

# Use of Stable Isotopes to Evaluate Bioefficacy of Provitamin A Carotenoids, Vitamin A Status, and Bioavailability of Iron and Zinc

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## ABSTRACT

The ability of nutrition scientists to measure the status, bioavailability, and bioefficacy of micronutrients is affected by lack of access to the parts of the body through which a nutrient may travel before appearing in accessible body compartments (typically blood or urine). Stable isotope–labeled tracers function as safe, nonradioactive tools to follow micronutrients in a quantitative manner because the absorption, distribution, metabolism, and excretion of the tracer are assumed to be similar to the unlabeled vitamin or mineral. The International Atomic Energy Agency (IAEA) supports research on the safe use of stable isotopes in global health and nutrition. This review focuses on IAEA's contributions to vitamin A, iron, and zinc research. These micronutrients are specifically targeted by the WHO because of their importance in health and worldwide prevalence of deficiency. These 3 micronutrients are included in food fortification and biofortification efforts in low- and middle-income regions of the world. Vitamin A isotopic techniques can be used to evaluate the efficacy and effectiveness of interventions. For example, total body retinol stores were estimated by using <sup>13</sup>C<sub>2</sub>-retinol isotope dilution before and after feeding Zambian children maize biofortified with β-carotene to determine if vitamin A reserves were improved by the intervention. Stable isotopes of iron and zinc have been used to determine mineral bioavailability. In Thailand, ferrous sulfate was better absorbed from fish sauce than was ferrous lactate or ferric ammonium citrate, determined with the use of different iron isotopes in each compound. Comparisons of one zinc isotope injected intravenously with another isotope taken orally from a micronutrient powder proved that the powder increased total absorbed zinc from a meal in Pakistani infants. Capacity building by the IAEA with appropriate collaborations in low- and middle-income countries to use stable isotopes has resulted in many advancements in human nutrition. *Adv Nutr* 2018;9:625–636.

**Keywords:** bioconversion, biofortification, compartmental modeling, fortification, micronutrients, retinol isotope dilution, stable isotopes

## Introduction

The WHO advises that many micronutrients are limited in human diets and may need public health interventions to address deficiency (1). Of particular concern, 3 essential micronutrients specifically targeted for improvement are vitamin A, iron, and zinc. Deficiencies in these micronutrients are largely due to a large proportion of the diet being composed of high-carbohydrate staple foods that are micronutrient poor. For example, in many African countries, >50% of available calories are from staple grains, roots, and tubers (2). In order to diminish deficiencies of these key

nutrients, they are often added to foods that have high-reaching coverage in a process called fortification (3). In addition, biofortification is a plant-breeding technique that naturally enhances micronutrient content of plant foods (4, 5). Both of these long-term, sustainable processes are aimed at lowering the need for direct supplementation to vulnerable groups. Supplementation is relatively expensive and requires continuous human resource inputs (6).

Measuring the micronutrient value of foods and the status of individuals and target groups is not always straightforward. Three terms associated with micronutrients are as follows: 1) bioaccessibility, defined as the quantity of a nutrient that is available for absorption after digestion; 2) bioavailability, defined as the proportion of a given nutrient that is absorbed and available for physiologic function (7); and 3) bioefficacy, defined as the proportion of a nutrient that is ultimately converted to the active form (e.g., retinol produced from dietary provitamin A carotenoids).

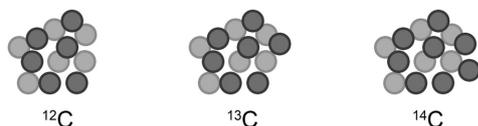
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Abbreviations used: EZP, exchangeable zinc pool; FeNaEDTA, ferric sodium EDTA; IAEA, International Atomic Energy Agency; ICP-MS, inductively coupled plasma–MS; IOM, Institute of Medicine; MRDR, modified relative dose response; RID, retinol isotope dilution; TIMS, thermal ionization MS.

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- Isotopes of an element differ in the number of neutrons (●) they contain but have the same number of protons (●)
- Stable isotopes occur naturally in the environment.
- Stable isotopes are completely safe to use.

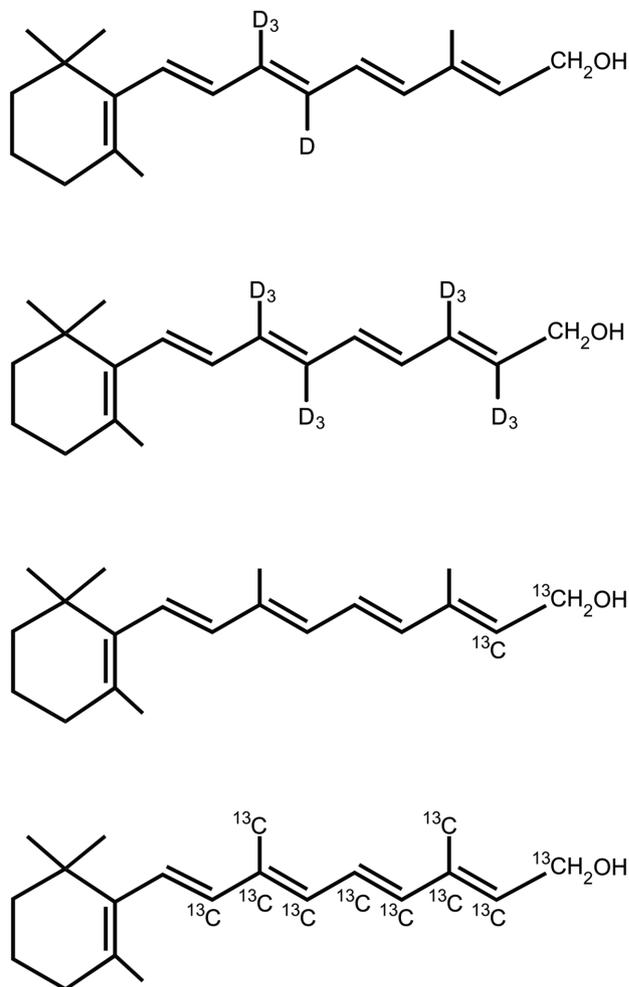
**FIGURE 1** The 3 isotopic forms of carbon.  $^{12}\text{C}$  is the most common of the isotopic forms (98.9%) and  $^{13}\text{C}$  is the stable form (1.1%);  $^{14}\text{C}$  is the radioactive form and is only found in trace amounts in nature.

