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Simple and fast ultrasound-assisted method for mineral content and bioaccessibility study in infant formula by ICP OES

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Infant formula (IF) constitutes the sole source of mineral intake for infants who are only fed IF. The assurance of the amount of minerals declared on the label and the mineral levels and their chemical forms present a major concern related to providing a good amount of nutrients for absorption by these children. Thus, the objectives of this study were: (i) to evaluate several sample preparation methods for minerals in IF; (ii) to validate an analytical method using an ultrasonic bath for simultaneous determination of the Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn contents in IF by ICP OES and (ii) to establish the optimum analytical conditions of the *in vitro* method to study the dialyzability of these minerals from IF. The ultrasound-assisted method was shown to conform to 'green chemistry principles', being simple, fast and low cost compared with reference methods. The results were similar to those obtained with reference methods (microwave-assisted acid digestion and dry ashing) with regard to selectivity, sensitivity and linearity (r^2 > 0.999). The accuracy and the precision were verified using certified reference materials, with recoveries and coefficients of variation ranging from 91 to 105% and from 1.1 to 5.2%, respectively. For *in vitro* dialyzability, the conditions established in this study allowed including an overnight step between the gastric and gastrointestinal stages (accuracy and precision ranging from 81 to 108% and 0.4 to 6.3%, respectively), contributing to establishing an *in vitro* digestion method suitable for infant gastrointestinal conditions.

Introduction

The first year of life is a critical period in child development and adequate nutrition is essential to ensure healthy growth. The efforts to produce infant formula (IF) as similar as possible to human milk are well known. However, newborn infants fed formula may present biological differences compared to those fed human milk.[1,2]

Mineral deficiency can affect child development, causing dysfunctions that can affect their lives.[3] The nutritional status can be understood to be the body condition after using food nutrients, leading to an equilibrium between ingested and assimilation, and can be influenced by factors such as sex and age of the individual.[4] Codex STAN 72 (ref. 5) defines infant formula (IF) as "substitutes for mother's milk, specially manufactured to satisfy, by themselves, the nutritional needs of babies during their first months of life, until the introduction of appropriate complementary food". The nutritional safety and adequacy of IF should be scientifically demonstrated to support growth and development of infants.

Mineral and inorganic contaminant determination in IF is usually performed using microwave-assisted digestion and dry ashing.[6–8] Ultrasound-assisted extraction stands out as an efficient alternative method, being simple, fast and low cost compared to the reference methods. Several studies were reported for milk, baby food, sugarcane juice and seafood analysis using this method.[7,9–13] Although mineral content determination in IF is important for the manufacturers and health authorities to guarantee the quality of their products, it is essential to evaluate if the levels of these elements are available for absorption for children fed IF.

In vivo models can be expensive or difficult to use due to ethical questions.[14] Compared to these models, *in vitro* methods present the advantages of being faster and less expensive and having no ethical restrictions.[15] Such methods express the quantity of a nutrient freed from the matrix and considered available for absorption, estimating the nutrient percentages available for absorption (bioaccessible content) and for physiological functions (bioavailable content).[16] The bioaccessible content can be determined from the soluble and/or dialyzed fraction. Dialyzability is determined by simulating the interaction of the elements with the intestinal wall through the addition of a dialysis membrane. The basic premise of this model is

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that dialyzable compounds will be available for absorption in the small intestine.[17]

Nevertheless, more study and refined methods need to be developed for a better understanding of the digestive kinetics.[18] The *in vitro* digestion models for adults generally consider three main phases: oral, gastric and duodenal, and fundamentally simulate a set of conditions close to the physiological situation of the population under study. However, to better evaluate digestion in newborn infants (aged zero to six months), the oral phase is usually omitted, since liquid milk-based meals have a limited residence time between food and mouth.[19] Since newborn infants present immature enzymatic and secretory functions during their first months of life, one must decrease the amounts of enzymes as compared to those in methods involving adults.[15,18–20] To the best of our knowledge, there is a lack of recent literature regarding *in vitro* digestion models which consider the amount of infant gastrointestinal enzymes.

Considering the importance of assuring the nutritional quality of infant formula intended for newborn infants, the main objectives of this study were to evaluate a simple and fast ultrasound-assisted method for Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn determination in IF by ICP OES. In addition, an *in vitro* digestion model was evaluated in order to provide a reliable method to assess the minerals' dialyzability.

Experimental section

Reagents, solutions and samples

Water and nitric acid were purified using a reverse osmosis system (Gehaka, São Paulo, Brazil) with a resistivity below 18.2 MΩ cm and by sub-boiling distillation (Distillacid, Berghof, Eningen, Germany), respectively. Hydrochloric acid 37% (m/v), hydrogen peroxide 30% (m/v) (Merck, Darmstadt, Germany) and polytetrafluorethylene (PTFE) 0.45 μm filters (Agilent Technologies, Brazil) were also employed in the assay. Sodium bicarbonate, pepsin from porcine gastric mucosa (enzyme activity of 250 U mg^{-1}, catalog no P7000), pancreatin from porcine pancreas (enzyme activity equivalent to 8× USP specifications, catalog no P7545), bile from bovine and ovine (bile acid mixture, catalog no B8381) and a dialysis bag with porosity of 25 Angstroms and a cut-off of 12 000 Da were all purchased from Sigma-Aldrich, Saint Louis, USA. The following certified reference materials (CRMs) were used to validate the proposed method: *Baby Food Composite* (NIST 2383a) and *Infant/Adult Nutritional Formula* (NIST 1849a).

