A stable isotope approach to accurately determine iron and zinc bioaccessibility in cereals and legumes based on a modified INFOGEST static in vitro digestion method

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Abstract

The establishment of the INFOGEST in vitro static digestion method, a standardized international consensus, was an important milestone in the field of food digestion. We evaluated the contribution of iron and zinc in reagents used in the INFOGEST method in relation to sample iron and zinc and the potential interference of reagent-derived iron and zinc with bioaccessibility measurements. In most cases, reagent-derived iron and zinc contributed more than 50% of the total iron or zinc in the digesta containing selected cereals and legumes. Moreover, the chemical behaviour of reagent-derived iron and zinc was matrix dependent such that the application of a blanket blank correction was not appropriate. We therefore propose an improved approach involving isotopic labelling of reagent iron and zinc in order to discriminate between reagent-derived and sample-derived iron and zinc in each matrix. This stable isotope approach could improve the accuracy and reliability of iron and zinc bioaccessibility studies.

Keywords: gastro-intestinal digestion, digestive enzymes, pancreatin, bile, minerals

1 Introduction

There is a rising interest in the use of in vitro methods to study the fate of food during digestion with more than 2,500 articles published in the last 40 years, of which 85% were published in the last two decades (Lucas-González, Viuda-Martos, Pérez-Alvarez, & Fernández-López, 2018). In vitro methods are increasingly being used to understand the digestibility, bioaccessibility, stability and structural changes undergone by foods under different conditions of the gastrointestinal tract (Hur, Lim, Decker, & McClements, 2011). Although in vitro methods cannot fully mimic the physiological and physiochemical events of digestion in vivo, they offer a cost-effective and rapid alternative to in vivo methods which are often costly, labour intensive and subject to ethical restrictions (Bohn et al., 2018). In some cases where large numbers of samples have to be analysed or where comprehensive analyses are needed, in vitro methods may be the only ethical alternatives.

Iron and zinc are mineral micronutrients of public health importance whose bioavailability is largely modulated by dietary factors. Bioavailability is thus an important aspect when considering the iron and zinc supply of foods. As an alternative to the difficult and expensive human absorption studies used to measure iron and zinc bioavailability, Miller, Schricker, Rasmussen, and Van Campen (1981) proposed an in vitro dialyzability assay, which involves a simulated gastrointestinal digestion followed by measurement of low molecular weight iron or zinc as bioavailability proxies. This method has been found to be in reasonable agreement with human absorption data, especially for iron (Aragón, Ortiz, & Pachón, 2012; Sandberg, 2005; Van Campen & Glahn, 1999). Since then, the dialyzability assay has been used extensively to understand the bioaccessibility of iron and zinc (meaning in vitro bioavailability) in foods. This rapid and low cost method is crucial to inform the large number of nutrition programs aimed at improving iron and zinc nutrition for vulnerable populations (Fairweather-Tait et al., 2005). Dialyzability assays are used to understand the many variables influencing iron and zinc bioavailability of foods, such as processing, formulation, fortification compounds and biofortification, among others (Aragón et al., 2012; Gabaza, Shumoy, Muchuweti,

67 Vandamme, & Raes, 2018; Guillem et al., 2000; Kapsokefalou, Alexandropoulou, Komaitis, & Politis,
68 2005; Kruger, Taylor, & Oelofse, 2012; Shumoy et al., 2017).

69 Despite the advancements made in this area, it is difficult to compare results across different
70 laboratories due to the numerous variations in methods used to simulate gastrointestinal digestion.
71 Hur et al. (2011) showed that in vitro digestion models used to study different components of foods,
72 including minerals, differed widely in: the occurrence and concentrations of digestive enzymes used,
73 duration of digestion, pH values and buffer concentrations achieved in the different phases of
74 digestion. Clearly, the use of a standardised method is important to enable easier comparability and
75 reproducibility of studies in this field as all these factors modify the extent to which minerals are
76 released. To address this problem, the COST Action INFOGEST network established an international
77 harmonised protocol for static simulation of gastrointestinal digestion of foods based on available
78 physiological data (Brodkorb et al., 2019; Minekus et al., 2014). Since the publication of this method,
79 it has been cited more than 1,000 times in Web of Science and a rising number of studies are in the
80 field of iron and zinc bioaccessibility. An important aspect resolved by this international consensus
81 protocol is the standardization of: (i) sources of enzymes, (ii) enzyme activity units to be achieved
82 during each digestion phase and (iii) assays to determine the enzyme activity. This makes it easier for
83 researchers to source enzymes from any suitable supplier, making this protocol applicable for
84 researchers globally (Verhoeckx et al., 2015).

85 Based on some preliminary unpublished findings, we hypothesise that the enzymes used to simulate
86 gastrointestinal digestion contain trace amounts of iron and zinc which may interfere with
87 bioaccessibility measurements. Quantities of enzymes recommended in the INFOGEST method are
88 greater than most in vitro digestion models, suggesting an even larger contribution of enzyme-derived
89 iron and zinc into the digestion system. Before non-haem iron is absorbed in vivo, it first enters a
90 common non-haem iron pool, which can include intrinsic and/or extrinsic iron sources. Iron that enters
91 this pool in the digestive tract is absorbed to the same extent depending on the balance of absorption

92 enhancers and inhibitors in the food consumed (Hurrell & Egli, 2010). The same mechanism of
93 absorption also exists between intrinsic and extrinsic zinc sources (Fredlund, Rossander-Hulthén,
94 Isaksson, Almgren, & Sandberg, 2002; Signorell et al., 2019). Similarly, during in vitro digestion, iron
95 and zinc from samples and reagents enter a common pool that is subjected to the same interactions
96 that influence bioaccessibility. Therefore, the bioaccessible iron and zinc measured after digestion is
97 potentially contributed by iron and zinc derived from both samples and reagents so that discrimination
98 between the two sources of minerals is needed for a reliable and accurate quantification.