Determination of these parameters is not trivial (8<sup>†</sup>). Stable isotope methods are the most powerful techniques available to scientists for evaluating vitamin A status and provitamin A bioefficacy (9<sup>†</sup>, 10, 11<sup>†</sup>, 12) and iron and zinc bioavailability from meals (13<sup>†</sup>). The International Atomic Energy Agency (IAEA) has invested in capacity building of laboratories worldwide, and one of their main objectives is to foster the safe use of stable isotopes in human nutrition and health. The purpose of this review is to inform the reader about stable isotope methods and their application to nutrition programming. It also serves to highlight some of the work that the IAEA has supported over the past 2 decades on these 3 micronutrients, denoted throughout this article by “†” after a citation number.

## Current Status of Knowledge

### Safety and natural abundance of stable isotopes

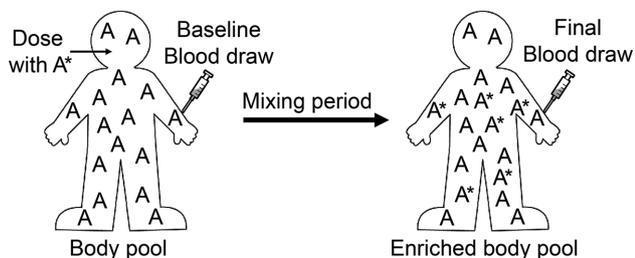
A common misconception when the term “isotope” is used when explaining nutrition research is that it refers to compounds that are radioactive. In fact, radioisotopes are far less abundant in nature than other stable isotopic forms. The difference in isotopic forms of an element relates to the number of neutrons in the nucleus; all isotopic forms have the same number of protons (e.g., 6 for carbon) (Figure 1). The distribution of an element among each form is called the natural abundance. For example, carbon is predominantly  $^{12}\text{C}$  (98.9%), but occurs in a stable form as  $^{13}\text{C}$  (1.1%), which is a common isotope used in nutrition studies (14). Radioactive carbon,  $^{14}\text{C}$ , is found only in trace amounts (0.0000000001%), and 11,12- $^{14}\text{C}_2$ -retinyl acetate was used in vitamin A nutrition studies in conjunction with accelerator MS, because  $^{14}\text{C}$  has a relatively long half-life, allowing it to be followed for months (15–17). The radioactive form of hydrogen [tritium,  $^3\text{H}$ ; trace amounts in nature ( $10^{-16}\%$ )] was introduced into 11,12- $^3\text{H}_2$ -retinyl acetate and used in the seminal work in humans for the determination of vitamin A requirements and half-life (18). In addition to  $^{13}\text{C}$ , the stable isotope of hydrogen,  $^2\text{H}$  [deuterium; 0.015% (14)], is used to label organic molecules, such as vitamin A, for nutrition studies (Figure 2). Researchers have used  $^2\text{H}$ -labeled water



**FIGURE 2** The 4 most common stable isotope–labeled vitamin A structures. These molecules are usually administered as the retinyl acetate compounds in retinol isotope dilution and compartmental modeling studies. Top to bottom: 10, 19, 19, 19- $\text{D}_4$ -retinol; 10, 19, 19, 14, 20, 20, 20- $\text{D}_8$ -retinol; 14,15- $^{13}\text{C}_2$ -retinol; and 8, 9, 10, 11, 12, 13, 14, 15, 19, 20- $^{13}\text{C}_{10}$ -retinol. D, deuterium.

to measure breast-milk intake volume in infants (19–21<sup>†</sup>). Vitamin A (20<sup>†</sup>) and zinc (21<sup>†</sup>) intakes were estimated in Mexican and Indian infants, respectively, by combining breast-milk intake with routine analysis of micronutrient content of the milk.

The stable isotopes of elemental iron and zinc can be used to study these essential minerals. The most common iron form is  $^{56}\text{Fe}$  (91.75%), and isotopes used for bioavailability studies include  $^{57}\text{Fe}$  (2.12%) and  $^{58}\text{Fe}$  (0.28%) (14). The most common zinc forms are  $^{64}\text{Zn}$  (49.17%) and  $^{66}\text{Zn}$  (27.73%). The stable isotopes used for nutrition research include  $^{67}\text{Zn}$  (4.04%),  $^{68}\text{Zn}$  (18.45%), and  $^{70}\text{Zn}$  (0.61%) (14). To date, there are no isotopic methods used to evaluate iron status. The utility of monitoring the size of the exchangeable zinc pool (EZIP) to reflect changes in zinc intake or absorption has been investigated (22).



**FIGURE 3** For vitamin A status assessment, a dose of vitamin A ester labeled with stable isotope is administered after a baseline blood sample. A period of the dose mixing with the vitamin A body pool is necessary before the follow-up blood sample is taken for analysis by MS (adapted from reference 28). Prediction equations use the data from the mass spectrometer, along with key assumptions about absorption, storage, and catabolism of dose, to estimate total body retinol stores (29).

### Vitamin A status assessment and provitamin A carotenoid bioavailability studies

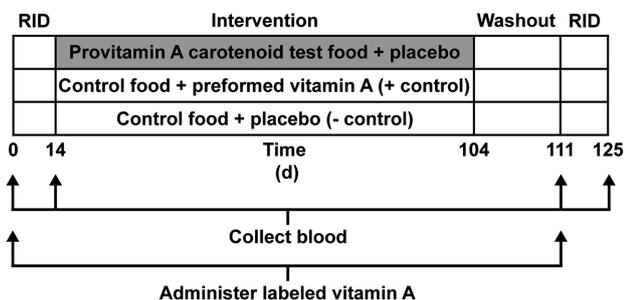
#### IAEA-funded nonisotopic work with vitamin A analogs.