The analytical curves were prepared by successive dilutions of 100 mg L^{-1} multielement standard solutions (Cu, Fe, Mn and Zn); a 10 000 mg L^{-1} P standard solution (Specsol, Quimlab, Jacareí, Brazil) and 10 000 mg L^{-1} Ca, Mg, K and Na standard solutions (Merck, Darmstadt, Germany) in 5% (v/v) HCl.

IF from brands A, B, C & D ($n = 5$) intended for 0 to 6 month old children was acquired from local markets in Campinas (Brazil) and Santiago (Chile). Some samples were described as "IF for suckling infants" (BR-A, BR-B & BR-C); "IF with iron for suckling infants" (CH–C) and "soybean based IF for suckling infants" (BR-D/SOY). Table 1 shows the identification of the samples according to the code, front panel words (label) and ingredients.

Instrumentation

Minerals were determined using an inductively coupled plasma optical emission spectrometer (ICP OES 5100 VDV, Agilent Technologies, Tokyo, Japan) and the operating conditions are described in Table 2. An ultrasonic bath (Easy 180H, Elma, Singen, Germany), a closed microwave system (Start D, Milestone, Sorisole, Italy) and a muffle furnace (Fornitec, São Paulo, Brazil) were used to determine the mineral contents in the samples. A shaking water bath (NT 230, Nova Tecnica, Piracicaba, Brazil) and a pH meter with a selective electrode (Starter 3100, Ohaus, Parsippany, USA) were used for the bioaccessibility assay.

Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn determination

IF samples were prepared according to the label instructions (15 g IF was reconstituted in 100 mL purified water) and three sample digestion methods were evaluated:

Table 1 Identification and labeling information (front panel and main ingredients) of IF samples ($n = 5$)

	Labelling	
Sample[a]	Front panel	Main ingredients
BR-A	IF for suckling infants. With lutein; DHA and ARA	Powdered skim milk; lactose; concentrated whey protein; hydrolyzed whey protein; ascorbic acid
BR-B	IF for suckling infants – with DHA and ARA	Lactose; powdered skim milk; concentrated whey protein; ascorbic acid
BR-C	IF for suckling infants, prebiotics; DHA and ARA; nucleotides	Demineralized whey; lactose; skimmed milk; galacto-oligosaccharides (prebiotics); ascorbic acid
CH–C	IF starting powder with Fe for suckling infants, adequate protein; Fe; omega 3 and 6	Whey; skimmed milk; lactose; ascorbic acid
BR-D/SOY	IF for soy-based infants. Contains no dairy proteins	Maltodextrin; soy protein; ascorbic acid

[a] BR: Brazil; CH: Chile; Brand: A, B, C and D; SOY: Soy protein.

Table 2 ICP OES operating conditions[a]

ICP OES conditions			
Radio frequency generator (MHz)		27	
Radio frequency power (kW)		1.2	
Plasma view		Axial (Cu, Fe, Mn, and Zn) and Radial (others)	
Argon flow (plasma/auxiliary/nebulizer) rate (L min^{-1})		12/1.0/0.7 (*Seaspray*)	
Spray chamber		Quartz, double-pass	
Number of replicates		03	
Wavelength (nm)		Analytical curves	
Ca(II); Na(I); P(I)	317.933, 589.592, 213.618	0.20–5.00	mg 100 mL^{-1}
Cu(I); Fe(II); Mn(II); Zn(II)	324.754, 259.940, 257.610, 206.200	0.5–200	μg 100 mL^{-1}
K(I)	766.491	0.20–10.00	mg 100 mL^{-1}
Mg(II)	279.553	0.20–2.00	mg 100 mL^{-1}

[a] (I): atomic emission line; (II): ionic emission line.

Method 1 – Acid digestion in a closed microwave-assisted system (based on Lima *et al.*[21]): 2 g of prepared IF was weighed in a PTFE vessel, 8 mL of HNO_3 was added and the mixture was allowed to react for approximately 15 minutes. 2 mL of H_2O_2 were added and the vessel was kept closed overnight. The flasks were then sealed and transferred to the microwave system and 1000 W power was applied at a maximum temperature of 170 °C for 25 minutes. The solutions were transferred to graduated tubes and purified water was used to bring the final volume to 25 mL.

Method 2 – Ultrasound-assisted extraction (based on Machado *et al.*,[7] Bermejo-Barrera *et al.*[9] and Cava-Montesinos *et al.*[10]): 2 g of prepared IF was weighed in a 50 mL graduated tube and 2 mL HNO_3 and 1 mL H_2O_2 were added. The tube was closed and placed in an ultrasonic bath for 30 minutes at 60 °C. After cooling, the volume was made up to 25 mL with purified water and filtered.

Method 3 – Dry ashing (based on Cámara *et al.*[22]): 3.50 g of prepared IF were weighed in a platinum capsule, heated on an electric plate and incinerated in a muffle furnace at 450 °C until the organic matter was destroyed. After cooling, 3 mL of purified water and 1.25 mL of HCl were added, the contents were carefully transferred to a graduated tube and the volume was made up to 25 mL with purified water.