99 In this paper, we assess the iron and zinc concentrations of reagents used in the INFOGEST method in
100 relation to the iron and zinc concentrations of cereals and legumes that are often targets of iron and
101 zinc nutrition programs. A suitable modification of enzyme and bile concentrations that limit the
102 contribution of reagent iron and zinc is thereby recommended. In addition, we propose isotopic
103 labelling of reagent iron and zinc as a strategy to trace the fate of reagent-derived iron and zinc during
104 digestion and compare this approach with conventional approaches of calculating bioaccessibility. The
105 reliability and accuracy of in vitro methods to predict the iron and zinc bioaccessibility of crops is
106 important as large investments are being made in the quest to improve their bioavailability in crops.

107

108 ## 2 Materials and methods

109 Pepsin from porcine gastric mucosa (specific activity, 3412 U/mg), α-amylase from *Bacillus*
110 *sp.* (specific activity, 1380 U/mg), pancreatin from porcine pancreas (specific activity, 4.3 U/mg trypsin
111 activity), bovine bile (specific activity, 1.410 mM/g), 1,4-piperazinediethanesulfonic acid disodium salt
112 (PIPES) and dialysis tubing (high retention seamless cellulose tubing, average flat width 23 mm,
113 molecular weight cut-off 12,400 kDa) were obtained from Sigma Aldrich, Dorset, UK. Concentrated
114 HNO_3 (PrimarPlus^{TM} grade) was obtained from Fisher Scientific, Loughborough, UK. Wheat flour
115 standard reference material (NIST 1567b) was procured from the National Institute of Standards and
116 Technology. Stable isotopes, ^{57}Fe and ^{70}Zn (95% enrichment) were purchased from Isoflex, USA.

Common bean, pearl millet and finger millet were procured from supermarkets while the rest of the cereals and legumes were kindly supplied by colleagues in UK and Malawi (maize, cowpea, velvet bean and wheat). All cereals and legumes were milled into flour before analysis.

2.1 Analysis of iron and zinc in reagents and samples

Iron and zinc concentrations of reagents and samples (cereals and legumes) were determined. Reagents analysed were: simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), enzymes and bile. For solid samples, 0.2 g of sample was weighed into microwave heating vessels and 6 mL concentrated HNO_3 was added. microwave heating (Microwave Pro, Anton Paar GmbH, Austria) was performed over 45 min in order to release minerals. The sample was heated over 10 min to reach 140^{o}C, held for 20 min at 140^{o}C and then cooled over 15 min to 55^{o}C. The solutions were diluted accordingly to achieve an acid concentration of less than 5% using Milli-Q water (18.2 MΩ cm) prior to analysis using a triple quadrupole inductively coupled plasma mass spectrometer (ICP-MS) (iCAP TQ, Thermo-Fisher Scientific, Bremen, Germany). Liquid samples were diluted 10× with 2% HNO_3 prior to analysis. Samples were introduced at a flow rate of 1.2 mL min^{-1} from an autosampler (ESI SC-4 DX FAST Autosampler) incorporating an ASXpress™ rapid uptake module through a perfluoroalkoxy (PFA) Microflow PFA-ST nebuliser (Thermo-Fisher Scientific). An internal standard of rhodium (5 µg L^{-1}), was introduced to the sample stream on a separate line with an equal flow rate via the ASXpress™ unit. A standard calibration was created by serial dilution of iron and zinc standards to give a concentration ranging from 0 to 100 µg L^{1}. A wheat certified reference material (CRM) was included for quality control of the microwave assisted heating of the dry flours. The iron and zinc reference concentrations of the wheat CRM were 14.11 ± 0.33 mg kg^{-1} and 11.61 ± 0.26 mg kg^{-1} respectively, and the recovery was 87.7 ± 2.39% for iron and 80 ± 8.33% for zinc. The LOD and LOQ were respectively: 0.014 and 0.042 µg L^{-1} for ^{56}Fe, 0.048 and 0.146 µg L^{-1} for ^{57}Fe, 0.014 and 0.043 µg L^{-1} for ^{66}Zn and 0.194 and 0.588 µg L^{-1} for ^{70}Zn. Based on the INFOGEST

gastro-intestinal in vitro digestion method, the amount of reagent-derived and sample-derived iron and zinc (expressed in mg) potentially present in a typical gastro-intestinal digestion was assessed.

2.2 In vitro digestion 1: To determine the effect of reagents and sample matrix on solubility of iron and zinc

Gastro-intestinal digestion was done by following the INFOGEST method according to Minekus et al. (2014) with some modifications at the intestinal stage of digestion. Based on observed iron and zinc concentrations in the pancreatin and bile, a modification to reduce their contribution to the iron and zinc assay was made. Pancreatin was added to achieve a concentration of 100 U mL^{-1} protease in the final digestion mixture instead of 100 U mL^{-1} trypsin activity. The pancreatin used in this study (8×USP) was estimated to have an activity of at least 200 U mg^{-1} protease according to the certificate of analysis from the manufacturer. Bile was added to achieve a final concentration of 2 mM instead of 10 mM and this was calculated based on a bile concentration of 1.410 mmols g^{-1}. All other parameters recommended in the INFOGEST method were maintained i.e. electrolyte solutions, SSF, SGF and SIF were prepared accordingly. Since substantial reagent iron and zinc was still present even after this modification, the aim of this experiment was to determine the matrix effect on solubility of extrinsic iron and zinc. Extrinsic iron and zinc in the form of ^{57}Fe and ^{70}Zn was applied at the beginning of digestion to achieve a concentration of 100 µg L^{-1} in the final digesta. The stable isotopes were applied in the reagent blank and in cereal and legume matrices (maize, finger millet, cowpea and velvet bean). After digestion, the samples were placed on ice for 15 min to stop enzyme activity before being centrifuged for 30 min at 4,500 × *g*. The supernatant was separated from the pellet and filtered through a 5 µm syringe filter. Analysis of iron and zinc concentrations by ICP-MS was done following the method described previously after microwave assisted heating of 3 mL of the supernatant with 3 mL of concentrated HNO_3. Isotopes monitored were ^{56}Fe (native iron), ^{57}Fe (applied iron isotope), ^{66}Zn (native zinc) and ^{70}Zn (applied zinc isotope). The proportion of ^{57}Fe and ^{70}Zn recovered in the supernatant after gastro-intestinal in vitro digestion in each sample matrix was then calculated.

