pieces together, Cell Metab. **7** (2008) 288–290.
[34] HEANEY, R.P., Factors influencing the measurement of bioavailability, taking calcium as a model, J. Nutr. **131** (2001) 1344S–1348S.
[35] SINGH, M., et al., Iron bioavailability, UK Food Standards Agency workshop report, Br. J. Nutr. **96** (2006) 985–990.
[36] FINCH, C.A., et al., Ferrokinetics in man, Medicine **49** (1970) 17–53.
[37] VITERI, F.E., WARREN, R., Considerations on the use of radioisotopes in human nutrition research, Food Nutr. Bull. **23** (2003) 7–16.
[38] REDDY, M.B., et al., The influence of different protein sources on phytate inhibition of nonheme-iron absorption in humans, Am. J. Clin. Nutr. **63** (1996) 203–207.
[39] HURRELL, R.F., et al., Meat protein fractions enhance nonheme iron absorption in humans, J. Nutr. **136** (2006) 2808–2812.
[40] BEISEIGEL, J.M., et al., Iron bioavailability from maize and beans: a comparison of human measurements with Caco-2 cell and algorithm predictions, Am. J. Clin. Nutr. **86** (2007) 388–396.

[41] COOK, J.D., The influence of different cereal grains on iron absorption from infant cereal foods, Am. J. Clin. Nutr. **65** (1997) 964–969.
[42] HURRELL, R.F., Degradation of phytic acid in cereal porridges improves iron absorption by human subjects, Am. J. Clin. Nutr. **77** (2003) 1213–1219.
[43] HALLBERG, L., The role of meat to improve the critical iron balance during weaning, Pediatrics **111** (2003) 864–870.
[44] VITERI, F.E., WARREN, R., Why and how to use radioisotopes in human nutrition research, Forum Nutr. **56** (2003) 307–309.
[45] TAYLOR, P.D.P., Determination of the absolute isotopic composition and atomic weight of a reference sample of natural iron, Int. J. Mass Spectrom. Ion Processes **121** (1992) 111–125.
[46] DE LAETER, J.R., et al., Atomic weights of the elements, Pure Appl. Chem. **75** (2003) 683–800.
[47] ABRAMS, S.A., Absorption by 1-year-old children of an iron supplement given with cow's milk or juice, Pediatr. Res. **39** (1996) 171–175.
[48] O'BRIEN, K.O., Prenatal iron supplements impair zinc absorption in pregnant Peruvian women, J. Nutr. **130** (2000) 2251–2255.
[49] LOWMAN, J.T., KRIVIT, W., New in vivo tracer method with the use of nonradioactive isotopes and activation analysis, J. Lab Clin. Med. **61** (1963) 1042–1048.
[50] TURNLUND, J.R., Use of enriched stable isotopes to determine bioavailability of trace elements in humans, Sci. Total Environ. **28** (1983) 385–392.
[51] BARRETT, J.F., et al., Comparison of stable isotopes and radioisotopes in the measurement of iron absorption in healthy women, Clin. Sci. **87** (1994) 91–95.
[52] TURNLUND, J.R., et al., Use of enriched stable isotopes to determine zinc and iron absorption in elderly men, Am. J. Clin. Nutr. **35** (1982) 1033–1040.
[53] MARTINEZ, C., et al., Evaluation of iron bioavailability in infant weaning foods fortified with haem concentrate, J. Pediatr. Gastroenterol. Nutr. **27** (1998) 419–424.
[54] MINIHANE, A.M., FAIRWEATHER-TAIT, S.J., Effect of calcium supplementation on daily nonheme-iron absorption and long-term iron status, Am. J. Clin. Nutr. **68** (1998) 96–102.
[55] FOMON, S.J., et al., Less than 80% of absorbed iron is promptly incorporated into erythrocytes of infants, J. Nutr. **130** (2000) 45–52.
[56] WHITTAKER, P.G., et al., Iron absorption during normal human pregnancy: a study using stable isotopes, Br. J. Nutr. **65** (1991) 457–463.
[57] ZIMMERMANN, M.B., et al., Plasma hepcidin is a modest predictor of dietary iron bioavailability in humans, whereas oral iron loading, measured by stable-isotope appearance curves, increases plasma hepcidin, Am. J. Clin. Nutr. **90** (2009) 1280–1287.
[58] MILLER, L.L., HAHN, P.F., The appearance of radioactive iron as hemoglobin in the red cell; the significance of "easily split" iron, J. Biol. Chem. **134** (1940) 585–590.
[59] HAHN, P.F., et al., The utilization of iron and the rapidity of hemoglobin formation in anemia due to blood loss, J. Exper. Med. **71** (1940) 731–736.
[60] PATTERSON, K.Y., VEILLON, C., Stable isotopes of minerals as metabolic tracers in human nutrition research, Exp. Biol. Med. **226** (2001) 271–282.
[61] INSTITUTE OF MEDICINE, Nutrition During Pregnancy, National Academy Press, Washington, DC (1990).