Ultrasound-assisted method validation (Quality assurance/Quality control): CRM *Infant/Adult Nutritional Formula* (NIST 1849a) and the IF sample "BR-A" were used to validate the proposed method with respect to the merit parameters: accuracy, precision, selectivity (or the matrix effect), linearity and limits of detection and quantification, considering the recommendations of INMETRO[23] and AOAC.[24]

In vitro digestion and method evaluation

For *in vitro* digestion, the dialysis method described by Perales *et al.*[20] was applied with modifications:

Gastric digestion: 15 g of prepared IF was weighed into a 250 mL Erlenmeyer flask, 30 mL of purified water was added and the flask was incubated at 37 °C for 15 minutes. The pH was adjusted to 2.0 ± 0.2 with 6 mol L^{-1} (v/v) HCl and measured after 15 minutes (readjusting, if necessary). An aliquot of 1.87 mL freshly prepared pepsin solution (0.16 g mL^{-1}) was added (0.02 g of pepsin per g of sample). The sample was made up to 100 g with purified water and incubated in a shaking water bath at 37 °C for 2 hours. After incubation, the sample was maintained overnight in a fridge at 8 °C. Five aliquots of 20 g of the gastric digest were then transferred to a 250 mL Erlenmeyer flask.

Determination of the titratable acidity: 0.75 mL of a freshly prepared pancreatin + bile solution, containing 0.004 g mL^{-1} pancreatin and 0.025 g mL^{-1} bile extract (providing 0.00015 g of pancreatin and 0.00094 g of bile salts per g of sample), were added in an Erlenmeyer flask containing 20 g of the gastric digest. This mixture was titrated with 1 mol L^{-1} (v/v) $NaHCO_3$ to pH 7.2 ± 0.2. The titration volume was used in dialysis bag preparation.

Gastrointestinal digestion: in a dialysis bag (25 cm), purified water and the $NaHCO_3$ volume used in the determination of titratable acidity were added to achieve a total volume of 20 mL. The dialysis bag was placed in the gastric digestion solution and the flasks were incubated in a shaking water bath at 37 °C for 30 minutes. Freshly prepared pancreatin + bile solution (0.75 mL) was added and the flasks were incubated for up to 2 hours. The dialysis bag contents (dialyzed fraction) were transferred to a graduated tube and acidified with HCl to provide a 5% (m/v) solution, and the mineral levels were determined by ICP OES.

Evaluation of the *in vitro* method (quality assurance/quality control): the *in vitro* method was analyzed with respect to the following merit parameters: selectivity (matrix effect), accuracy and precision, considering the recommendations and definitions established by INMETRO[23] and AOAC.[24]

Statistical analyses

All the experiments were carried out with a minimum of three replicates and blank experiments were also performed. Statistical analyses (Tukey's test) were carried out using XLSTAT

software (Addinsoft, France), considering a 95% confidence level.

Results and discussion

Method evaluation and validation for Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn contents in IF

For method evaluation, the CRM Baby Food Composite (NIST 2383a) was analyzed employing Methods 1 (microwave-assisted digestion), 2 (ultrasound-assisted extraction) and 3 (dry ashing). In Fig. 1, the results obtained for recovery methods (ratio, in percentage, between the obtained and certified values) are presented. According to INMETRO[23] and AOAC,[24] the recovery ranges can vary from 90% to 107% for Ca, Mg, Na and P; from 80% to 110% for Cu, Mg, Fe and Zn; and from 95% to 105% for K (according to the mineral levels). Method 2 presented results within these parameters for all the elements (88 to 102%) whereas Method 3 presented results below the recovery range for Zn (74 ± 7%). This behavior was also verified analyzing a real IF sample (Table 3): at 95% significance, mineral levels did not differ significantly, except for the Zn result using Method 3 (dry ashing). The differences found may be related to the variations of the methods themselves.

Although the efficiency of the ultrasound-assisted extraction method could be affected by diverse variables, such as the concentrations and quantities of acid added,[9,25,26] the results obtained for Method 2 (ultrasound-assisted extraction) were similar to those of the reference methods usually described in the literature. In this method, the analyte is extracted into the acidic solution by ultrasonic irradiation (cavitation phenomenon).[26] Compared to microwave-assisted digestion, the ultrasound-assisted method stands out as an alternative method,[10] presenting similar residual carbon (0.33 ± 0.01%, in this study) with a lower cost and shorter time of analysis (less than 1 hour).

The method validation results are shown in Table 4. Accuracy and precision were evaluated using the CRM *Infant/Adult Nutritional Formula* (NIST 1849a) (0.2 mg of the CRM and n = 7 analytical repetitions). The recovery mean values ranged from 91 ± 2% (Zn) to 105 ± 2% (Mg), being within the ranges defined by INMETRO[23] and AOAC.[24] The limits of detection and quantification were calculated using LOD = 3.3* (s/b) and LOQ = 10* (s/b), where s = the standard deviation of the concentration of 10 blank experiments and b = slope (angular coefficient). The LODs and LOQs obtained were adequate for the determination of the 9 elements in IF, varying from 0.1 μg 100 mL^{-1} (Cu, Mn and Zn) to 0.08 mg 100 mL^{-1} (Ca).

Selectivity (matrix effect) was verified using two analytical curves: one curve for the acidic medium (HCl 5% (v/v), blank – group 1) and the other for the matrix medium (IF sample – group 2), where the same concentrations were added in at least 5 different levels considering the ranges of each element (n = 3).