2.3 In vitro digestion 2: improved stable isotope approach

A modified version of the INFOGEST method was used, involving isotopic labelling of reagent iron (^{57}Fe) and zinc (^{70}Zn) in order to discriminate between reagent-derived and sample-derived iron and zinc in the different sample matrices. Electrolyte solutions were prepared according to the procedure described in the INFOGEST method, i.e. (SSF), (SGF) and (SIF). After this, complete simulated digestion fluids or master mixes of solutions required at each phase of digestion, were prepared by including in the simulated electrolyte solutions, the respective enzyme, $CaCl_2$ (only for gastric and intestinal phase as it caused precipitation in the SSF), the stable isotopes for isotopic labelling and Milli-Q water to achieve the required concentrations. Four solutions were prepared as illustrated in **Table 1**, namely: simulated salivary fluid (SSF complete), simulated gastric fluid (SGF complete), simulated pancreatin fluid (SPF complete) and simulated bile fluid (SBF complete). To determine the amount of ^{57}Fe and ^{70}Zn to add to the digestion solutions, the total native ^{57}Fe and native ^{70}Zn of these solutions were determined first. The stable isotopes were then applied to each digestion solution at a level 10× their concentration in the respective solution. The final complete digestion mixtures for each phase of digestion were placed in a shaking water bath at 20^{o}C, overnight, to allow for complete isotopic equilibration. Isotopic equilibration was considered complete when the ratio of native Fe/applied Fe (or native Zn/applied Zn) was the same before centrifugation and in all fractions after centrifugation. In the previous experiment, complete isotopic exchange was not achieved during gastro-intestinal digestion so it was necessary to attain this prior to digestion. Preliminary trials showed that complete isotopic equilibration occurred after at least 6 hours of incubation at 20^{o}C. Enzyme activity was determined according to the standard procedures outlined in the INFOGEST protocol and there was no loss in activity after overnight incubation. After equilibration, the complete digestion fluids were placed on ice before commencing the digestion. Digestion was performed on unprocessed cereal and legume flour samples (maize, wheat, finger millet, pearl millet and common bean). To begin the oral phase of digestion, 2.5 g of cereal or legume flour slurry (flour mixed with Milli-Q water to make a 30%

194 dry flour slurry) was mixed with 2.488 mL SSF complete and 0.012 mL $CaCl_2$ (75 U mL^{-1} amylase activity
195 in final digestion mixture). The pH was adjusted to 7.0 and the mixture was incubated at 37°C, in a
196 shaking water bath for 2 min. For the gastric digestion, 5 mL of SGF complete was added (2,000 U mL^{-1}
197 pepsin activity in final digestion mixture) and the pH was corrected to 3.0 followed by incubation for
198 90 min. Dialysis tubing containing 17.5 mL of 0.05 M PIPES buffer (pH 6.7) was added to the sample
199 digestion tubes, except for the reagent blanks, and the tubes were incubated for a further 30 min.
200 Finally, intestinal digestion was followed by adding 5 mL of SPF complete and 5 mL of SBF complete
201 and adjusting the pH to 7 where necessary. The tubes were incubated again for 2 hours before being
202 placed on ice for 15 min to stop enzyme activity. The dialysis membranes were removed and the
203 dialysate (solution in the dialysis membranes - bioaccessible fraction) was carefully transferred into
204 clean storage tubes. Analysis of iron and zinc concentrations by ICP-MS was done following the
205 method described previously after microwave-assisted heating of 4 mL of the dialysate with 2 mL of
206 50% HNO_3 or 3 mL of the soluble non dialysed fraction with 3 mL of concentrated HNO_3. The insoluble
207 fraction, or pellet, was dried and also analysed for iron and zinc after microwave-assisted heating.
208 Again, the isotopes ^{56}Fe (native iron), ^{57}Fe (applied iron isotope), ^{66}Zn (native zinc) and ^{70}Zn (applied
209 zinc isotope) were monitored. Since the total intrinsic and extrinsic iron and zinc concentration in the
210 reagent blanks was needed for the calculation, the reagent blanks were not centrifuged because
211 centrifuging caused a proportion of the minerals to partition into the insoluble fraction.

212

213 **2.4 Data processing and statistical analysis**

214 Blank and drift corrections were done on raw intensity data (counts per second) obtained after ICP-
215 MS analysis. Standard calibrations of ^{56}Fe, ^{57}Fe, ^{66}Zn and ^{70}Zn were used to convert intensity data into
216 concentration data (µg L^{-1}). The concentration of native Fe (Fe_{native}) and Zn (Zn_{native}) was calculated
217 from the measurement of ^{56}Fe and ^{66}Zn respectively. On the other hand, the concentration of ^{57}Fe and
218 ^{70}Zn represents the total ^{57}Fe and ^{70}Zn which includes a contribution from the applied stable isotopes

and a small proportion from the native iron and zinc according to their isotopic abundances i.e. 0.2119% for ^{57}Fe and 0.061% for ^{70}Zn (Meija et al., 2016). Therefore, to obtain the concentration of only the applied ^{57}Fe or ^{70}Zn, the concentration of native ^{57}Fe ($^{57}Fe_{native}$, µg L^{-1}) or ^{70}Zn ($^{70}Zn_{native}$, µg L^{-1}) was calculated first. Equation 1 below shows the calculation for Fe:

$$^{57}Fe_{native} = Fe_{native} * \left(\frac{^{57}Fe_M}{Fe_M}\right) * {}^{57}Fe_{IA} \qquad (1)$$

Where Fe_{native} is the concentration of native Fe expressed in µg L^{-1}, $^{57}Fe_M$ is atomic mass of ^{57}Fe (56.935), Fe_M is average atomic mass of Fe (55.845), and $^{57}Fe_{IA}$ is the isotopic abundance of ^{57}Fe (0.002119). A mass correction was used to account for mass differences of the iron isotopes. Applied iron ($Fe_{applied}$, µg L^{-1}) was then calculated using Equation 2:

$$Fe_{applied} = {}^{57}Fe_{tot} - {}^{57}Fe_{native} \qquad (2)$$

Where $^{57}Fe_{tot}$ is the total concentration of ^{57}Fe (µg L^{-1}). Iron concentration in the dialysate fraction ($Fe_{dialysate}$, µg L^{-1}) was calculated using Equation 3 below:

$$Fe_{dialysate} = Fe_{native} - \left(\frac{Fe_{applied}}{Fe_{applied-tot}} * Fe_{reagents}\right) \qquad (3)$$

