J. Dairy Sci. 99:9405–9414
http://dx.doi.org/10.3168/jds.2016-11405

Availability of zinc from infant formula by in vitro methods (solubility and dialyzability) and size-exclusion chromatography coupled to inductively coupled plasma-mass spectrometry

Beatriz Gomez Gomez, M. Teresa Perez-Corona, and Yolanda Madrid[1]
Departamento de Química Analítica, Facultad de Ciencias Químicas, Universidad Complutense de Madrid, 28040 Madrid, Spain

ABSTRACT

Zinc bioaccessibility from infant formula was estimated by in vitro methods (solubility and dialyzability) and size-exclusion chromatography (SEC) coupled to inductively coupled plasma-mass spectrometry (ICP-MS). Infant formula samples were first characterized in terms of Zn bound to lipids and proteins and Zn distribution in the aqueous soluble protein fraction. We found that Zn is not incorporated into the lipid fraction of the samples, being mainly associated with the protein fraction (around 100%). Fractionation of Zn-containing proteins in the soluble protein fraction was achieved by SEC-ICP-MS after performing protein extraction with a solution of 100 m*M* (pH 6.8) Tris-HCl. The percentages of zinc in the soluble protein fraction in the soy-based and lactose-free infant formula were very low, around 7 and 24%, respectively, whereas the content of Zn in the soluble protein fraction of milk-based formula was around 90%. By SEC-ICP-MS, we found that Zn is associated with low-molecular weight compounds (around 10 kDa) in all the infant formulas tested. The percentages of Zn estimated in the in vitro gastrointestinal digests of the infant formula ranged from 30 to 70% and from 1 to 10% for solubility and dialyzability assays, respectively. The dialyzability test resulted in lower than expected scores, as SEC-ICP-MS analysis of the gastrointestinal extracts revealed that Zn is bound to biomolecules with a molecular weight ranging from 1 to 7 kDa, which suggests that dialysis data should be interpreted with caution. Speciation studies are a valuable tool for establishing availability of nutrients and for validating data from dialyzable in vitro methods.

Key words: zinc, infant formula, in vitro bioaccessibility, speciation

INTRODUCTION

Zinc is an essential nutrient (Harmaza and Slobozhanina, 2014) with a recommended daily intake dependent on age and sex, ranging from 2.6 to 9.3 mg/d in children, from 6.8 to 14.5 mg/d in adolescents, and from 8.0 to 14.0 mg/d in adults (EFSA, 2014). Although zinc is one of the most abundant essential elements in the human body, its deficiency is very common, particularly in areas where cereal proteins are primary components of the local diets. Cereal proteins contain high amount of an organic phosphate compound, phytate, which complexes zinc and makes it unavailable for absorption. The groups more vulnerable for Zn deficiency are pregnant and lactating women and young children (WHO, 2009). The pathological signs of zinc deficiency in young children are growth failure, alteration of cognitive functions, increases of the incidence rate of infectious diseases, and impaired parturition (dystocia; Prasad, 1998).

The first 2 yr of a child's life are particularly important, as optimal nutrition during this period lowers morbidity and mortality, reduces the risk of chronic disease, and fosters better development overall. Although breast milk is the best nutritional choice for infants, when breastfeeding is not possible infant formula is a healthy alternative. Infant formula has to fulfil the nutritional requirements of infants for optimum development (EFSA, 2009). These products are based on cow milk or on edible constituents of plant origin that have been proven to be suitable for infant feeding; for instance, soy formula has become popular as an alternative formula in infants who are allergic to cow milk. The main goal of the composition of infant formula is to make it similar to that of human milk. However, infant formula and human milk present differences in the protein content, supply of EAA, and content of essential trace elements (Lönnerdal, 2014). Moreover, the composition of breast milk changes dynamically during the period of lactation. In contrast, infant formulas have an established composition that only varies depending on the type of infant formula (Brätter et al., 1998).