Dose-response tests are a simpler alternative or complementary technique to stable isotope approaches, but these tests only qualitatively define liver retinol stores, whereas isotope methods are quantitative. The dose response is based on the accumulation of *apo*-retinol-binding protein in the liver during times of vitamin A depletion and rapid release of the *holo*-retinol-binding protein after a challenge dose (23). The modified relative dose response (MRDR) test, which determines a serum ratio of orally consumed 3,4-didehydroretinol (vitamin A<sub>2</sub>) to retinol, similar to tracer dilution, can detect the degree of vitamin A deficiency in a population (10) but does not quantify total body retinol stores in an individual or group (9<sup>†</sup>). For example, the MRDR test was used to evaluate high-dose supplementation (24<sup>†</sup>) and a green leafy vegetable intervention (25<sup>†</sup>) in Ghanaian lactating women. Furthermore, the MRDR test was used in Senegal to determine a high prevalence (73.5%) of low liver stores of vitamin A (MRDR  $\geq 0.06$ ) among 6-mo-old breastfed infants, whereas plasma retinol concentrations only identified 15% as deficient ( $< 0.7 \mu\text{mol/L}$ ) (26<sup>†</sup>). The MRDR test was used to determine baseline vitamin A liver status of 7- to 9-mo-old infants in Ghana before an intervention (27<sup>†</sup>), whereas after the intervention, retinol isotope dilution (RID; discussed below) was used to determine that body stores did not differ between infants receiving a vitamin A supplement or placebo (mean  $\pm$  SD:  $436 \pm 303$  and  $434 \pm 186 \mu\text{mol}$ , respectively). Both MRDR and RID analyses diagnosed the cohort as having adequate vitamin A status.

**Stable isotope methods.** RID can be applied to evaluate the vitamin A status of groups with the use of <sup>2</sup>H- or <sup>13</sup>C-labeled retinyl acetate (Figure 2). After the subject consumes the labeled dose of retinyl ester, the retinol is distributed and diluted into the vitamin A pool after absorption (Figure 3). The enrichment in the blood with the labeled compound is determined after a mixing period, which is typically

$> 10$  d. Studies in rats suggest that mixing has not fully occurred before 10 d, especially when the RID test is applied to individuals with adequate vitamin A status (30). The measured ratio of labeled tracer to endogenous vitamin A is used to estimate total body stores and total liver reserves of vitamin A after correcting for natural abundance, absorption, and catabolism of the dose (29). A baseline blood sample measured for isotopic natural abundance is not necessarily required in all subjects because it is often considered to be nonenriched in the case of <sup>2</sup>H or a mean baseline <sup>13</sup>C natural abundance of a subsample is used (27<sup>†</sup>, 31<sup>†</sup>) to account for interindividual variation. It is recommended to measure infection or inflammation status by analyzing serum concentrations of the acute-phase reactant C-reactive protein and convalescence reactant  $\alpha_1$ -acid glycoprotein (32, 33). Participants with fever should not be enrolled because active infection decreases the amount of dose absorbed (34), causes enhanced retinol losses in urine (35), and may influence the ratio of isotopic enrichment of retinol in serum to that in liver (36), variables that affect estimated retinol stores. RID has been applied in a number of countries including Cameroon, China, Ghana, Mexico, Thailand, and Zambia to determine vitamin A status or changes in status during interventions (37<sup>†</sup>). More studies are currently underway in other African countries.

A variety of methods to analyze the isotopic enrichment of samples are available, and the tracer used depends upon the type of mass spectrometer available to the researcher. It is essential that collaborating partners know the type of mass spectrometer that will be used in the final analysis in order to familiarize themselves and understand the advantages and limitations of the system, to determine the number of atoms needed to be labeled with stable isotope in the molecule for appropriate detection postdosing, and to evaluate the amount of serum needed for the analysis. Moreover, the amount of stable isotope dose to be administered needs to be calculated on the basis of the sensitivity of the instrument, the anticipated pool size, and the amount of retinol estimated in the sample for analysis. For example, if the mass spectrometer available is a GC-combustion-isotope-ratio mass spectrometer, the number of labeled atoms needed is fewer (2 carbons needed as <sup>13</sup>C) than that used by GC-MS, which requires  $\geq 4$  atoms labeled as <sup>2</sup>H or <sup>13</sup>C to provide detectable enrichment in serum (38<sup>†</sup>). The mass of dose also differs, with recent doses as low as  $1.0 \mu\text{mol}$  <sup>13</sup>C<sub>2</sub>-retinyl acetate given to children for vitamin A assessment using the GC-combustion-isotope-ratio MS analysis (31<sup>†</sup>);  $1.75$  or  $2.95 \mu\text{mol}$  <sup>13</sup>C<sub>10</sub>-retinyl acetate for stable isotope reference methods using LC-atmospheric pressure MS (39), or LC-tandem MS (40), respectively; and  $28 \mu\text{mol}$  to women for vitamin A assessment with the use of GC-MS analysis (41<sup>†</sup>). Researchers reduced the dose amount to  $6.0 \mu\text{mol}$  <sup>2</sup>H<sub>4</sub>- or <sup>2</sup>H<sub>8</sub>-retinyl acetate in studies underway in African children supported in part by the IAEA with the use of GC-MS with electron capture detection. These unique features among isotope methods, mass spectrometers, and labeling underscore the importance of knowing the research question



**FIGURE 4** Study design for the paired-isotope dilution method in a highly controlled, 90-d feeding intervention with a high provitamin A-containing food, such as vegetables, a fortified food, or a biofortified staple crop. Negative (placebo) and positive (supplemental preformed vitamin A) control groups are recommended. If both control groups are included, a bioefficacy factor can be estimated for provitamin A-containing foods. RID, retinol isotope dilution.

asked and the methodology available to the research team for the final analysis.