Where:

Fe_{native} is the native iron concentration in dialysate fraction (µg L^{-1})

$Fe_{applied}$ is the concentration of remaining applied iron in the dialysate fraction (µg L^{-1}) obtained in Equation 2

$Fe_{applied\text{-}tot}$ is the total applied iron obtained from the reagent blank (µg L^{-1})

$Fe_{reagents}$ is the total native reagent derived iron obtained from reagent blank (µg L^{-1})

The iron and zinc concentrations of the dialysate fractions were then converted to a gravimetric basis based on the weight and volume used for the digestion to obtain bioaccessible iron Fe_{bio}(mg kg^{-1}) or zinc Zn_{bio}(mg kg^{-1}). Iron and zinc bioaccessibility was also calculated relative to the total iron and zinc

in the sample to obtain Fe_{bio}(%) and Zn_{bio}(%) respectively. This stable isotope approach was compared with conventional approaches (1 and 2 below) used to calculate mineral bioaccessibility after in vitro digestion without a discrimination of reagent and sample derived iron and zinc.

Approach 1: A blanket reagent blank correction was done in order to obtain the iron or zinc concentration in the dialysate fraction, then bioaccessibility was calculated relative to the total iron and zinc in the sample (Wolfgor, Drago, Rodriguez, Pellegrino, & Valencia, 2002).

Approach 2: A reagent blank correction was not done. Iron and zinc concentration was determined in all fractions obtained after digestion, i.e. dialysate, soluble non dialyzed fraction and pellet. Bioaccessibility was calculated relative to the total recovered iron and zinc (Greffeuille et al., 2011).

Comparison of means was conducted using one-way ANOVA ($p < 0.05$) and Tukey's Honest Significant Difference where applicable, in R (Version 3.5.2; R Core Team, 2017).

3 Results and discussion

3.1 Iron and zinc concentrations of reagents in relation to samples according to INFOGEST method

The iron and zinc concentrations of reagents used in the INFOGEST gastro-intestinal digestion method were determined and are presented in **Table 2.** The iron and zinc concentrations in the electrolyte solutions were low (not more than 20 μg L^{-1}) and were estimated to contribute negligible levels of iron and zinc due to a dilution effect during digestion. In terms of enzymes and bile, the α-amylase had the lowest iron and zinc concentrations while substantial levels were present in the rest. The amount of iron or zinc contributed by the enzymes in the digesta can only be understood based on the amount of enzyme added. The amount of enzyme to be added depends on its specific activity and the desired activity units to be achieved in the final gastro-intestinal digestion mixture. Using an example provided by Brodkorb et al. (2019) of enzyme amounts needed for digestion of 5 g of food based on the

INFOGEST method, an estimate of the iron and zinc contents potentially contributed by the enzymes was calculated (**Table 3**).The amount of iron and zinc contributed from the enzymes depends on the iron and zinc concentration in the enzyme and the amount of enzyme used. For example, pepsin with 226 mg kg^{-1} iron, contributes only 0.003 mg iron compared to pancreatin with a lower iron concentration of 78 mg kg^{-1} but contributing at least ten times higher iron than pepsin. This is because only 13.34 mg of pepsin needs to be added in comparison to pancreatin where 667 mg must be added. In general, pancreatin and bile introduce much greater amounts of iron and zinc (more than 90% of the total reagent iron and zinc) to the digestion because more of these are needed to achieve the recommended activity units in the final digestion mixture.

Cereals and legumes are important sources of iron and zinc for low income countries, as such they are amongst the most studied crops in terms of their iron and zinc bioaccessibility. **Table 4** shows the iron and zinc concentrations of some cereals and legumes used in this study, together with an estimate of the amount of iron and zinc that will be present in a digesta of 5 g food sample with dry flour content ranging between 30 – 100%. The range of dry flour contents that can potentially be in the digesta were based on the wide variation of products that can be produced from cereals and legumes ranging from thin porridges (20 – 30% dry matter) and drier products such as roasted or popped products (~12% moisture which is equivalent to 100% dry flour in the digesta). If we consider the estimates in **Table 3 and 4** of the reagent and sample iron and zinc contribution per digestion respectively, the total amount of iron and zinc that can potentially be present in the digestion can be calculated. This is crucial to understand the proportion of minerals of interest in the reagents compared to the samples. According to these estimates, for a food with 30% dry flour, reagent iron can contribute 53 – 77% of total iron in the digesta while for 100% dry flour, it can contribute 25 – 53% of the total iron. In most cases, reagent iron is greater than sample iron. Although reagent iron is mostly lower than sample iron in the samples with greater iron concentration when 100% dry flour is considered, it still contributes substantial levels of iron (at least 25% of the total iron in the digesta). Similarly, for a food with 30% dry flour, reagent zinc can range between 83 – 93% of total zinc in digesta and 59 – 77% for

294 a food comprising 100% dry flour. In all scenarios, reagent zinc is always greater than sample zinc. The
295 proportion of reagent-derived iron or zinc can be assumed to be even higher than estimated because
296 not all the iron or zinc in the sample is released into solution during gastro-intestinal digestion. This
297 shows that the reagent blank based on the INFOGEST method as it is, will most likely contribute a
298 greater amount of iron and zinc than samples, although the reagent blank should contain trace levels
299 of the analyte of interest.