[62] INSTITUTE OF MEDICINE, Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium and Zinc, National Academy Press, Washington, DC (2001).
[63] KASTENMAYER, P., et al., A double stable isotope technique for measuring iron absorption in infants, Br. J. Nutr. **71** (1994) 411–424.
[64] BJORN-RASMUSSEN, E., et al., Measurement of iron absorption from composite meals, Am. J. Clin. Nutr. **29** (1976) 772–778.
[65] FIGUEROA-COLON, R., et al., Failure to label baboon milk intrinsically with iron, J. Pediatr. Gastroenterol. Nutr. **9** (1989) 521–523.
[66] GISLASON, J., et al., Iron absorption differs in piglets fed extrinsically and intrinsically ^{59}Fe-labeled sow's milk, J. Nutr. **122** (1992) 1287–1292.
[67] HICKS, P.D., et al., Iron deficiency, but not anemia, upregulates iron absorption in breast-fed Peruvian infants, J. Nutr. **136** (2006) 2435–2438.
[68] DOMELLOF, M., et al., Iron absorption in breast-fed infants: effects of age, iron status, iron supplements, and complementary foods, Am. J. Clin. Nutr. **76** (2002) 198–204.
[69] DAVIDSSON, L., et al., Influence of lactoferrin on iron absorption from human milk in infants, Pediatr. Res. **35** (1994) 117–124.
[70] JIN, F., et al., Extrinsic labeling method may not accurately measure Fe absorption from cooked pinto beans (*Phaseolus vulgaris*): comparison of extrinsic and intrinsic labeling of beans, J. Agric. Food Chem. **56** (2008) 6881–6885.
[71] BOZA, J.J., et al., The validity of extrinsic stable isotopic labeling for mineral absorption studies in rats, J. Nutr. **125** (1995) 1611–1616.
[72] DONANGELO, C.M., et al., Iron and zinc absorption from two bean (*Phaseolus vulgaris* L.) genotypes in young women, J. Agric. Food Chem. **51** (2003) 5137–5143.
[73] COOK, J.D., et al., Food iron absorption measured by an extrinsic tag, J. Clin. Invest. **51** (1972) 805–815.
[74] BJORN-RASMUSSEN, E., et al., Food iron absorption in man. I. Isotopic exchange between food iron and inorganic iron salt added to food: studies on maize, wheat, and eggs, Am. J. Clin. Nutr. **25** (1972) 317–323.
[75] BJORN-RASMUSSEN, E., et al., Food iron absorption in man. II. Isotopic exchange of iron between labeled foods and between a food and an iron salt, Am. J. Clin. Nutr. **26** (1973) 1311–1319.
[76] BJORN-RASMUSSEN, E., Food iron absorption in man. IV. Validity of the extrinsic tag two-pool method for measurement of dietary non-heme iron absorption in patients with various clinical disorders, Scand. J. Gastroenterol. **8** (1973) 645–650.
[77] ERTL, A.C., et al., Techniques used for the determination of blood volume, Am. J. Med. Sci. **334** (2007) 32–36.
[78] IMAI, T., et al., Accuracy and repeatability of blood volume measurement by pulse dye densitometry compared to the conventional method using ^{51}Cr-labeled red blood cells, Intensive Care Med. **26** (2000) 1343–1349.
[79] SILVER, H.M., et al., Red cell volume determination using a stable isotope of chromium, J. Soc. Gynecol. Investig. **4** (1997) 254–258.
[80] SILVER, H.M., et al., Comparison of total blood volume in normal, preeclamptic, and nonproteinuric gestational hypertensive pregnancy by simultaneous measurement of red blood cell and plasma volumes, Am. J. Obstet. Gynecol. **179** (1998) 87–93.