The paired RID technique in which total body retinol pool size is estimated before and after an intervention can be used to determine the efficacy or bioefficacy of provitamin A carotenoids consumed with careful study design (42<sup>†</sup>). An efficacy study in Chinese children showed that green and yellow vegetables maintained total body retinol stores (43<sup>†</sup>). Another efficacy study in Filipino children showed that vitamin A status inversely affected bioconversion of provitamin A carotenoids to retinol (44<sup>†</sup>). To calculate bioefficacy factors, appropriate control groups are required (Figure 4). Bioefficacy is estimated as the ratio of the change in stores in the study group to that in the positive control group, adjusted for the difference between the total reference dose consumed and the total carotenoid consumed during the intervention, corrected for loss or maintenance estimated in the negative control group. When the paired RID test is used, it is necessary to determine a baseline serum retinol isotopic natural abundance postintervention because some of the baseline isotopic tracer may still be measurable and the intervention itself could change isotope enrichment depending on foods consumed. Specifically, provitamin A-biofortified C4 plants, such as maize, sorghum, and millet, are enriched in <sup>13</sup>C, and therefore vitamin A produced from these provitamin A sources could change the serum <sup>13</sup>C-enrichment of retinol in the subjects when consumed (45). This effect has been proposed as a biomarker for biofortified C4 crop intervention effectiveness (45), discussed later in this review.

To gain further information about vitamin A body kinetics, researchers have used stable isotope-labeled vitamin A data to perform compartmental modeling (46). Similar to the RID methods, subjects consume an isotopically labeled dose of retinyl acetate, and then blood is ideally collected several times on the first day and at specific time points thereafter. The changing fraction of the dose in the serum is

mathematically modeled by constructing several theoretical compartments within the body by using kinetic constants that describe the movement of retinol throughout the body and its irreversible degradation. Attempts have been made to use this model to develop RID equations that could be applied as early as 3–5 d postdose by estimating the ratio of isotopic enrichment of retinol in plasma and stores reached in the body at any given time (47<sup>†</sup>, 48<sup>†</sup>). Some studies make use of a composite model (i.e., “super child” or “super woman”) in which data points from multiple subjects, who each contribute to a fraction of the total number of time points, are combined to form a population-level compartmental model (49<sup>†</sup>, 50). These super models are used to estimate mean total body retinol stores and kinetic parameters of the associated groups. Recently, a “super child” approach was used in 15 Mexican preschool children to evaluate the bioefficacy of provitamin A carotenoids from intrinsically labeled  $\beta$ -carotene in plants grown with <sup>2</sup>H<sub>2</sub>O; the estimated bioefficacy factor was 3.3  $\mu$ g  $\beta$ -carotene to 1  $\mu$ g retinol (51<sup>†</sup>).

RID has been modified since its inception and will likely continue to change as analytical techniques advance, and a focus is placed on adapting the methods for accessibility and ease of use in low-income regions performing large-scale population surveys. To this end, research recommendations were suggested to improve future RID applications (52<sup>†</sup>, 53<sup>†</sup>), including making the method more field-friendly and confirming its applicability in diverse populations.

#### Evaluation of vitamin A fortification efforts with the use of stable isotopes

The first known evaluation of a vitamin A-fortification program with the use of stable isotopes used the paired RID technique to evaluate changes in vitamin A status 1 y after the introduction of vitamin A-fortified sugar in Nicaragua (54). The children had adequate liver retinol concentrations at baseline (0.52  $\mu$ mol/g liver), and 1 y later liver concentrations had significantly increased and 43% of the children were above the cutoff considered hypervitaminotic [i.e., 1  $\mu$ mol/g liver (10)]. The paired RID method was also used to evaluate the change in vitamin A status of Mexican children fed fortified milk (55<sup>†</sup>). Liver vitamin A concentrations of the children fed the fortified milk were  $\sim$ 2 times higher than the control group after 3 mo of feeding (55<sup>†</sup>). The method has similarly been used to evaluate total body retinol stores of Thai children fed fortified rice for an average of 54 d (31<sup>†</sup>). Total body stores almost doubled in this short time frame despite not showing any significant change in serum retinol concentrations (31<sup>†</sup>). These findings underscore the importance of continued monitoring in populations who have adopted fortification of staple foods with preformed retinol. When stores are adequate, excess preformed vitamin A will accumulate in the liver (56) and may have detrimental effects on bone health (57).

## Biofortification efforts to improve population vitamin A status

The bioefficacy of provitamin A carotenoids to retinol, referred to as a retinol activity equivalent, is currently defined by the Institute of Medicine (IOM) as 12  $\mu\text{g}$   $\beta$ -carotene (or 24  $\mu\text{g}$   $\alpha$ -carotene and  $\beta$ -cryptoxanthin) to 1  $\mu\text{g}$  retinol in healthy humans, by assigning a static bioconversion factor to pure  $\beta$ -carotene in oil of 2  $\mu\text{g}$ :1  $\mu\text{g}$  retinol, multiplied by an average absorption factor of 6 due to plant matrix effects (58). Biofortification initiatives use the IOM's bioefficacy factor to establish staple-crop target levels for provitamin A carotenoids, usually based on  $\beta$ -carotene (59). However, a static bioefficacy factor may not reflect what actually happens in vivo when provitamin A carotenoids are fed to different groups of humans with different vitamin A statuses and carotenoid-processing genotypes (60). Despite the IOM's recommendation of 12:1 and the maximum theoretical value of 0.94:1, bioefficacy values ranging from 2:1 to 28:1 have been reported (39, 61), showing how this value changes with current vitamin A status, due to feedback inhibition of absorption and processing of carotenoids (62), as well as genotype and plant matrix effects. Inaccuracies are also possible when using nonisotopic methods (12), such as changes in serum retinol concentration (61). The IOM's bioefficacy factor is used for predictions; however, researchers can calculate an actual bioefficacy factor on the basis of experimental outcomes when applying stable isotope techniques. The vitamin A value of a given plant can be determined by growing the plant on a stable isotope substrate, such as heavy water ( $^2\text{H}_2\text{O}$ ), feeding the labeled plant, and taking appropriate blood samples (63<sup>†</sup>). The bioefficacy of the provitamin A in the labeled plant source is calculated by comparing the carotenoid-derived retinol in a subject consuming the plant with that from a reference dose of stable isotope-labeled retinyl acetate.