300

301 The levels of pancreatin and bile were modified in order to reduce both reagent iron and zinc
302 contributions and their interference in mineral binding. Saturated solutions of pancreatin and bile are
303 used in the INFOGEST method as is, and these precipitate during centrifugation with the potential to
304 adsorb metals into the solid phase. Rousseau et al. (2019), showed that zinc bioaccessibility was
305 drastically reduced when the complexity of the in vitro digestion model was increased by adding bile
306 salts in comparison to enzymes which had no effect. They concluded that bile salts may interact with
307 zinc thereby reducing zinc bioaccessibility. According to the INFOGEST method, pancreatin must be
308 added to achieve trypsin activity of 100 U mL^{-1} in the final digestion mixture. Trypsin activity of
309 pancreatin was 6 U mg^{-1} for the batch described by Brodkorb et al. (2019); in the current study we
310 measured trypsin activity of 4.3 U mg^{-1}. Based on this specific activity, a high quantity of pancreatin is
311 needed to achieve the required 100 U mL^{-1} in the final digestion mixture. Instead, the amount of
312 pancreatin added was calculated to achieve a protease activity of 100 U mL^{-1} in the final digestion
313 mixture, based on a specific activity of 200 U mg^{-1} protease as specified by the supplier. Based on this
314 specific activity, pancreatin solution with a concentration of 2 mg mL^{-1} was added instead of 133 mg
315 mL^{-1}.

316 Bile amount was calculated to reach 2 mM bile salt concentration in the final digestion mixture instead
317 of 10 mM based on a specific activity of 1.410 mmols g^{-1}. Likewise, bile solution with a concentration
318 of 19 mg mL^{-1} was added instead of 200 mg mL^{-1}. The reduction in the amount of pancreatin and bile

added reduced reagent iron and zinc by more than 50% thereby reducing their interference in the chemical processes occurring between minerals and mineral binders during intestinal digestion. Other in vitro digestion models used to study mineral bioaccessibility also use much lower concentrations of pancreatin (c.1.4 mg mL^{-1}) and bile (c.8.6 mg mL^{-1}) than proposed in the INFOGEST method (Glahn, Cheng, & Giri, 2015; Miller et al., 1981; Wolfgor et al., 2002). Most of the iron and zinc in foods is released during the gastric phase of digestion where isotopic exchange between intrinsic and extrinsic iron and zinc sources occurs (Petry & Hurrell, 2015). Iron and zinc bioaccessibility in the intestinal phase is then influenced by the intestinal pH, the balance and interaction of mineral binding compounds present in the matrix.

3.2 Effect of reagents and sample matrix on solubility of iron and zinc after in vitro digestion

The reagent blank should contain trace levels of the analyte of interest and, most importantly, the chemical behaviour of the analyte of interest in the reagent blank should be consistent in all sample matrices. In this regard, it is important to determine whether a matrix-dependence exists in order to validate the use of a blanket reagent blank correction. **Figure 1** shows the results of the stable isotope experiment in which ^{57}Fe and ^{70}Zn were applied to reagent blanks and different food matrices at the beginning of gastro-intestinal digestion. When ^{57}Fe and ^{70}Zn were added to a reagent blank, only 65% and 47% of ^{57}Fe and ^{70}Zn were recovered, respectively. This shows that despite reducing the concentration of pancreatin and bile, the enzymes and bile still exhibit a significant mineral binding effect as not all of the ^{57}Fe and ^{70}Zn was recovered. In this study, we did not investigate the binding effect of the enzymes and bile salts individually, as such it was not possible to determine whether the binding effect was from specific enzymes or bile salts or their combination. Although Rousseau et al. (2019) found a zinc binding effect from bile salts and not from enzymes, they used different enzymes to ours such that an enzyme binding effect cannot be ruled out, especially from pancreatin which was

particularly difficult to dissolve. The iron and zinc binders present in the reagent blank are most likely associated with the pancreatin and bile considering their high iron and zinc contribution to the gastro-intestinal digesta. When a cereal and legume sample was added, there was variable recovery of the ^{57}Fe and ^{70}Zn depending with the matrix. The recoveries of ^{57}Fe and ^{70}Zn from all the sample matrices were significantly lower than the recovery in reagent blanks suggesting an increased mineral binding effect when samples were added. The recovery of both ^{57}Fe and ^{70}Zn was lowest in maize, followed by cowpea and finger millet and greatest in velvet bean. This shows that during in-vitro gastro-intestinal digestion, the mineral binding effect in the system is a function of the total interactions of the reagents with a specific sample matrix. Cereals and legumes contain strong mineral chelators, in particular, phytic acid, phenolic compounds and dietary fibres (Gabaza, Shumoy, Louwagie, et al., 2018). The variable recoveries of the ^{57}Fe and ^{70}Zn in the cereal and legume matrices are most likely dependent on the amount of mineral binders in the matrix, their kinetics of release and competition for minerals between sample-derived and reagent-derived mineral binders.

3.3 Recovery of reagent-derived iron and zinc from cereals and legumes after in vitro digestion

To calculate iron and zinc bioaccessibility accurately and reliably, it is important to know the reagent iron and zinc that remains in the bioaccessible fraction of each sample matrix. This discrimination between reagent and sample iron and zinc can be effectively done by using stable isotopes as tracers of reagent iron and zinc. Stable isotopes can also be used as tracers for sample iron and zinc, but it has been shown previously that extrinsic isotopic labelling of whole grain cereals and legumes does not always result in complete equilibration with the intrinsic iron (Consaul & Lee, 1983; Glahn et al., 2015). Similarly, findings from the previous experiment indicated lack of isotopic equilibration between the $Fe_{applied}$ or $Zn_{applied}$ and Fe_{native} or Zn_{native} in both the sample and reagent iron and zinc during gastro-intestinal digestion. Reagent iron and zinc was thus isotopically labelled with ^{57}Fe and ^{70}Zn at least six

hours before digestion. **Figure 2** shows the percentage of recovered reagent iron and zinc in the bioaccessible fractions (in dialysates) of the different food matrices after gastro-intestinal digestion. In all cases, there were significant differences in the proportion of recovered reagent iron and zinc in the different food matrices in comparison with the reagent blank. Finger millet consistently showed the least reagent iron and zinc recovery. There was a stark contrast in the reagent iron and zinc recovered in beans, with a low recovery of reagent iron, but much greater reagent zinc recovery than other crops including the reagent blank. The greater protein content in beans than cereals caused more reagent zinc to be recovered as zinc has a strong binding affinity for soluble peptides (Udechukwu, Downey, & Udenigwe, 2018). On the other hand, the low recovery of reagent iron in finger millet and beans is likely because they contain substantial amounts of phytic acid and mineral binding phenolic compounds which are both potent mineral binders (Gabaza, Shumoy, Louwagie, et al., 2018; Glahn et al., 2015). Based on these results, it is clear that applying a blanket reagent blank correction is not appropriate when determining iron and zinc bioaccessibility.