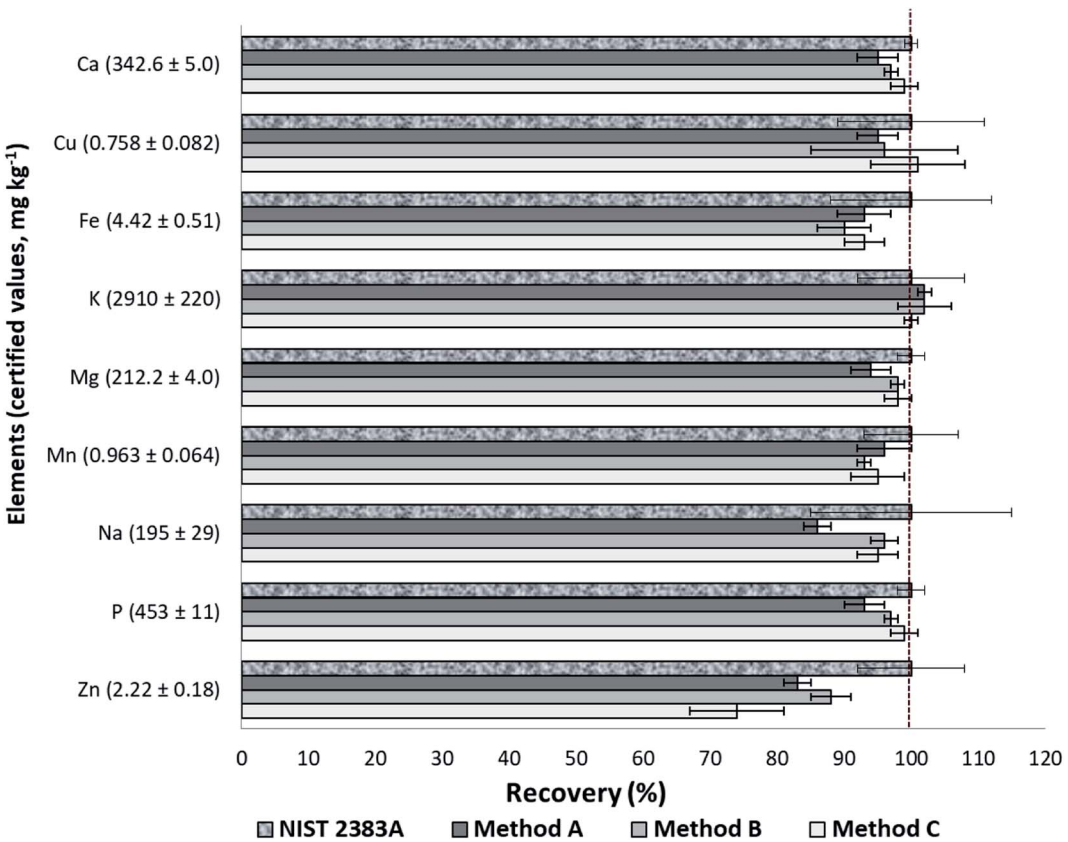

Fig. 1 CRM Baby Food Composite (NIST 2383a) evaluation using Method 1 (microwave-assisted digestion), Method 2 (ultrasound-assisted extraction) and Method 3 (dry ashing); n = 8.

Table 3 Method evaluation using a real infant formula sample (mean ± SD, $n = 7$)[a]

	Concentration (mg 100 mL^{-1})		
Elements	Method 1 (microwave-assisted digestion)	Method 2 (ultrasound-assisted extraction)	Method 3 (dry ashing)
Ca	42.1 ± 3.3[a]	44.1 ± 0.5[a]	43.5 ± 1.1a
Cu	0.054 ± 0.002[a]	0.055 ± 0.001[a]	0.052 ± 0.004a
Fe	0.73 ± 0.06[a]	0.73 ± 0.01[a]	0.71 ± 0.03a
K	65.8 ± 4.4[a]	65.4 ± 2.3[a]	67.8 ± 1.7a
Mg	4.5 ± 0.3[a]	4.7 ± 0.1[a]	4.5 ± 0.1a
Mn	0.015 ± 0.001[a]	0.015 ± 0.001[a]	0.016 ± 0.001a
Na	16.2 ± 1.0[a]	15.8 ± 0.6[a]	16.7 ± 0.4a
P	21.7 ± 1.6[a]	22.2 ± 0.4[a]	22.4 ± 0.5a
Zn	0.61 ± 0.03[a]	0.63 ± 0.01[b]	0.71 ± 0.07a

[a] [a,b] Mean values between different columns with the same letter are not significantly different at $p > 0.05$, according to Tukey's test.

Table 4 Validation results for the ultrasound-assisted method ($n = 7$) using the CRM Infant/Adult Nutritional Formula (NIST 1849a)[a]

	Accuracy and precision (NIST 1849a)							
		AOAC[23] thresholds		Ultrasound-assisted method				
Element	Certified values (mg kg^{-1})	REC (%)	CV (%)	Obtained values (mg kg^{-1})	REC (%)	CV (%)	LOD (mg 100 mL^{-1})	LOQ (mg 100 mL^{-1})
Ca	5253 ± 51	95–105	3.7	5147 ± 72	98 ± 1	1.4	0.03	0.08
Cu	19.78 ± 0.26	80–110	7.3	20.6 ± 1.1	104 ± 5	5.2	0.0001	0.0002
Fe	175.6 ± 2.9	90–107	5.3	163 ± 5	93 ± 3	3.3	0.0002	0.0005
K	9220 ± 110	95–105	3.7	9580 ± 104	104 ± 1	1.1	0.02	0.05
Mg	1648 ± 36	95–105	3.7	1727 ± 39	105 ± 2	2.2	0.01	0.04
Mn	49.59 ± 0.97	80–110	7.3	45.4 ± 0.5	92 ± 1	1.2	0.0001	0.0002
Na	4265 ± 83	95–105	3.7	4279 ± 52	100 ± 1	1.2	0.02	0.07
P	3990 ± 140	95–105	3.7	3808 ± 115	95 ± 3	3.0	0.02	0.06
Zn	151.0 ± 5.6	90–107	5.3	137 ± 3	91 ± 2	2.1	0.0001	0.0004

[a] REC = Recovery (%), CV = Coefficient of variation, LOD = Limit of detection, LOQ = Limit of quantification.