The success of provitamin A-biofortification programs may require target populations to adopt different-colored staples than what are traditionally and culturally acceptable (64). For example, Zambians typically consume white maize and most varieties of biofortified maize are orange. Nutritionists have demanded that biofortified crops be evaluated for their impact on the nutritional status of target groups (65). Stable isotope methods are among the most powerful to evaluate bioefficacy (10, 11<sup>†</sup>, 12) and could support effectiveness studies if applied to subgroups of populations in areas where the crops have been disseminated.

**Sweet potato.** The first biofortified crop targeted for dissemination was orange-fleshed sweet potato because of its widespread availability and high achievable  $\beta$ -carotene concentrations (66). Stable isotope techniques have been used to estimate  $\beta$ -carotene bioefficacy from these crops. The vitamin A value of  $\beta$ -carotene from sweet potato determined in Bangladeshi men was  $\sim 13$   $\mu\text{g}$   $\beta$ -carotene to 1  $\mu\text{g}$  retinol with the use of paired RID with  $^2\text{H}$ -vitamin A before and after a 60-d intervention, which was based on the increase in total body retinol stores (67). More recent work

in Bangladeshi women who were fed orange-fleshed sweet potato, however, did not result in a net gain of total body retinol stores but did contribute to higher circulating serum  $\beta$ -carotene concentrations (41<sup>†</sup>).

Effectiveness studies are used to follow improvement in status after the crop of interest has been broadly released for an extended period. A 2-y integrated effectiveness study was undertaken in Mozambique that included the introduction of sweet potato vines into households and monitoring for 2 agricultural cycles. Children in intervention households ate more orange-fleshed sweet potato and had higher blood-spot retinol concentrations than controls (68). However, it should be noted that, after this study, many of the children still had very low serum retinol concentrations, defined as  $<0.7$   $\mu\text{mol/L}$  by the WHO (69). In an effectiveness study of orange sweet potato in Uganda, vitamin A status was improved in children but only after making corrections and shifting the deficiency cutoff value from 0.7 to 1.05  $\mu\text{mol}$  retinol/L (70<sup>†</sup>). Thus, more quantitative measures of vitamin A status in populations of interest are needed to better predict actual vitamin A status (10), especially when evaluating agriculture-based interventions.

**Provitamin A-biofortified maize.** Studies with biofortified maize began with evaluation in animal models (71–73), because traditionally bred maize did not have high levels of provitamin A when the research first began (74). Favorable bioefficacy factors for provitamin A carotenoids (i.e., more efficient than the 12:1 value recommended by the IOM) in the maize were obtained in animal models, and human studies followed. A conversion factor of  $6.5 \pm 3.5$   $\mu\text{g}$   $\beta$ -carotene to 1  $\mu\text{g}$  retinol was obtained in 6 women in the United States who consumed biofortified maize porridge, which was determined by HPLC with electrochemical detection in reference to retinyl palmitate (75). A bioefficacy factor of  $3.0 \pm 1.5$ :1 was determined in 9 healthy Zimbabwean men by feeding  $^2\text{H}_2\text{O}$ -labeled maize (76). Although similar to the values in the animal studies, these values are estimates of bioefficacy factors with a single-exposure meal in healthy adults and may not reflect what would occur with longer feeding trials in target populations. For example, in a study feeding biofortified maize to Zambian children for 3 mo, the estimated bioefficacy factor was 10.4:1 with the use of paired RID (56).

Long-term maize intake studies in children have been completed to determine if these favorable bioefficacy factors can translate into improved liver concentrations of vitamin A (56, 77). In the first of these controlled feeding studies in Zambia, 3- to 5-y-old children adapted very quickly to eating the orange maize and enjoyed freshly harvested maize much more than maize that was held in storage (77); however, the  $\beta$ -carotene content of the maize was not adequate to maintain vitamin A stores after a high-dose vitamin A supplement, resulting in a higher MRDR value after the trial (78). The need for quantitative evaluation of orange maize interventions led to a second trial 2 y later, which evaluated the impact of 90 d of feeding orange maize to

5- to 7-y-old children with the use of  $^{13}\text{C}_2$ -RID to estimate the total body retinol pool before and after the intervention (56). The total body retinol stores were similarly increased in the children consuming the orange maize or receiving a preformed vitamin A supplement (an increase of 84 and 98  $\mu\text{mol}$ , respectively), which was much more than the 13- $\mu\text{mol}$  increase in children consuming their usual low-carotenoid white maize during the same time frame. These results showed that consuming orange maize could be efficacious in these settings.

A change in the natural enrichment of  $^{13}\text{C}$  in serum retinol may be a way to evaluate interventions with provitamin A carotenoids (79). This phenomenon was studied in overweight individuals when a negative shift was measured in the ratio of  $^{13}\text{C}$  to  $^{12}\text{C}$  in serum retinol after a 3-mo vegetable intervention (79). In the first Zambian study mentioned above, the change in natural abundance of  $^{13}\text{C}$  in serum retinol was positively shifted in the 3- to 5-y-old children eating orange maize for 46 d (45), showing that the  $\beta$ -carotene from this maize is efficacious and contributed to the vitamin A requirements of these children, even though liver stores did not increase. Similarly, gerbils fed an orange maize feed showed an increase in  $^{13}\text{C}$  enrichment in serum retinol compared with controls (80).

Effectiveness studies are needed to measure the generational effects of feeding  $\beta$ -carotene-enhanced staple crops to population groups (7). Feeding orange maize to sows during gestation and lactation improved HPLC-determined liver concentrations of vitamin A in offspring compared with sows fed white maize and administered a high-dose vitamin A supplement (81). Sensitive techniques, such as RID, applied to subgroups of populations who adopt biofortified crops will provide useful data to public health administrators. Nutrition education campaigns will also likely improve acceptance and willingness to pay in target populations for biofortified crops (82).