3.4 Stable isotope approach to determine iron and zinc bioaccessibility

A specific blank correction was applied for each food matrix (Equation 3) and bioaccessibility was calculated and compared with two conventional approaches of calculation as described in the methods section. The iron and zinc bioaccessibility results are shown in **Tables 5 and 6**. According to the improved approach, the Fe_{bio}(%) was in the order finger millet, beans, pearl millet < maize, wheat while Fe_{bio}(mg kg^{-1}), was in the order finger millet < maize, pearl millet, beans < wheat. The same order was also observed when Approaches 1 and 2 were used for calculation. However, in terms of the magnitude of response among the three approaches, significant differences were observed for almost all the crops. For example, Fe_{bio}(%) of finger millet was 1.10% with the stable isotope approach, in comparison with 0.64% with Approach 1 and 0.70% with Approach 2 indicating an underestimation of iron bioaccessibility of up to 42%. In terms of Fe_{bio}(mg kg^{-1}), Approach 1 resulted in underestimation

(0.20 mg kg^{-1} for finger millet), while Approach 2 resulted in an overestimation (0.38 mg kg^{-1}) compared to 0.35 mg kg^{-1} for the stable isotope approach. Approach 1 consistently resulted in an underestimation of both the Fe_{bio}(%) and Fe_{bio}(mg kg^{-1}) while Approach 2 resulted in an underestimation of the Fe_{bio}(%) with a slight overestimation of Fe_{bio}(mg kg^{-1}) (only significantly different for finger millet).

The Zn_{bio}(%) was in the order finger millet < maize, pearl millet < wheat < beans for the stable isotope approach and this was the same when Approach 1 was used. For Approach 2, Zn_{bio}(%) was in the order finger millet < maize, pearl millet, wheat < beans. In this case, wheat was considered to have comparable bioaccessibility with maize and pearl millet which was not the case according to the stable isotope approach. The use of the stable isotope method is particularly important when studying samples with small differences which may not be captured with the conventional approaches of calculation as observed for Zn_{bio}(%) of wheat which was higher than that of maize and pearl millet with the stable isotope approach but this difference was not seen when Approach 2 was used. Pertaining to Zn_{bio}(mg kg^{-1}), it was in the order finger millet < maize, wheat < pearl millet < beans and this order was the same for all methods. As seen for iron bioaccessibility, the magnitude of response for all the approaches was significantly different across all crops. The Zn_{bio}(%) of beans was 31.7% with the stable isotope approach compared to 34.5% with Approach 1 and 24.8% for Approach 2 causing an underestimation of up to 22%. For finger millet, Zn_{bio}(%) was 5.11% with the stable isotope approach, compared to 3.46% with Approach 2 while a negative value was obtained with Approach 1. The application of a blanket reagent blank correction using Approach 1 can lead to negative values when the reagent blank mineral concentration is higher than the sample mineral concentration. This is more likely when the sample has low mineral concentrations in relation to the reagent blank coupled with a very strong mineral binding effect. The same trend observed for iron bioaccessibility was also observed for zinc bioaccessibility; i.e. an underestimation of Zn_{bio}(%) according to Approaches 1 and 2 and an underestimation of Zn_{bio}(mg kg^{-1}) according to Approach 1 followed by an overestimation according to Approach 2.

The use of in vitro methods of digestion to determine mineral bioaccessibility offer an excellent tool to screen, rank or categorize foods in terms of their mineral bioaccessibility (Etcheverry, Grusak, & Fleige, 2012) providing information necessary for food formulation, human nutrition trials and crop germplasm screening among many other applications. The use of both Approaches 1 and 2 to calculate bioaccessibility can result in inconsistent direction and magnitude of response because of the inability to correctly account for matrix specific reagent-derived iron and zinc. This inconsistency can have adverse consequences for hypothesis building and the shaping of ideas around the subject of iron and zinc bioaccessibility and can mislead future research with potential losses in funding investments.

Approach 1 is used by many researchers to calculate mineral bioaccessibility but this approach is fundamentally erroneous as it does not consider the matrix dependence of reagent-derived iron and zinc bioaccessibility. This error can be mitigated if studying samples with much greater iron and zinc concentrations than samples used in our study such that reagent iron and zinc is negligible. However, this is not likely to be the case when studying cereals and legumes. Approach 2 provides an alternative when Approach 1 cannot be used particularly when analysing samples such as finger millet which result in higher reagent blank mineral concentrations than sample mineral concentrations. However, the accuracy of this method of calculation is premised on complete isotopic equilibration of the reagent-derived and sample-derived iron and zinc, meaning that the proportion of reagent iron or zinc to sample iron or zinc must be the same in all fractions after gastro-intestinal digestion. Our findings suggested that this is not the case. Based on our findings, isotopic labelling of reagent iron and zinc used for in vitro digestion results in accurate and reliable iron and zinc bioaccessibility measurements. Researchers must therefore carefully consider the ramifications of potential errors in quantifying iron and zinc bioaccessibility before deciding on the approach to use.

4 Conclusion

The establishment of the INFOGEST static gastro-intestinal digestion method, a standardized international consensus, was an important milestone in the field of food digestion. However, the enzymes used in this method contain significant concentrations of iron and zinc leading to interferences in iron and zinc bioaccessibility measurements. Isotopic labelling of reagent iron and zinc allowed the discrimination of reagent and sample derived iron and zinc resulting in accurate and reliable quantification of bioaccessibility. Traditional approaches of calculating mineral bioaccessibility can either overestimate or underestimate iron and zinc bioaccessibility and this can have a profound effect on how results are interpreted and could potentially misdirect the trajectory of future research.