The analytical curves were visually parallel (Fig. 2) and the slopes were compared using the *t*-test. For all the elements, the values for $t_{calculated}$ (0.494 to 1.472) were lower than those for $t_{critical}$ (2.037 to 2.160) indicating an absence of the matrix effect (Fig. 2). The linearity was evaluated using the correlation coefficient (r^2) and the values (0.999 to 1.000) demonstrated that the method has linearity for the work ranges.

In vitro digestion method evaluation for mineral bioaccessibility in infant formula

The bioaccessibility assays involve numerous steps that can significantly influence the final results[27] and, to the best of our knowledge, few studies have dealt with the optimization of these methods under infant gastrointestinal conditions. Hence the present study evaluated some method variables that could contribute to the mineral dialyzed fraction in IF. In order to minimize the errors, some variables were rigorously controlled and verified (such as incubation time, pH, and final volume) and a single sample was evaluated. The tests were: Test A which included an overnight step after gastric digestion, Test B which included an ice bath step after gastric digestion and Test C which considered an alternative IF preparation with a similar procedure to that in Test A using 3 g of IF with the final solution made up to 20 mL; in this case, no aliquots were used and all replicates were performed individually. The results observed in these tests are presented in Table 5.

In general, the results obtained for Tests A, B and C which involved the gastric digestion step showed no significant differences at a confidence level of 95%. The results directly indicate that inactivation of pepsin overnight in a fridge (Test A) or an ice bath (Test B) has similar effects on mineral dialyzability in IF. On the other hand, the use of an alternative preparation (Test C) may decrease the K and P dialysis – which presented higher values in Test A.

In general, Test A presented advantages due to which the analytical procedure could be carried out carefully in two days; titratable acidity was determined under similar conditions to those for gastrointestinal digestion.

In vitro digestion method validation for mineral bioaccessibility in infant formula

The selectivity (matrix effect) was verified using two analytical curves: one curve for the matrix medium (dialyzed sample solution) and the other for the acidic medium (dialyzed blank