**Provitamin A-biofortified cassava.** The progression of studies with biofortified cassava mirrored those with maize. In vitro (83) and animal (84) studies were conducted in parallel. In studies conducted in animals with yellow biofortified cassava, the bioefficacy factor was 3.7  $\mu\text{g}$   $\beta$ -carotene to 1  $\mu\text{g}$  retinol (84). Two human studies that used the retinyl palmitate response in the plasma TG-rich lipoprotein fractions were completed in women with the use of biofortified cassava in porridge (85, 86). The bioefficacy factors for  $\beta$ -carotene in these studies were  $2.80 \pm 1.77$  and  $4.5 \pm 3.1$   $\mu\text{g}$   $\beta$ -carotene to 1  $\mu\text{g}$  retinol, indicating readily bioaccessible  $\beta$ -carotene. Larger human studies should consider stable isotope methods, especially during effectiveness evaluations (45, 56).

**Golden Rice.** Transgenic approaches to enhance provitamin A concentrations in the rice endosperm were undertaken, producing high- $\beta$ -carotene (1.6  $\mu\text{g}/\text{g}$ ) “Golden Rice” (87) and the improved ( $26 \pm 5$   $\mu\text{g}/\text{g}$ ) “Golden Rice 2” (88). In

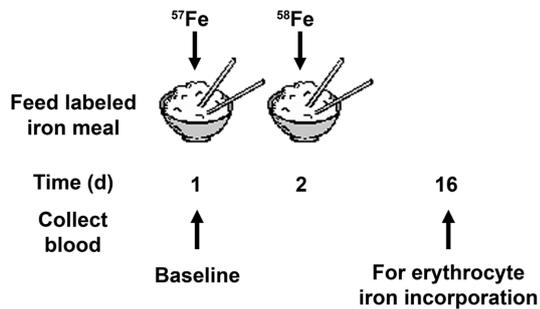
5 healthy adults, the conversion factor for Golden Rice  $\beta$ -carotene (with the use of a particular genotype containing 20  $\mu\text{g}$   $\beta$ -carotene/g) to retinol was  $3.8 \pm 1.7$   $\mu\text{g}$  to 1  $\mu\text{g}$ , with a range of 1.9–6.4:1, and was determined by using rice grown in heavy water ( $^2\text{H}_2\text{O}$ ) and  $^{13}\text{C}_{10}$ -retinyl acetate as a reference dose (89). In Chinese children fed pure  $\beta$ -carotene, Golden Rice, or spinach, the  $\beta$ -carotene to retinol bioefficacy factors were 2.0, 2.1, and 7.3  $\mu\text{g}$  to 1  $\mu\text{g}$ , respectively, which were evaluated by using a combination of  $^2\text{H}$ - and  $^{13}\text{C}$ -labeled compounds (39). This efficient bioefficacy factor for Golden Rice, similar to pure  $\beta$ -carotene in oil, likely reflects the low level of  $\beta$ -carotene in the rice as well as favorable bioaccessibility from this matrix.

### Iron and zinc stable isotope studies

To date, iron and zinc isotopes are not routinely used for assessing the status of individuals. Iron has many biomarkers of status, and typically, population surveys use hemoglobin, hematocrit, soluble transferrin receptor, and ferritin (90). Interventions to improve iron status include low-dose supplementation, fortification, and promotion of heme iron sources or enhancers of iron absorption (91, 92).

The recommended method to assess population zinc status is the prevalence of plasma zinc concentration less than the age-, sex- and time of day-specified cutoffs (93). A prevalence  $>20\%$  of the group studied below the specified cutoff should call for an intervention to improve zinc status (94<sup>†</sup>). Zinc interventions include supplements as an adjunctive therapy for selected infections, fortification, dietary diversification, biofortification, and behavior modification strategies, such as the promotion of breastfeeding (95<sup>†</sup>). Low zinc intakes occur in the absence of these programs and in populations with low rates of breastfeeding, even when breast-milk zinc concentrations are adequate. Adequate zinc concentrations were measured in Indian women, but low breast-milk consumption resulted in low zinc intakes by their infants (21<sup>†</sup>).

**Iron stable isotope bioavailability tests.** Studies with iron stable isotopes are focused on determining differences in bioavailability due to dietary factors by using test foods that have been fortified, biofortified, or contain a potential inhibitor [e.g., phytic acid (8<sup>†</sup>)] or enhancer [e.g., ascorbic acid (96)] of absorption. Isotopes used for labeling test meals in bioavailability studies include  $^{57}\text{Fe}$  (2.12%) and  $^{58}\text{Fe}$  (0.28%) (14). Typically, the tests and controls are performed within the same individual for optimal quality control, because iron status tightly regulates iron absorption. Thus, it is important to measure relative bioavailability of iron sources within the same individual. The basic study design for stable iron incorporation into erythrocytes (RBCs) for bioavailability studies is detailed in an IAEA manual [Figure 5 (97<sup>†</sup>)]. Applications of this isotopic technique in women include the evaluation of bioavailability of different iron fortificants from fish sauce in Thailand (98<sup>†</sup>) and the influence of ascorbic acid on iron absorption in Switzerland (99).

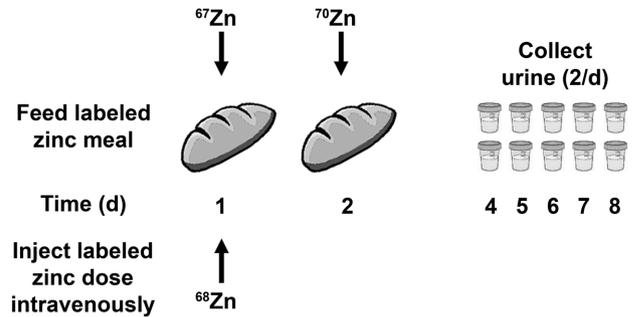


**FIGURE 5** Design used for iron bioavailability studies to compare the quantities of iron absorbed and used between 2 test meals (based on references 97<sup>†</sup>, 98<sup>†</sup>, and 99). Two blood samples are required to determine the incorporation of the isotopes into the erythrocytes. After sample preparation, the iron is analyzed with an appropriate mass spectrometer and ratios of the stable isotopes are compared before and after dosing to determine the amount of iron incorporated into the RBCs. † following a citation number indicates a work that the International Atomic Energy Agency has supported on stable isotopes.