Received May 5, 2016.
Accepted August 23, 2016.
[1]Corresponding author: ymadrid@quim.ucm.es

It is well known that only a certain amount of all nutrients in food are used effectively by the organism. For this reason, a correct evaluation of nutrients effect implies not only the determination of their total concentration, but also their bioavailability and, when possible, the chemical form present in the soluble fractions. Bioavailability is defined as the amount of an ingested nutrient that is absorbed and available for physiological functions. This parameter is dependent on digestion, the food matrix, absorption by intestinal cells, and transport to body cells (Etcheverry et al., 2012). Bioaccessibility is the amount of an ingested nutrient that is potentially available for absorption and is dependent only on digestion and release from the food matrix (Fernández-García et al., 2009). The bioavailability and bioaccessibility can be assessed by in vivo or in vitro techniques. In vivo methodology is the most appropriate, but it is time-consuming, costly, and difficult to perform (Perales et al., 2006). In vitro methods are a good alternative for evaluating bioaccessibility or bioavailability. Four principal in vitro methods exist: solubility, dialyzability, and gastrointestinal models (e.g., TIM, TNO intestinal model) for bioaccessibility, and Caco-2 models for bioavailability (Etcheverry et al., 2012). For solubility assays, the food studied is generally subjected to a simulation of gastrointestinal digestion including 3 steps: mouth digestion, gastric digestion, and intestinal digestion (Alzate et al., 2010). The in vitro dialyzability methods involve a 2-step digestion process simulating the gastric and intestinal phase and dialysis through a semipermeable membrane with a selected molecular weight cutoff (Moreda-Piñeiro et al., 2012).

Based on these dietary considerations, the aim of our study was first to characterize different types of infant formula (4 of them based on cow milk and 1 on soy protein) in terms of Zn bound to lipids and proteins and Zn distribution in the aqueous soluble protein fraction, and second to investigate the solubility and dialyzability of Zn from selected infant formulas. Moreover, Zn species in the resulting extracts were determined by size-exclusion chromatography (**SEC**) on line coupled to a UV-visible (UV-VIS) and inductively coupled plasma-mass spectrometry (**ICP-MS**) detectors.

MATERIALS AND METHODS

Instrumentation

A flame atomic absorption spectrophotometer (**FAAS**; Perkin Elmer 1100, Waltham, MA) was used for Zn analysis. A Zn hollow cathode lamp was run under operational conditions suggested by the manufacturer (Perkin Elmer, Norwalk, CT), shown in Table 1. All measurements were performed with an air-acetylene flame. The experimental conditions are compiled in Table 1.

Before total zinc determination, samples were microwave digested in double-walled advanced composite vessels using a 1,600-W microwave sample preparation

Table 1. Operating instrumental conditions

Instrument and characteristic	Conditions
Inductively coupled plasma-mass spectrometry parameters	
Radiofrequency power (W)	1,250
Plasma gas flow rate (mL/min)	15.0
Ar auxiliary flow rate (mL/min)	1.26
Carrier gas flow rate (mL/min)	1.1
Nebulizer	Meinhard (glass concentric nebulizer)
Spray chamber	Scott (double-pass or reversed-flow type)
Acquisition mode	Continuous
Isotopes monitored	^{66}Zn
Size-exclusion chromatography chromatographic parameters	
Columns	Superdex 200[1] (10–600 kDa) Superdex 75 (3–70 kDa) Superdex peptide (0.1–7 kDa)
Mobile phases	Tris-HCl 50 m*M*, KCl 0.05 m*M* (pH 6.8)
Mode	Isocratic
Flow rate (mL/min)	0.7
Injection volume (μL)	100
Flame atomic absorption spectrophotometer parameters	
Wavelength (nm)	213.9
Band pass (nm)	0.2
Lamp current (%)	75
Signal	Continuous
Fuel flow rate (acetylene/air; mL/min)	2.5/4.0
Flame type	Air-acetylene

[1]GE Healthcare Bio-Sciences AB, Uppsala, Sweden.

system microwave oven (CEM, Matthews, NC) equipped with temperature and pressure feedback controllers and 12 high-pressure vessels with 100 mL of inner volume. An incubator Heraeus D-6450 (Hanau, Germany) and an Eppendorf 5804 F34–6-38 ultracentrifuge were used in vitro simulation procedures.