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563 **Table 1: Preparation of complete simulated digestion fluids**

Constituent	SSF complete	SGF complete	SPF complete	SBF complete
Simulated electrolyte fluid[a]	SSF	SGF	SIF	SIF
Volume of simulated electrolyte fluid (mL)	50	100	100	100
Enzyme/bile	α-amylase	Pepsin	Pancreatin	Bile
Enzyme weight (mg)	0.681	146.5	250	710
0.3 M $CaCl_2$ (mL)[b]	-	0.062	0.500	-
^{57}Fe (mL) (8,944 µg L^{-1})[c]	0.022	0.118	0.590	1.775
^{70}Zn (mL) (2,386 µg L^{-1})[c]	0.024	0.048	2.235	0.480
Milli-Q water (mL)	12.454	24.772	21.675	22.745
Total volume (mL)	62.5	125	125	125
pH	7	3	7	7

564 SSF: Simulated salivary fluid, SGF: simulated gastric fluid, SIF: Simulated intestinal fluid, SPF: Simulated
565 pancreatin fluid, SBF: Simulated bile fluid.

566 [a]Simulated electrolyte fluids were prepared according to Brodkorb et al. (2019) and Minekus et al. (2014)

567 [b]$CaCl_2$ was not added to SSF complete as it caused precipitation.

568 [c]^{57}Fe and ^{70}Zn were added at a level 10× their concentration in the respective digestion mixture.

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577 **Table 2: Mineral contents of reagents used in the static INFOGEST in vitro digestion method**

Reagent	Iron	Zn
SSF (μg L^{-1})	10.3 ± 0.09	11.4 ± 1.08
SGF (μg L^{-1})	16.5 ± 0.80	7.62 ± 0.38
SIF (μg L^{-1})	13.0 ± 0.06	5.43 ± 0.45
α-amylase (mg kg^{-1})	16.6 ±0.42	13.2 ± 0.70
Pepsin (mg kg^{-1})	226 ± 3.74	75 ± 1.85
Pancreatin (mg kg^{-1})	78.0 ± 0.07	253 ± 3.44
Bovine bile (mg kg^{-1})	111 ± 6.71	10.3 ± 1.62

578 SSF: Simulated salivary fluid, SGF: simulated gastric fluid, SIF: Simulated intestinal fluid, values are shown as
579 mean ± standard deviation, n=2.

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Table 3: Estimated iron and zinc contents in digestion mixtures based on INFOGEST recommended enzyme activity units

Parameter	α - amylase[a]	Pepsin	Pancreatin	Bovine bile
Specific activity (U mg^{-1})	1380	3,000	6	0.667 mM g^{-1}
Volume added per digestion (mL)	0.75 (0.725 mg mL^{-1})	0.667 (20 mg mL^{-1})	5 (133 mg mL^{-1})	3 (200 mg mL^{-1})
Enzyme weight per digestion (mg)	0.54	13.34	667	600
Estimated reagent iron per digestion (mg) (total ~ 0.121 mg)	<0.001	0.003	0.052	0.066
Estimated reagent zinc per digestion (mg) (total ~ 0.175 mg)	<0.001	<0.001	0.169	0.006

[a]Values for α-amylase were recalculated using α-amylase from *Bacillus sp.* instead of human salivary amylase used by Brodkorb et al. (2019).

608 **Table 4: Iron and zinc concentrations of some cereals and legumes studied**

Crop type	Fe (mg kg^{-1})	Estimated Fe per digestion (mg)[a]	Zn (mg kg^{-1})	Estimated Zn per digestion (mg)[a]
Maize	20.6 ± 1.26	0.031 – 0.103	18.6 ± 0.45	0.028 – 0.093
Wheat	31.9 ± 0.95	0.048 – 0.160	12.7 ± 0.75	0.019 – 0.064
Finger millet	31.5 ± 1.27	0.048 – 0.158	11.7 ± 0.67	0.018 – 0.059
Pearl millet	47.1 ± 0.24	0.071 – 0.236	21.4 ± 0.72	0.032 – 0.107
Common beans	72.2 ± 1.26	0.108 – 0.361	23.8 ± 0.42	0.036 – 0.119

609 [a]Estimated Fe and Zn was calculated based on 5 g sample per digestion with minimum 30% dry flour and

610 maximum 100% dry flour content.

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Table 5: Bioaccessibility of iron based on the stable isotope approach in comparison with two other conventional approaches of calculation

Crop type	Stable isotope approach	Approach 1	Approach 2
	Bioaccessible Fe (%)		
Maize	4.15 ± 1.39^{b}	3.86 ± 1.50^{b}	3.74 ± 1.14^{b}
Wheat	4.94 ± 0.4^{bB}	4.76 ± 0.46^{bB}	3.18 ± 0.27^{bA}
Finger millet	1.10 ± 0.03^{aB}	0.64 ± 0.04^{aA}	0.70 ± 0.02^{aA}
Pearl millet	1.86 ± 0.11^{aC}	1.57 ± 0.11^{aB}	1.16 ± 0.07^{aA}
Beans	1.55 ± 0.08^{aA}	1.35 ± 0.08^{aB}	0.98 ± 0.05^{aC}
	Bioaccessible Fe (mg kg^{-1})		
Maize	0.85 ± 0.29^{b}	0.79 ± 0.31^{b}	0.97 ± 0.31^{b}
Wheat	1.57 ± 0.14^{c}	1.52 ± 0.16^{c}	1.70 ± 0.15^{c}
Finger millet	0.35 ± 0.01^{aB}	0.20 ± 0.01^{aA}	0.38 ± 0.01^{aC}
Pearl millet	0.88 ± 0.06^{bB}	0.74 ± 0.05^{bA}	0.91 ± 0.05^{bB}
Beans	$1.12\pm0.06^{b,AB}$	$0.98\pm0.06^{b,A}$	$1.15\pm0.06^{b,B}$

Approach 1: blanket blank correction, bioaccessibility was calculated based on the amount of iron and zinc in sample. Approach 2: no blank correction, bioaccessibility was calculated based on recovered iron and zinc from all fractions. Values with different small superscript letters within columns are significantly different, values with different capital superscript letters across rows are significantly different, $p < 0.05$, n=3.