Foods to be evaluated for iron bioavailability can either be extrinsically labeled by adding the isotope to the test meal or intrinsically labeled by growing the food in an isotopically enriched environment. As with intrinsically labeled provitamin A carotenoid studies, the advantage of this approach is that the isotope is located in the same components as the predominant naturally occurring <sup>56</sup>Fe. Iron isotope enrichment is determined by 2 MS methods: inductively coupled plasma–MS (ICP-MS) and the more sensitive, but costlier, thermal ionization MS (TIMS). ICP-MS of iron isotopes, however, suffers from interferences from other molecules, such as nickel and argon compounds. Therefore, these interferences must be accounted for by standardization with TIMS (100). Thus, similar to vitamin A MS, familiarity with instrumentation and methodology is important.

**Zinc stable isotope tests.** Unlike iron and vitamin A, zinc does not have a well-defined marker of body stores. Most studies interested in evaluating zinc status measure plasma zinc, which responds quickly to dietary intake or supplement usage (101). Zinc has many naturally occurring stable isotope forms including <sup>64</sup>Zn (most abundant), <sup>66</sup>Zn, <sup>67</sup>Zn, <sup>68</sup>Zn, and <sup>70</sup>Zn, with the less abundant forms (i.e., <sup>67</sup>Zn, <sup>68</sup>Zn, and <sup>70</sup>Zn) preferred as tracers because they result in a lower background signal. The same techniques used to measure iron are used for zinc (i.e., ICP-MS and TIMS), along with the same limitations due to interfering compounds (102).

Similar to iron, zinc stable isotopes can be used to evaluate absorption efficiency. However, the regulation of zinc absorption by zinc status is much less strong, but still occurs (103), and absorption is affected by the dose size administered or the amount consumed. Therefore, the difference in total zinc absorbed from 2 different dietary sources is most relevant to determining the superiority of



**FIGURE 6** Design for a zinc-bioavailability study to determine the difference in absorption of zinc from typical wheat and a zinc-enhanced variety [based on a study evaluating tortillas (104<sup>†</sup>)]. Zinc isotopes are administered orally with the test food on days 1 and 2 and intravenously on the first day. Urine samples are collected on days 4 through 8 (2/d) and analyzed with an appropriate mass spectrometer. † following a citation number indicates a work that the International Atomic Energy Agency has supported on stable isotopes.

one source or another, whereas the fractional absorbed zinc is most relevant when comparing a standard dose of 2 fortificants or supplements. To determine bioavailability, a subject consumes a test meal (with the same considerations as iron) containing one zinc stable isotope while a second, different zinc stable isotope is injected intravenously and assumed to be equivalent to 100% absorption. The ratio of the 2 isotopes in urine (or plasma can be used) is examined after an appropriate mixing time (Figure 6). After correcting for dose size and enrichment, fractional absorbed zinc and total zinc absorption from the test meal can be determined (22). For example, a zinc-absorption study provided women with typical wheat and zinc-biofortified wheat tortillas containing different zinc isotopes (<sup>67</sup>Zn and <sup>70</sup>Zn), and compared the enrichment in urine with an intravenous dose of <sup>68</sup>Zn [Figure 6 (104<sup>†</sup>)]. More zinc was absorbed from the biofortified wheat tortillas (104<sup>†</sup>).

Although much of zinc is not accessible to measurement in the body due to its incorporation into proteins and function in cellular processes, ~20% of body zinc exchanges rapidly with plasma and is called the exchangeable zinc pool or EZP, which has been suggested as a biomarker of zinc status (22). One study determined the change in EZP after supplementation, where it was responsive (105<sup>†</sup>). Further research on the topic is warranted because EZP could prove to be a more robust estimate of zinc stores than plasma concentrations alone. Stable isotope compartmental modeling can also be used to examine the kinetics of the zinc pool (22), similar to the type of modeling described for vitamin A research earlier in this review.

### Fortification of staple foods with iron and zinc

In the processing of grains, minerals are often discarded with the hull and the endosperm. Therefore, iron is often one of the components added back during the fortification process. However, this may not be feasible in some countries where

the crops are locally grown and consumed and not processed at centralized mills. Countries that want to improve mineral nutrition need to choose a widely consumed food that is centrally processed and inexpensive for target groups. In Thailand, this vehicle was identified as fish sauce. To evaluate bioavailability, iron absorption from the intrinsically labeled compounds was determined via erythrocyte incorporation of  $^{57}\text{Fe}$  and  $^{58}\text{Fe}$ . Different fortificants were explored, and absorption from the fish sauce was significantly lower with the use of ferrous lactate and ferric ammonium citrate than from ferrous sulfate (98<sup>†</sup>). The same method was used to compare absorption of the iron fortificants ferric ammonium citrate or a 2:1 mixture of ferrous sulfate ( $\text{FeSO}_4$ ) and ferric sodium EDTA ( $\text{FeNaEDTA}$ ) in a rice dessert in Thai children aged 8–24 mo (106<sup>†</sup>). The study showed that, although either approach was sufficient to provide adequate iron, absorption was significantly higher in the  $\text{FeSO}_4 + \text{FeNaEDTA}$  group. Similarly,  $\text{FeNaEDTA}$  alone showed ~40% better absorption than ferrous fumarate in Haitian women and children, and a mixture of the 2 iron sources was not better than ferrous fumarate alone (107<sup>†</sup>). In both the Thai and Haitian studies, it was shown that, although not as bioavailable as  $\text{FeNaEDTA}$ , cheaper iron sources, such as ferric ammonium citrate or ferrous fumarate, were effective at improving iron status.

Another fortification approach was undertaken in Brazil with the use of drinking water as a vehicle after a study identified a significant prevalence of anemia assessed by hemoglobin concentration (29.3%), and iron deficiency assessed by mean corpuscular volume, transferrin saturation, and RBC distribution width (75%) among preschool children who attended full-time daycare centers (108<sup>†</sup>). The intervention showed that by fortifying the childcare center's drinking water with both iron and ascorbic acid, functional iron improved [defined as mean corpuscular volume (109)] and iron deposits increased [defined as ferritin concentrations (109)] compared with an unfortified control water in Brazilian children (110<sup>†</sup>).