[81] IIJIMA, T., et al., Circulating blood volume measured by pulse dye-densitometry: comparison with ^{131}I-HSA analysis, Anesthesiology **89** (1998) 1329–1335.
[82] DWORKIN, H.J., et al., Comparison of red cell and whole blood volume as performed using both chromium-51-tagged red cells and iodine-125-tagged albumin and using I-131-tagged albumin and extrapolated red cell volume, Am. J. Med. Sci. **334** (2007) 37–40.
[83] NIELSEN, M.H., NIELSEN, N.C., Spectrophotometric determination of Evans blue dye in plasma with individual correction for blank density by a modified Gaeblers method, Scand. J. Clin. Lab Invest. **14** (1962) 605–617.
[84] CLARK, A.C., GAIRDNER, D., Postnatal plasma shift in premature infants, Arch. Dis. Child. **35** (1960) 352–354.
[85] SISSON, T.R., et al., The blood volume of infants: I. The full-term infant in the first year of life, J. Pediatr. **55** (1959) 163–179.
[86] SISSON, T.R., WHALEN, L.E., The blood volume of infants, J. Pediatr. **56** (1960) 43–47.
[87] DAVIDSSON, L., "Trace-elements in infants and pregnant or lactating women", Advances in Isotope Methods for the Analysis of Trace Elements in Man (JACKSON, M., LOWE, N., Eds), CRC Press, New York (2001) 167–183.
[88] MAERTZDORF, W.J., et al., Circulating blood volume in appropriate and small for gestation age full term and preterm polycythaemic infants, Acta Paediatr. Scand. **80** (1991) 620–627.
[89] ETCHEVERRY, P., et al., Production of stable-isotope-labeled bovine heme and its use to measure heme-iron absorption in children, Am. J. Clin. Nutr. **85** (2007) 452–459.
[90] LINDERKAMP, O., et al., Estimation and prediction of blood volume in infants and children, Eur. J. Pediatr. **125** (1977) 227–234.
[91] BROWN, E., et al., Red cell, plasma, and blood volume in healthy women measured by radiochromium cell-labeling and hematocrit, J. Clin. Invest. **41** (1962) 2182–2190.
[92] WENNESLAND, R., et al., Red cell, plasma and blood volume in healthy men measured by radiochromium (^{51}Cr) cell tagging and hematocrit: influence of age, somatotype and habits of physical activity on the variance after regression of volumes to height and weight combined, J. Clin. Invest. **38** (1959) 1065–1077.
[93] O'BRIEN, K.O., et al., Influence of prenatal iron and zinc supplements on supplemental iron absorption, red blood cell iron incorporation, and iron status in pregnant Peruvian women, Am. J. Clin. Nutr. **69** (1999) 509–515.
[94] WHITTAKER, P.G., et al., The erythrocyte incorporation of absorbed non-haem iron in pregnant women, Br. J. Nutr. **86** (2001) 323–329.
[95] HOSAIN, F., et al., Blood ferrokinetics in normal man, J. Clin. Invest. **46** (1967) 1–9.
[96] HUNT, J.R., ROUGHEAD, Z.K., Adaptation of iron absorption in men consuming diets with high or low iron bioavailability, Am. J. Clin. Nutr. **71** (2000) 94–102.
[97] HUNT, J.R., High-, but not low-bioavailability diets enable substantial control of women's iron absorption in relation to body iron stores, with minimal adaptation within several weeks, Am. J. Clin. Nutr. **78** (2003) 1168–1177.
[98] ZIMMERMANN, M.B., et al., Iron metabolism in heterozygotes for hemoglobin E (HbE), alpha-thalassemia 1, or beta-thalassemia and in compound heterozygotes for HbE/beta-thalassemia, Am. J. Clin. Nutr. **88** (2008) 1026–1031.