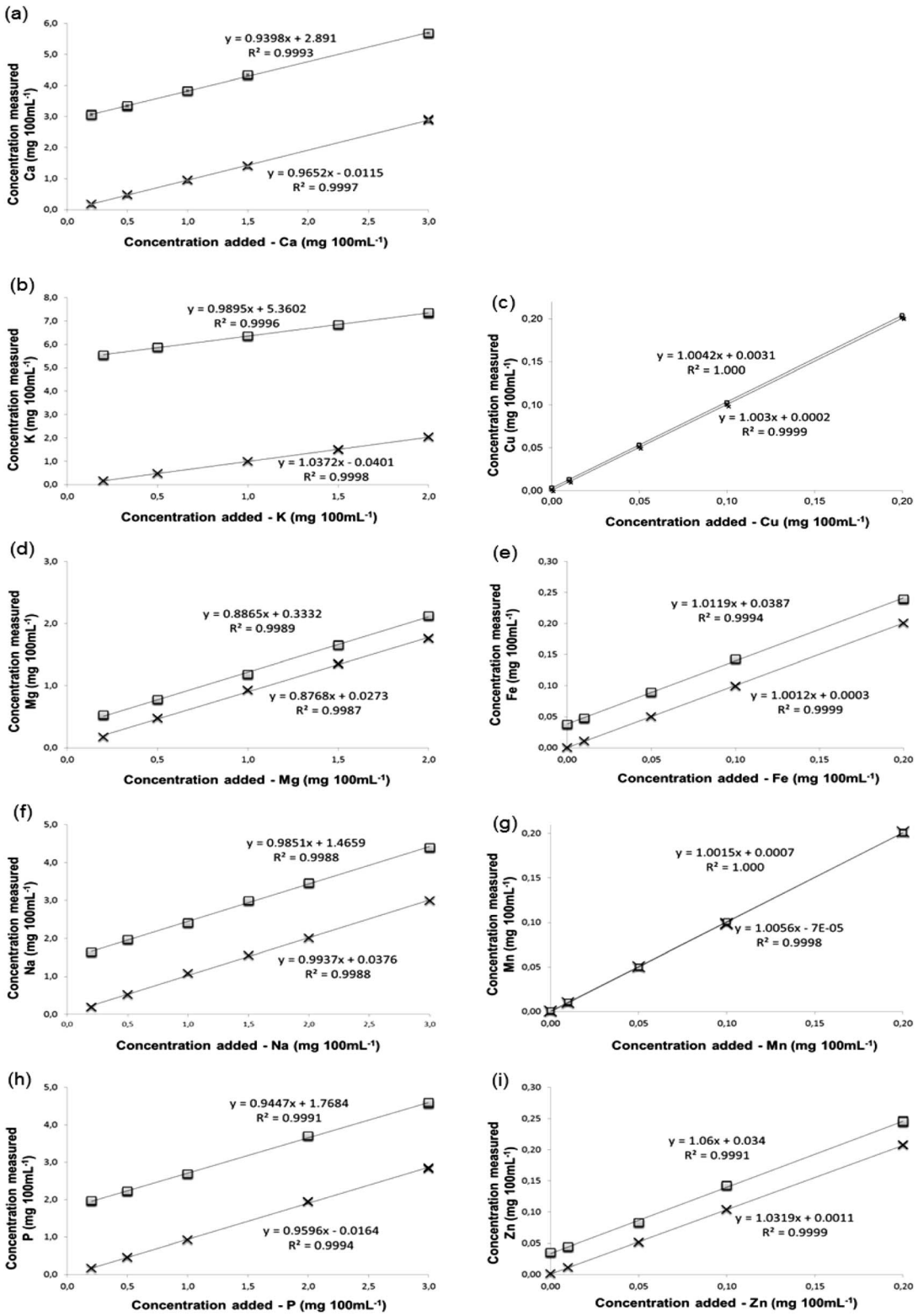

Fig. 2 Selectivity evaluation using analytical curves obtained in acid (Group 1) and in the matrix medium (Group 2), $n = 3$; Group 1: Blank + Analyte added; Group 2: sample of IF + Analyte.

experiment), where the same concentrations of analytes were added at 3 different concentration levels (considering the mineral levels and analytical curve ranges). The slopes were compared using the *t*-test and all values for $t_{calculated}$ were lower than those for $t_{critical}$, indicating the absence of matrix effects. For linearity, the correlation coefficients (r^2) ranged from 0.993

Table 5 Results from *in vitro* digestion method evaluation (mean ± SD, $n = 2$)[a]

Element	Method modification		
	Test A	Test B	Test C
Ca (mg 100 mL^{-1})	8.19 ± 0.18[a]	8.85 ± 0.74[a]	7.69 ± 0.18[a]
Cu (μg 100 mL^{-1})	17.75 ± 3.00[a]	14.20 ± 0.28[a]	16.80 ± 1.13[a]
Fe (μg 100 mL^{-1})	0.13 ± 0.05[a]	0.12 ± 0.00[a]	0.12 ± 0.00[a]
K (mg 100 mL^{-1})	43.66 ± 0.93[ab]	45.21 ± 0.50[b]	39.80 ± 1.82[a]
Mg (mg 100 mL^{-1})	2.54 ± 0.09[a]	2.53 ± 0.03[a]	2.32 ± 0.10[a]
Mn (μg 100 mL^{-1})	2.90 ± 0.28[a]	2.80 ± 0.42[a]	2.75 ± 0.21[a]
P (mg 100 mL^{-1})	13.52 ± 0.30[ab]	14.04 ± 0.24[b]	12.26 ± 0.46[a]
Zn (mg 100 mL^{-1})	0.07 ± 0.01[a]	0.06 ± 0.00[a]	0.07 ± 0.01[a]

[a] [a,b] The same letters for Tests A, B and C indicate no significant difference at the 95% confidence level ($p < 0.05$); Test A = overnight step after gastric digestion; Test B = ice bath step after gastric step; Test C = 3 g of IF with the final solution made up to 20 mL.

(K) to 1.000 (P) and they were considered adequate for Ca, Cu, Fe, K, Mg, Mn, P and Zn determination in the dialyzable fraction by ICP OES.

Accuracy and precision (repeatability) were verified using spiked trials at 3 different levels in triplicate. Recovery was calculated according to the following equation: Rec (%) = $(C_1 - C_2) \times 100/C_3$, where C_1 is the mineral level in the spiked dialyzed solution, C_2 is the mineral level in the dialyzed solution and C_3 is the spiking level. The thresholds (recoveries: 80% to 110% and CV < 7.3% for Ca, K, Mg and P and CV < 11% for Cu, Fe, Mn and Zn) were accepts considering the recommendations and definitions established by INMETRO[23] and AOAC[24]. From Table 6 one can observe that the results in all the spiked experiments are below the recovery and coefficient of variation thresholds established, demonstrating that the proposed method has adequate precision and accuracy for Ca, Cu, Fe, K, Mg, Mn, P and Zn determination by the dialyzability assay for infant formula.

Method application in real samples

Table 7 presents the mineral contents (in mg or μg 100 mL^{-1}) found in the five IF samples analyzed. The results showed that Na was the only element that did not differ significantly, at a 95% significance level, in the infant formula samples analyzed.

On comparing the results obtained with the Codex Alimentarius[5] (mg or μg 100 kcal^{-1}, Table 8) all the elements presented values above the minimum and maximum or in agreement with the guidance upper level (GUL) values established. Different from the Codex Alimentarius, Brazilian regulation[28] establishes a maximum value for Fe (1.3 mg 100 kcal^{-1}) in IF for 0 to 6 month old infants. The Brazilian IF samples (BR-A, BR-B, BR-C and BR-D/SOY) presented results within this limit.

The results observed in the dialyzability assays are summarized in Table 9. In all samples, different mineral contents were observed, the lowest levels of the mineral dialyzability being found in soy-based IF (Sample BR-D/SOY). Dialyzed fractions were calculated as the ratio, in percentage, between the dialyzed content and the mineral content in IF prepared samples. For Fe, the highest variation in the amount dialyzed (10 to 34%) was verified, especially in the iron-fortified sample (CH–C). Related

Table 6 Evaluation of accuracy (recovery trials) and precision in the *in vitro* digestion method[a]