An example of zinc fortification is point-of-use fortification by including zinc in micronutrient powders that are added to traditional foods shortly before consumption. In a trial in Pakistani infants, total absorbed zinc from a zinc-containing micronutrient powder sprinkled on rice and lentils increased when compared with a control powder ( $1.2 \pm 0.5$  and  $0.1 \pm 0.1$  mg, respectively) and the EZP was enhanced ( $4.5 \pm 1.0$  and  $3.7 \pm 0.6$  mg/kg, respectively) (105<sup>†</sup>).

### Iron and zinc in staple crops

Many studies have been conducted with in vitro methodology evaluating the bioaccessibility of minerals from staple crops. However, due to whole-body regulatory systems involved in mineral metabolism, the consensus was that human studies will ultimately be needed to assess the impact of biofortified crops on zinc and iron nutrition (111<sup>†</sup>).

**Iron-biofortification efforts.** Rice and beans are among several crops that have been biofortified with iron and

tested in humans. Considering the global prevalence of iron deficiency anemia and the billions of people who consume rice and other staples, iron-biofortified crops could make an impact. For example, due to inadequate intake of dietary iron and poor bioavailability, prevalences of anemia and iron deficiency in India were 39% and 62%, respectively (112<sup>†</sup>). Biofortified rice could improve iron intakes among consumers who consume rice as a staple, such as in India. The first study, to our knowledge, to show the potential of iron-biofortified rice, which contained 3.21 mg Fe/kg, to improve iron body stores in humans was orchestrated in Filipino women, where it was compared with a local rice variety that only contained 0.57 mg/kg (113). The study used hemoglobin and serum ferritin, which are traditional biomarkers of iron status. After controlling for baseline iron stores in these women, calculated body iron was higher in the biofortified rice group than in the control group (113).

High-iron beans were studied in Rwandese women by determining the differences in bioavailability on the basis of phytic acid and polyphenol content (114<sup>†</sup>). When  $^{57}\text{Fe}$  and  $^{58}\text{Fe}$  incorporations into RBCs were determined after the consumption of mixed meals, high-iron beans did not seem to overcome the inhibitory effects of phytic acid and polyphenols on bioavailability. Therefore, the recommendation was to select beans that had high iron, low phytate, and low polyphenols for future studies (114<sup>†</sup>).

**Zinc-biofortification efforts.** Most research studies on zinc-biofortified crops have used zinc isotopic bioavailability techniques. In the zinc-biofortification study described earlier (Figure 6), it was determined that meals that included wheat tortillas made with zinc-biofortified wheat resulted in 0.5 mg/d higher absorption than control wheat (104<sup>†</sup>). The bioavailability of zinc from rice-based diets that provided 4.8 mg Zn/d did not have any more absorbed zinc than a conventional rice-based diet at 3.8 mg Zn/d, but the quantity of zinc absorbed increased from fortified rice containing 6.03 mg Zn/d (115<sup>†</sup>). Thus, either zinc needs to be increased further in biofortified varieties to match fortified levels or phytate levels need to be decreased (115<sup>†</sup>). Since then, a biofortified maize variety with 34  $\mu\text{g}$  Zn/g grain was able to provide amounts of zinc similar to a fortified maize with 60  $\mu\text{g}$  Zn/g grain ( $1.1 \pm 0.5$  and  $1.2 \pm 0.4$  mg Zn/d, respectively), significantly more than the control maize with 21  $\mu\text{g}$  Zn/g grain ( $0.6 \pm 0.2$  mg Zn/d) in young rural Zambian children (116<sup>†</sup>). The biofortified variety was able to meet the zinc requirements for this vulnerable population (116<sup>†</sup>).

As biofortification efforts move forward, developing cultivars that have multiple micronutrients should be pursued (64). Pearl millet that was biofortified with iron and zinc was shown to provide significantly increased quantities of these nutrients compared with a test meal (117<sup>†</sup>). Quality-protein maize often has increased levels of zinc (118), and it is known that the synergistic effects between vitamin A and zinc lead to enhanced overall nutrient metabolism (7) and may reduce malaria morbidity when supplemented together (119<sup>†</sup>). Therefore, maize bred with quality protein,

enhanced zinc, and increased provitamin A carotenoids may supply better nutrition than any single-nutrient approach for populations that have high staple-crop intakes.

Simulations have shown that biofortification of rice with zinc could readily affect zinc intakes of women and children in Bangladesh (120) and adults following traditional eating patterns in China (121). Manipulating both iron and zinc in rice may be feasible (122). Furthermore, depressed nutrient intakes occur in children who have repeated infections, such as endemic malaria, and therefore reevaluating micronutrient target levels may be important in regions that adopt biofortified staple crops (123).

## Conclusions

Worldwide efforts to improve micronutrient status include fortification and biofortification. These food-based approaches have the potential to reach nearly all sectors of the population, including, but not limited to, low-income groups who are vulnerable to micronutrient deficiency. In this regard, population nutrient assessment needs to move beyond rural populations and include urban and affluent groups. This is particularly true for preformed vitamin A-fortification programs that have populationwide coverage where excessive intakes could lead to hypervitaminosis A. Mineral absorption is more highly regulated than preformed vitamin A, and therefore the risk of mineral toxicity is much less from high intakes of mineral-fortified or biofortified foods.

In summary, the RID methods discussed here are powerful tools to examine provitamin A carotenoid bioefficacy and vitamin A status from deficiency to toxicity in a diverse range of populations. Iron isotopes have been valuable in recommending appropriate fortificants for foods and defining the interaction with inhibitors and enhancers of absorption. Zinc isotopes are also valuable for absorption studies, and developments that use EZP may yield new insights in evaluating zinc status. Further refinement of these techniques to increase their accuracy, accessibility, and cost-effectiveness will help guide fortification efforts in the future. The use of these techniques to evaluate food-based interventions will inform nutrition scientists, public health professionals, and policy makers, especially with regard to biofortification.

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