[99] ROE, M.A., et al., Iron absorption in male C282Y heterozygotes, Am. J. Clin. Nutr. **81** (2005) 814–821.
[100] TONDEUR, M.C., et al., Determination of iron absorption from intrinsically labeled microencapsulated ferrous fumarate (sprinkles) in infants with different iron and hematologic status by using a dual-stable-isotope method, Am. J. Clin. Nutr. **80** (2004) 1436–1444.
[101] WALCZYK, T., The potential of inorganic mass spectrometry in mineral and trace element nutrition research, Fresenius J. Anal. Chem. **370** (2001) 444–453.
[102] TOFFOLO, G., et al., "Tracer-to-tracee ratio for compartmental modeling of stable-isotope tracer data", Advances in Isotope Methods for the Analysis of Trace Elements in Man (LOWE, N.M., JACKSON, M.J., Eds), CRC Press, New York (2001) 43–57.
[103] HUNT, J.R., High-, but not low-bioavailability diets enable substantial control of women's iron absorption in relation to body iron stores, with minimal adaptation within several weeks, Am. J. Clin. Nutr. **78** (2003) 1168–1177.
[104] ROUGHEAD, Z.K., HUNT, J.R., Adaptation in iron absorption: iron supplementation reduces nonheme-iron but not heme-iron absorption from food, Am. J. Clin. Nutr. **72** (2000) 982–989.
[105] ZLOTKIN, S.H., et al., Determination of iron absorption using erythrocyte iron incorporation of two stable isotopes of iron (^{57}Fe and ^{58}Fe) in very low birthweight premature infants, J. Pediatr. Gastroenterol. Nutr. **21** (1995) 190–199.
[106] MCDONALD, M.C., et al., Iron absorption and red blood cell incorporation in premature infants fed an iron-fortified infant formula, Pediatr. Res. **44** (1998) 507–511.
[107] DOHERTY, C.P., et al., Iron incorporation and post-malaria anaemia, PLoS ONE **3** (2008) e2133.
[108] BAILIE, G.R., et al., Hypersensitivity reactions and deaths associated with intravenous iron preparations, Nephrol. Dial. Transplant. **20** (2005) 1443–1449.
[109] FAICH, G., STROBOS, J., Sodium ferric gluconate complex in sucrose: safer intravenous iron therapy than iron dextrans, Am. J. Kidney Dis. **33** (1999) 464–470.
[110] FRIEL, J.K., et al., Intravenous iron administration to very-low-birth-weight newborns receiving total and partial parenteral nutrition, J. Parenter. Enteral. Nutr. **19** (1995) 114–118.
[111] TAYLOR, P., et al., Relationships among iron absorption, percent saturation of plasma transferrin and serum ferritin concentration in humans, J. Nutr. **118** (1988) 1110–1115.
[112] LYNCH, M.F., et al., Iron absorption is more closely related to iron status than to daily iron intake in 12- to 48-mo-old children, J. Nutr. **137** (2007) 88–92.
[113] WALTER, T., et al., Bioavailability of elemental iron powder in white wheat bread, Eur. J. Clin. Nutr. **58** (2004) 555–558.
[114] COOK, J.D., et al., Assessment of the role of nonheme-iron availability in iron balance, Am. J. Clin. Nutr. **54** (1991) 717–722.
[115] HALLBERG, L., Perspectives on nutritional iron deficiency, Ann. Rev. Nutr. **21** (2001) 1–21.
[116] HALLBERG, L., HULTHEN, L., Prediction of dietary iron absorption: an algorithm for calculating absorption and bioavailability of dietary iron, Am. J. Clin. Nutr. **71** (2000) 1147–1160.
[117] MELLON, F.A., SANDSTRÖM, B., (Eds) Stable Isotopes in Human Nutrition, Inorganic Nutrient Metabolism, Academic Press, London (1996).