The HPLC-ICP-MS measurements were carried out using a PU-2089 LC pump (Jasco, Tokyo, Japan) fitted with a 6-port injection valve (model 7725i, Rheodyne, Rohner Park, CA) with a 100-μL injection loop for the chromatographic separations. Chromatographic separations were performed using different size exclusion Superdex (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) columns ranged from 600 to 0.1 kDa. The outlet of the column was connected directly to the conical nebulizer of the ICP-MS with Peek tubing (Shimadzu, Tokyo, Japan). The SEC columns were also coupled to a Jasco LC-NET II/ADC UV-VIS spectrophotometer equipped with a photodiode array detector MD-2018 to monitor the UV absorption (280 nm) of proteins. The operating parameters are compiled in Table 1.

Reagents and Standards

Five types of commercial infant formula (NSL, NH1, BPF, and NC3, based on cow milk; and NS, based on soy protein) were used in our study. Characteristics of the selected infant formulas are detailed in Table 2. Chemicals and reagents were of analytical grade and solutions were prepared with deionized water (18 MΩ·cm) from a Milli-Q water purification system unit (Millipore, Bedford, MA).

The 1,000 mg/L zinc standard solution was obtained from Sigma-Aldrich (St. Louis, MO). Working solutions were prepared daily by appropriately diluting the concentrate standard in Milli-Q water. Stock solutions were stored at 4°C. To avoid metal contamination, all glassware and plastic ware were kept in 10% (vol/vol) nitric acid and then rinsed several times with ultrapure water before use. Both HNO_3 (64%) and H_2O_2 (35%) from Panreac (Castellar de Valles, Spain) were used to digest the samples. Infant formula defatting was performed using cyclohexane (Scharlab, Barcelona, Spain).

For gastrointestinal simulated digestion, α-amylase (Merck, Kenilworth, NJ) was used in the mouth digestion step, 6% porcine pepsin (Sigma-Aldrich) and 0.15 *M* NaCl at pH 1.8 adjusted with 2 *M* HCl (35%, Merck) were used in the gastric digestion step, and 1.5% porcine pancreatin (Merck), 0.15% of biliary salts (Sigma), and 0.15 *M* NaCl were used in the intestinal digestion step. Digestive enzymes such as α-amylase from *Bacillus subtilis* powder, pepsin from porcine gastric mucosa, bile salts, pancreatin from porcine pancreas, and sodium hydrogen carbonate were purchased from Sigma-Aldrich. Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) disodium and cellulose dialysis tubing (molecular weight cutoff ≈ 12 kDa, 30 cm in length and 21 mm in diameter) were also purchased from Sigma-Aldrich.

The following peptides and proteins of different molecular weights were used for calibrating molecular weights for SEC: albumin from bovine serum (66 kDa), ovoalbumin (43 kDa), ribonuclease A from bovine pancreas (13.7 kDa), cytochrome C (12 kDa), aprotinin (7 kDa), and glycine (75 Da; all from Sigma-Aldrich). Potassium chloride (Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany) and Tris-HCl (Bio-Rad, Hercules, CA) were used for preparing the mobile phase.

Determination of Zn in Infant Formula by FAAS

Around 1.0 g of samples were accurately weighed and then digested with 3 mL of a mixture of nitric acid (65%) and hydrogen peroxide (35%; 3:1) in an analytical microwave oven. The solutions were then transferred to volumetric flasks and appropriately diluted to a final

Table 2. Total, soluble, and dialyzate zinc contents (mean ± SD; n = 10)

Sample	Age given	Description	Zn (mg/100 g of milk)	Solubility[1] (%)	Dialysis[1] (%)
NSL[2]	From 1 d	Lactose-free formula	4.6 ± 0.4	68 ± 4	7.0 ± 0.1
NH1[2]	From 1 d	Extensively hydrolyzed proteins formula with nucleotides, prebiotics, and docosahexaenoic acid.	4.6 ± 0.4	62 ± 5	11 ± 3
NS[3]	From 1 d	Soy-based formula	4.6 ± 0.2	25 ± 6	7.0 ± 0.1
BPF[2]	From 6 mo	Hydrolyzed proteins formula for infants with allergies or intolerant to milk proteins	4.0 ± 0.4	63 ± 9	6 ± 2
NC3[2]	From 1 yr	Standard milk-based formula enriched with calcium, iron, and vitamins	2.7 ± 0.7	64 ± 9	1.0 ± 0.1

[1]Solubility or dialysis = 100 × soluble or dialyzate content/total zinc content.