Element	Dialyzed fraction	Spike levels	Dialyzed fraction + spike level	REC (%)	CV (%)
Ca (mg 100 mL^{-1})	3.93 ± 0.01	0.2	4.13 ± 0.01	100	5.0
		1.0	4.94 ± 0.02	101	2.2
		2.0	5.9 ± 0.1	98	3.8
Cu (μg 100 mL^{-1})	11.43 ± 0.06	1	12.31 ± 0.04	88	4.0
		10	19.82 ± 0.02	84	0.2
		100	113 ± 2	102	2
Fe (mg 100 mL^{-1})	82.11 ± 0.49	1	83.05 ± 0.03	94	3.1
		10	91.4 ± 0.4	93	3.7
		100	181 ± 2	99	2
K (mg 100 mL^{-1})	31.98 ± 0.31	1.0	32.9 ± 0.1	92	5.1
		2.0	34.1 ± 0.1	106	3.2
		5.0	37.1 ± 0.1	103	1.4
Mg (mg 100 mL^{-1})	2.17 ± 0.01	0.1	2.27 ± 0.01	100	6.3
		1.0	3.16 ± 0.02	99	1.9
		2.0	4.1 ± 0.1	95	3.0
Mn (μg 100 mL^{-1})	0.92 ± 0.01	1.0	1.95 ± 0.01	103	0.7
		10	9.0 ± 0.3	81	0.3
		100	87 ± 1	86	1
P (mg 100 mL^{-1})	13.77 ± 0.02	1.0	14.82 ± 0.01	105	0.5
		2.0	15.64 ± 0.03	93	1.3
		5.0	18.32 ± 0.02	91	0.4
Zn (mg 100 mL^{-1})	28.05 ± 0.18	1.0	29.1 ± 0.1	108	5.9
		10	36.4 ± 0.4	84	4.4
		100	119 ± 1	91	1

[a] REC = Recovery; CV = coefficient of variation.

Table 7 Mineral content in Infant formulas from Brazil and Chile ($n = 3$)[a]

	Mineral levels in infant formula samples				
Elements	BR-A	BR-B	BR-C	CH–C	BR-D/SOY
Ca (mg 100 mL^{-1})	57.5 ± 0.7^{d}	46.6 ± 1.3^{b}	40.7 ± 0.7^{a}	46.8 ± 1.1^{b}	49.7 ± 0.4^{c}
Cu (μg 100 mL^{-1})	62.7 ± 0.8^{c}	42.8 ± 1.3^{b}	62.7 ± 1.9^{c}	61.3 ± 0.8^{c}	36.0 ± 0.4^{a}
Fe (mg 100 mL^{-1})	0.64 ± 0.02^{a}	0.83 ± 0.02^{c}	0.77 ± 0.02^{b}	0.97 ± 0.01^{d}	0.82 ± 0.01^{bc}
K (mg 100 mL^{-1})	81.8 ± 1.3^{b}	97.4 ± 2.9^{c}	77.6 ± 2.1^{ab}	115.3 ± 4.5^{d}	72.0 ± 1.3^{a}
Mg (mg 100 mL^{-1})	6.1 ± 0.1^{b}	6.1 ± 0.2^{b}	8.7 ± 0.2^{d}	7.3 ± 0.2^{c}	5.5 ± 0.1^{a}
Mn (μg 100 mL^{-1})	21.8 ± 0.3^{d}	4.5 ± 0.1^{a}	14.2 ± 0.3^{b}	15.4 ± 0.1^{c}	33.7 ± 0.4^{e}
Na (mg 100 mL^{-1})	21.0 ± 0.3^{a}	21.3 ± 0.7^{a}	21.0 ± 0.7^{a}	22.6 ± 0.9^{a}	21.5 ± 0.4^{a}
P (mg 100 mL^{-1})	32.2 ± 0.5^{c}	35.8 ± 1.2^{d}	23.6 ± 0.7^{a}	30.0 ± 0.8^{b}	29.7 ± 0.4^{b}
Zn (mg 100 mL^{-1})	0.61 ± 0.01^{b}	0.81 ± 0.03^{d}	0.75 ± 0.01^{c}	0.72 ± 0.01^{c}	0.49 ± 0.01^{a}

[a] [a,b,c,d,e] Same letters in different samples – no significant difference at 95% confidence level ($p < 0.05$).

Table 8 Minimum (min), maximum (max) and upper reference limit (LSR) levels recommended by Codex Alimentarium,[4] and mineral content in infant formulas

	Codex		Mineral levels in infant formula samples				
Elements	Min–Max*	LSR	BR-A	BR-B	BR-C	CH–C	BR-D/SOY
Ca (mg 100 kcal^{-1})	50	140	85.8 ± 1.0	69.6 ± 1.9	60.7 ± 1.1	69.9 ± 1.6	74.2 ± 0.5
Cu (μg 100 kcal^{-1})	35	120	93.6 ± 1.2	63.9 ± 2.0	93.5 ± 2.9	91.5 ± 1.2	53.7 ± 0.7
Fe (mg 100 kcal^{-1})	0.5	—	0.96 ± 0.02	1.24 ± 0.04	1.16 ± 0.02	1.45 ± 0.01	1.22 ± 0.01
K (mg 100 kcal^{-1})	60–180	—	122 ± 2	145 ± 4	116 ± 3	172 ± 7	108 ± 2
Mg (mg 100 kcal^{-1})	5	15	9.1 ± 0.1	9.1 ± 0.2	13.0 ± 0.3	10.9 ± 0.2	8.2 ± 0.1
Mn (μg 100 kcal^{-1})	1	100	32.5 ± 0.4	6.7 ± 0.1	21.2 ± 0.4	22.9 ± 0.1	50.3 ± 0.6
Na (mg 100 kcal^{-1})	20–60	—	31.4 ± 0.4	31.7 ± 1.0	31.3 ± 1.00	33.7 ± 1.3	32.0 ± 0.6
P (mg 100 kcal^{-1})	25	100	48.1 ± 0.7	53.5 ± 1.8	35.2 ± 1.00	44.7 ± 1.2	44.3 ± 0.5
Zn (mg 100 kcal^{-1})	0.5	1.5	0.92 ± 0.02	1.21 ± 0.04	1.11 ± 0.01	1.08 ± 0.01	0.73 ± 0.01