[118] YERGEY, A.L., Analytical instruments for stable isotopic tracers in mineral metabolism, J. Nutr. **126** (1996) 355S–361S.
[119] YERGEY, A.L., et al., Isotope ratio measurements of urinary calcium with a thermal ionization probe in a quadrupole mass spectrometer, Anal. Chem. **52** (1980) 1811–1814.
[120] HEUMANN, K.G., Isotope-dilution ICP-MS for trace element determination and speciation: from a reference method to a routine method? Anal. Bioanal. Chem. **378** (2004) 318–329.
[121] WALCZYK, T., TIMS versus multicollector-ICP-MS: coexistence or struggle for survival? Anal. Bioanal. Chem. **378** (2004) 229–231.
[122] DAVIDSSON, L., et al., Influence of ascorbic acid on iron absorption from an iron-fortified, chocolate-flavored milk drink in Jamaican children, Am. J. Clin. Nutr. **67** (1998) 873–877.
[123] THANKACHAN, P., et al., Iron absorption in young Indian women: the interaction of iron status with the influence of tea and ascorbic acid, Am. J. Clin. Nutr. **87** (2008) 881–886.
[124] TUNTIPOPIPAT, S., et al., Chili, but not turmeric, inhibits iron absorption in young women from an iron-fortified composite meal, J. Nutr. **136** (2006) 2970–2974.
[125] CHEN, Z., et al., High resolution inductively coupled plasma mass spectrometry allows rapid assessment of iron absorption in infants and children, J. Nutr. **135** (2005) 1790–1795.
[126] STURUP, S., The use of ICPMS for stable isotope tracer studies in humans: a review, Anal. Bioanal. Chem. **378** (2004) 273–282.
[127] DAUPHAS, N., ROUXEL, O., Mass spectrometry and natural variations of iron isotopes, Mass Spectrom. Rev. **25** (2006) 515–550.
[128] JANGHORBANI, M., TING, B.T., Stable isotope methods for studies of mineral/trace element metabolism, J. Nutr. Biochem. **1** (1990) 4–19.
[129] CREWS, H.M., et al., Mass spectrometric methods for studying nutrient mineral and trace element absorption and metabolism in humans using stable isotopes. A review, Analyst **119** (1994) 2491–2514.
[130] TURNLUND, J.R., KEYES, W.R., Isotope ratios of trace elements in samples from human nutrition studies determined by TIMS and ICP-MS: precision and accuracy compared, Food Nutr. Bull. **23** (2002) 129–132.

GLOSSARY

abundance. The proportion of an isotope as compared with the total number of atoms of that element (a fraction, as in atom fraction or atom per cent) or that compared with the number of atoms of the major isotope of that element (a ratio). The abundance of stable iron isotopes can be measured using thermal ionization mass spectrometry or inductively coupled plasma mass spectrometry.

accuracy. Describes the correctness of a result, that is, how close the measured value is to the 'true' value. Accuracy is expressed in terms of either absolute error or relative error. The absolute error of the mean of a set of replicate analyses equals measured value minus true value. The true value represents an accepted value of the quantity being measured. It is often useful to express accuracy in terms of relative error where relative error = (measured – true)/true × 100%.

atom per cent (at.%). The number of atoms of a particular isotope expressed as a proportion of the total number of atoms of that element, for example:

$$\text{at.\% } {}^{58}\text{Fe} = [{}^{58}\text{Fe}/({}^{54}\text{Fe} + {}^{56}\text{Fe} + {}^{57}\text{Fe} + {}^{58}\text{Fe})] \times 100$$

bioavailability. The degree to which a particular nutrient can be absorbed and utilized from the food source in question.

blood volume. The amount of blood found in an individual's circulatory system.