[2]Cow milk-based infant formulas.

[3]Formula derived from soy protein-based infant formula.

volume of 10 mL with Milli-Q water. These solutions were used in the Zn determination by FAAS under the experimental conditions listed in Table 1. By using this protocol, the concentration of Zn in the solution underwent the analysis was suitable to the method sensitivity (0.059 mg/L). A similar procedure was used for determining Zn in gastrointestinal-simulated extracts, in vitro dialyzable extracts, and the protein-soluble fraction.

Infant Formula Defatting

This was carried out according to the defatting procedure of Palomo et al. (2014). Approximately 2.5 g of sample were defatted using 6 mL of cyclohexane followed by stirring during 10 min and subsequent centrifugation at 7,728 × *g* for 10 min at 20°C. The supernatant was discharged and the precipitate was subjected twice to the same procedure by adding fresh cyclohexane. Defatted samples were dried in an oven at 37°C.

Protein Extraction Procedure and Quantification Using the Bradford Method

Protein precipitation was performed by overnight incubation of 0.5 g of infant formula with 9 mL of acetone 80% (vol/vol), cooling at −20°C, and subsequent centrifugation at 14,610 × *g* for 10 min at 4°C. The resulting precipitate was collected and washed with 3 mL of pure acetone at −20°C; the process was repeated twice. The protein pellet obtained was subjected to acid digestion and subsequently measured by FAAS to determine Zn concentration.

The soluble protein fraction was obtained by treating 50 mg of the protein pellet with 2.5 mL of a solution containing 100 m*M* (pH 6.8) Tris-HCl. The mixture was sonicated for 2 min at 40% power using an ultrasound probe. The supernatant was collected after centrifugation at 12,074 × *g* for 25 min at 25°C. The obtained soluble protein fraction was divided into 2 portions for total Zn determination and protein analysis, respectively. Protein in the soluble fractions was quantified using the Bradford reagent (Coomassie Brilliant Blue G-250) in combination with UV-VIS measurements at 595 nm. Bovine serum albumin was used as the standard in a concentration range of 0 to 20 mg/L.

In Vitro Solubility Assay and In Vitro Dialyzable Process

The in vitro solubility assay was carried out by simulating the digestion conditions that occur in the mouth, stomach, and intestine, and by following the experimental procedure described by Alzate et al. (2010) and Moreda-Piñeiro et al. (2012). Briefly, about 2.5 g of infant formula was placed into 12-mL polystyrene tubes containing 1 mL of 1.7 mg/mL α-amylase solution. The mixture was incubated for 15 min at 37°C. Subsequently, 7.5 mL of freshly prepared gastric juice that consisted of 12 mg/mL pepsin in 0.15 *M* NaCl at pH 1.8 adjusted with 6 *M* HCl was added. The mixture was kept in a thermostatic bath at 37°C for 4 h. After gastric digestion, 5 mL of pancreatin-bile solution (3 mg/mL of pancreatin and 0.3 mg/mL of bile salts in 0.15 *M* NaCl and saturated $NaHCO_3$ solution) was added to simulate the intestinal digestion. The mixture was then left at 37ºC for 4 h in a thermostatic bath, shaking periodically every 10 min. Once digestion was finished, samples were centrifuged at 4,347 × *g* for 30 min at 4°C. The supernatant was filtered through a 0.22-μm nylon filter and kept at 4°C until analysis. This fraction, called the soluble fraction, was used further for determining both the concentration of Zn and the presence of Zn-containing biomolecules. The procedure was performed in triplicate. Blanks of gastrointestinal digestion were prepared in parallel.

The in vitro dialyzable procedure was performed by repeating the in vitro digestion assay with the exception of placing a dialysis membrane (≈12 kDa) filled with 0.15 *N* piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) disodium solution inside the polystyrene tubes before adding the intestinal solution. After 120 min of contact time, the membrane was removed and its outer surface was rinsed with deionized water. The content of Zn in the dialyzed fraction was measured by FAAS after digesting the samples in a microwave oven with H_2O_2 or HNO_3. Blanks were prepared in parallel.