Table 9 Mineral bioaccessibility in infant formula from Brazil and Chile ($n = 4$)[a]

	Infant formula samples									
Elements	BR-A	Dialyzed fraction (%)	BR-B	Dialyzed fraction (%)	BR-C	Dialyzed fraction (%)	CH–C	Dialyzed fraction (%)	BR-D/SOY	Dialyzed fraction (%)
Ca (mg 100 mL^{-1})	5.97 ± 0.43^{d}	10%	2.16 ± 0.19^{c}	4.6%	0.88 ± 0.01^{ab}	2.2%	1.28 ± 0.29^{b}	2.7%	0.58 ± 0.18^{a}	1.2%
Cu (μg 100 mL^{-1})	11.78 ± 1.56^{c}	19%	9.69 ± 1.19^{b}	23%	12.42 ± 0.45^{c}	20%	13.23 ± 0.45^{c}	22%	7.30 ± 0.43^{a}	20%
Fe (mg 100 mL^{-1})	0.093 ± 0.005^{bc}	15%	0.083 ± 0.007^{b}	10%	0.096 ± 0.001^{c}	12%	0.331 ± 0.009^{d}	34%	0.015 ± 0.001^{a}	1.9%
K (mg 100 mL^{-1})	32.34 ± 0.82^{c}	40%	37.74 ± 0.68^{d}	39%	29.11 ± 0.40^{b}	38%	44.27 ± 0.82^{e}	38%	25.78 ± 0.71^{a}	36%
Mg (mg 100 mL^{-1})	1.83 ± 0.04^{c}	30%	1.34 ± 0.01^{b}	22%	2.88 ± 0.06^{e}	33%	2.57 ± 0.06^{d}	35%	0.80 ± 0.05^{a}	15%
Mn (μg 100 mL^{-1})	2.14 ± 0.21^{c}	10%	0.61 ± 0.02^{b}	14%	1.91 ± 0.11^{c}	13%	3.06 ± 0.17^{d}	20%	0.19 ± 0.04^{a}	0.6%
P (mg 100 mL^{-1})	9.39 ± 0.28^{c}	29%	11.34 ± 0.24^{d}	32%	7.26 ± 0.10^{b}	31%	9.75 ± 0.22^{c}	33%	5.55 ± 0.15^{a}	19%
Zn (mg 100 mL^{-1})	0.044 ± 0.003^{b}	7%	0.045 ± 0.002^{b}	5.6%	0.052 ± 0.003^{c}	7%	0.079 ± 0.005^{d}	11%	0.004 ± 0.001^{a}	0.8%

[a] [a,b,c,d,e] The same letters for different samples – no significant difference at the 95% confidence level ($p < 0.05$); BR-A, BR-B, BR-C = Infant formula for suckling infants; CH–C = Infant formula fortified with iron for suckling infants; BR-D/SOY = Soybean based infant formula for suckling infants.

studies for the dialyzed fractions from milk based IF presented similar values: 7.24 to 24.1% for Ca,[20,29,30] 15.9 to 32.6% for Cu,[31] 1.28 to 10.96% for Fe and 6.7 to 9.6% for Zn.[29–31]

The Ca percentages in the dialyzed fractions (2.2% to 10%) were low when compared to the values reported in the literature. Perales *et al.*[20] reported that in milk-based IF the composition of the protein fraction affects calcium dialyzability, particularly when casein is the main protein. For Cu (19% to 23%), Fe (10% to 34%) and Zn (5.6% to 11%), the values were within or higher than the ranges reported in the literature. For K, Mg, Mn and P dialyzability in IF, to the best of our knowledge, no studies have been reported in the literature.

Conclusions

The ultrasound-assisted method for mineral determination in IF was shown to be an alternative to the reference methods (microwave digestion and dry ashing), being faster, simpler, and low cost, requiring lower amounts of chemical reagents and agreeing with the 'green chemistry principles'. Method validation presented satisfactory results for selectivity, linearity, LOD, LOQ, precision (CV <7.3%) and accuracy (88 to 102%) for all elements. The *in vitro* digestion tests demonstrated that the modification, the overnight step between gastric and gastrointestinal digestion, did not affect the dialyzed fractions and allowed performing the bioaccessibility assay with adequate sensitivity, accuracy and precision. The IF samples studied presented mineral levels below the Brazilian regulation thresholds and the values suggested in the Codex Alimentarius. The highest percentages found in the dialyzed fraction were obtained for the minerals K (36–40%), Mg (15–35%) and P (19–33%) and the lowest for Ca (1.2–10%), Zn (0.8–11%) and Mn (0.6–20%). The IF with iron for suckling infants (Sample CH–C) presented levels 3× higher than the other samples in dialyzed fractions, and the soy-based IF (Sample BR-D/SOY) presented lower contents than the other IF samples for all the elements, demonstrating the need for more studies on mineral bioaccessibility in different types of IF.

Author contributions

All experimental work, analysis and writing were carried out by MIAF, RFM, ELP and MAM. All authors proof-read the manuscript and approved the final version.

Conflicts of interest

There are no conflicts to declare.

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