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

erythrocyte iron incorporation. A term used to denote the amount (or fraction) of absorbed or intravenously administered iron isotope that is incorporated into red blood cells within 10–12 d.

exchangeable pools. Also known as miscible pools. Iron atoms in a chemical matrix or system (such as the diet or the body) that interchange rapidly and completely. For example, dietary iron is absorbed from two separate exchangeable pools, haem and non-haem iron.

extrinsic labelling. A measured quantity of label is added directly to the test meal before consumption.

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. For instance, lighter isotopes of iron tend to evaporate from the filament faster than the heavier isotopes when using thermal ionization mass spectrometry.

haemoglobin. An iron containing protein used to transport oxygen in blood.

intrinsic labelling. The iron isotope is incorporated into the food of interest in a way that is as similar as possible to the way that iron 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, ^{58}Ni can cause an isobaric interference for ^{58}Fe.

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. Iron has four stable isotopes:

^{54}Fe has 26 protons and 28 neutrons
^{56}Fe has 26 protons and 30 neutrons
^{57}Fe has 26 protons and 31 neutrons
^{58}Fe has 26 protons and 32 neutrons

limit of detection. The lowest quantity of a substance that can be distinguished from the absence of that substance. The limit of detection is typically set at three times the relative standard deviation of the mean ratio measurement of the natural abundance (unenriched) sample of interest.

limit of quantitation. The lowest quantity of a substance that can be reliably quantified with an acceptable level of precision and accuracy. The limit of quantitation is typically set at ten times the relative standard deviation of the mean ratio measurement of the natural abundance (unenriched) sample of interest.

major isotope. The most abundant stable isotope is sometimes referred to as the major isotope. For iron, this is ^{56}Fe.

minor isotopes. The less abundant stable isotopes are sometimes referred to as the minor isotopes.

natural abundance. The fraction or proportion of atoms of a stable isotope relative to the other atoms of the same element found naturally. The natural abundance of the stable iron isotopes in atom per cent (not weight) is detailed below:

^{54}Fe: 5.845at.%
^{56}Fe: 91.754at.%
^{57}Fe: 2.1191at.%
^{58}Fe: 0.2819at.%

normalization factor. The normalization factor is a correction factor used to account for the fact that lighter iron isotopes evaporate from the filament faster than heavier iron isotopes. A normalization factor corrects the observed ratio of two iron isotopes to the expected ratio for those elements to adjust the data to account for the isotopic fractionation.

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 must be tested for pyrogenicity to ensure that they are safe for use in human studies.

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. Two radioactive isotopes of iron that have been used in nutrition research are ^{55}Fe and ^{59}Fe.

red blood cell. Otherwise known as an erythrocyte. This cell is comprised almost entirely of haemoglobin and does not contain a nucleus or mitochondria. It is shaped as a biconcave disc to increase surface area and facilitate oxygen delivery throughout the body.

reference isotope. An isotope with relatively high natural abundance that is used as a reference to express the relative abundance of the minor isotope in an isotope ratio.

stable isotopes. Isotopes that have stable nuclei which 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. Iron has four stable isotopes: ^{54}Fe, ^{56}Fe, ^{57}Fe and ^{58}Fe.

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.

LIST OF ABBREVIATIONS

AAS	atomic absorption spectrophotometry
CEDE	committed effective dose equivalent
Dcytb	duodenal cytochrome b
DMT1	divalent metal transport 1
FDA	US Food and Drug Administration
FPN	ferroportin
HEPH	hephaestin
HSA	human serum albumin
ICP	inductively coupled plasma
ICP-AES	inductively coupled plasma-atomic emission spectrometry
ICPMS	inductively coupled plasma mass spectrometry
LOD	limit of detection
LOQ	limit of quantitation
PTFE	polytetrafluoroethylene
RBC	red blood cell
RSD	relative standard deviation
TIMS	thermal ionization mass spectrometry
TOF	time of flight
WHO	World Health Organization

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