Table 6: Bioaccessibility of zinc based on the stable isotope approach in comparison with two other conventional approaches of calculation

Crop type	Stable isotope approach	Approach 1	Approach 2
	Bioaccessible zinc (%)		
Maize	11.39 ± 0.17^{bB}	8.77 ± 0.44^{bA}	9.17 ± 0.02^{bA}
Wheat	16.18 ± 0.99^{cB}	14.13 ± 1.08^{cB}	10.15 ± 0.54^{bA}
Finger millet	5.11 ± 0.21^{aC}	0^{aA*}	3.46 ± 0.16^{aB}
Pearl millet	11.53 ± 0.44^{bB}	9.56 ± 0.50^{bA}	9.43 ± 0.36^{bA}
Beans	31.73 ± 0.77^{dB}	34.47 ± 0.69^{dC}	24.86 ± 0.46^{cA}
	Bioaccessible zinc (mg kg^{-1})		
Maize	2.12 ± 0.03^{bB}	1.63 ± 0.08^{bA}	2.35 ± 0.08^{bC}
Wheat	2.06 ± 0.13^{bA}	$1.79\pm0.14^{b,cA}$	$2.52\pm0.13^{b,cB}$
Finger millet	0.60 ± 0.03^{aB}	0^{aA*}	0.74 ± 0.03^{aC}
Pearl millet	2.47 ± 0.09^{cB}	2.05 ± 0.11^{cA}	2.77 ± 0.11^{cC}
Beans	7.55 ± 0.18^{dA}	8.20 ± 0.16^{dB}	8.92 ± 0.16^{dC}

Approach 1: blanket blank correction, bioaccessibility was calculated based on the amount of iron and zinc in sample. Approach 2: no blank correction, bioaccessibility was calculated based on recovered iron and zinc from all fractions. *Negative value was obtained. Values with different small superscript letters within columns are significantly different, values with different capital superscript letters across rows are significantly different, $p < 0.05$, n=3.

Figures

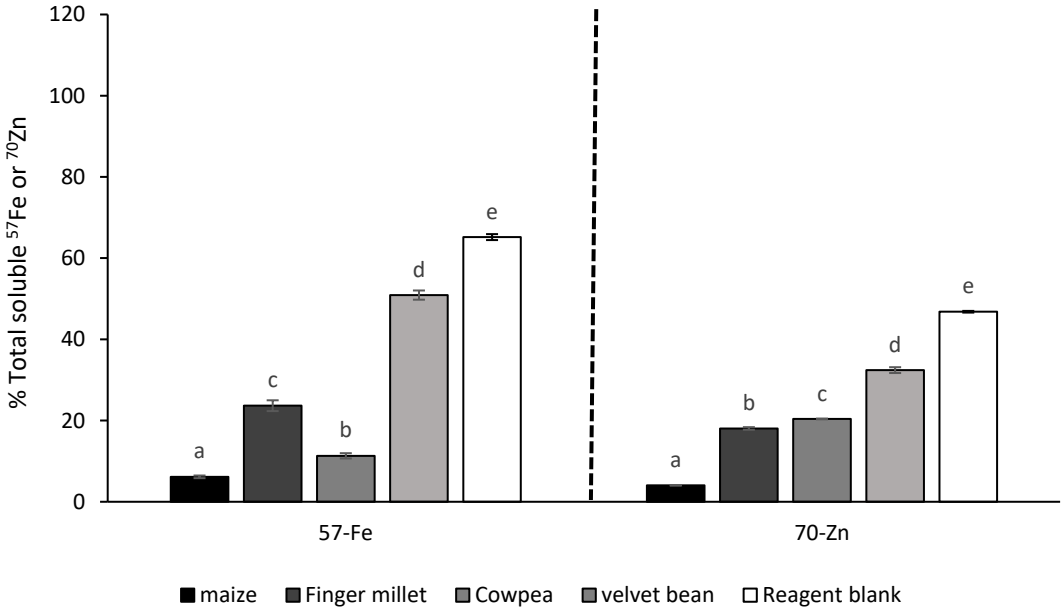
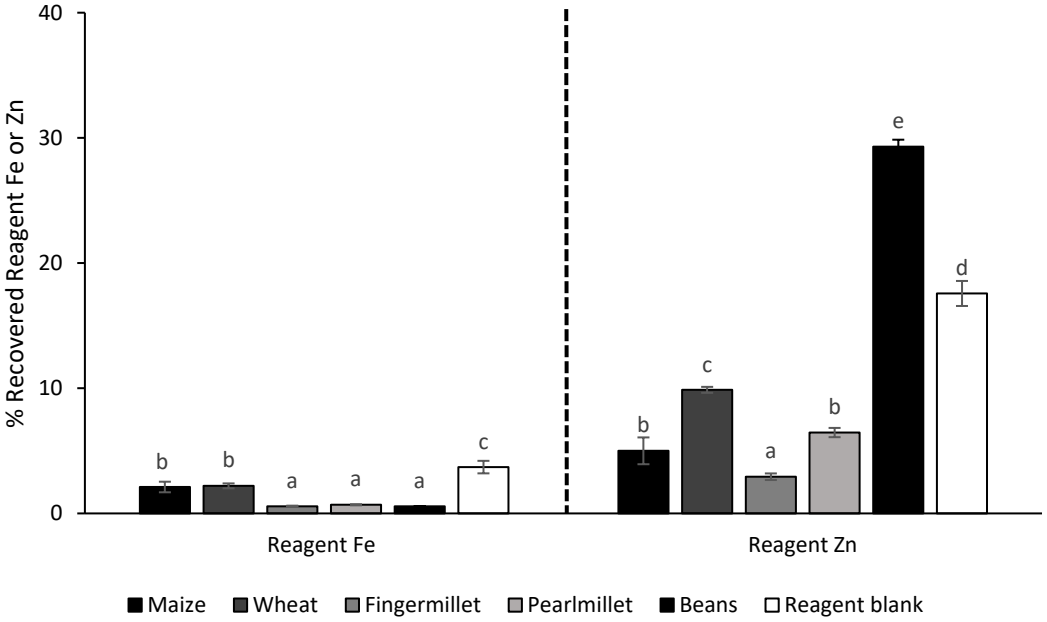


Figure 1: Proportion of total soluble ^{57}Fe and ^{70}Zn recovered in reagent and different sample matrices. Bars with different letters are significantly different, $p < 0.05$, $n = 3$.



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667 Figure 2: Reagent derived iron and zinc recovered from different food matrices in the bioaccessible
668 fraction. Bars with different letters are significantly different, $p < 0.05$, $n = 3$.

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