Determination of Zn-Containing Biomolecules by SEC with UV and ICP-MS Detectors

Zinc-containing biomolecules were detected in the solubility extracts, dialyzable extracts, and in the protein-soluble fraction by SEC-UV-ICP-MS using 3 SEC columns (listed in Table 1) with different molecular weights. Optimal separation conditions are summarized in Table 1. The following peptides and proteins of molecular weights from 0.75 to 67 kDa were used for the determination of molecular weights: 8 mg/mL albumin (67 kDa), 5 mg/mL ribonuclease A (13.7 kDa), 1.5 mg/mL cytochrome C (13.6 kDa), 2 mg/mL aproptinin (1.4 kDa), 2.5 mg/mL ovalbumin (4.3 kDa), and 1 mg/mL glycine (75 Da). A solution consisting of 50 m*M* Tris-HCl and 0.05 m*M* KCl at pH 6.8 was used as the mobile phase. The mass calibration curve for the SEC was determined by UV (280 nm) by plotting log (molecular weight) versus the retention time. The column was

regularly washed according to the recommendations of the manufacturer to remove adsorbed material.

RESULTS AND DISCUSSION

Total Zn Content in Infant Formula

Zinc concentration in infant formula was determined by FAAS after microwave acid digestion of the samples following the procedure described in the Materials and Method section. Infant formula of different brands and characteristics (4 based on cow milk and 1 on soy protein) were acquired in local supermarkets. As noted previously, the main source of Zn in infants (aged 0–12 mo) is human milk or infant formulas. The addition of Zn compounds during manufacture is a common practice when preparing infant formula, with the aim of reaching the levels of recommended daily intake. The Zn compounds allowed by European legislation (European Commission, 1991) are zinc acetate, zinc chloride, zinc sulfate, zinc gluconate, and zinc oxide (Guillem et al., 2000). According to the information given by the manufacturers, all the infant formula tested in our study includes Zn as zinc sulfate; however, no information about the level of Zn enrichment is provided. As shown in Table 2, the concentrations of Zn in the infant formulas tested were quite similar except for infant formula developed for infants at least 1 yr old. Results are expressed as mean ± standard deviation (SD; n = 3 replicates). The zinc contents found were in agreement with the contents reported on the label by the manufacturer.

The analytical methodology used was validated by analyzing a reference material certified for Zn (skim milk powder certified reference material; BCR-063R). No significant differences were observed between the certified value (49.0 ± 0.4 mg/kg) and the experimental value (50.0 ± 0.8 mg/kg) of Zn at the 95% of confidence level.

Lipid Fraction-Bound Zn

Determination of the Zn content present in the lipid fraction of the infant formula was carried out by FAAS analysis of the solid residue obtained after sample defatting. The percentage of Zn found was lower than 1%, indicating that Zn was not incorporated into the lipid fraction of the infant formula.

Protein-Bound Zn

The determination of Zn in the protein pellet by FAAS after microwave acid digestion revealed that around 100% of Zn present in all the infant formulas tested was bound to the protein fraction.

Fractionation of Proteins in Infant Formula by SEC-UV-VIS and SEC-ICP-MS

Zinc determination in the Tris-soluble protein fraction by FAAS showed that the amount of Zn was of around 95% of the total Zn concentration. In contrast, the percentages of zinc found in the soluble protein fraction in the soy-based (NS) and lactose-free (NLS) infant formula were very low, around the 7 and 24%, respectively.

Subsequently, the soluble protein extracts from infant formula were analyzed by SEC-UV-VIS and SEC-ICP-MS. To improve the resolution of SEC when fractionating biomolecules, 3 SEC columns of different molecular weights were applied: Superdex 200 (10–600 kDa), Superdex 75 (3–70 kDa), and Superdex Peptide (0.1–7 kDa). The UV-VIS chromatographic profile from Superdex 200 column is shown in Figure 1a. Most of the biomolecules extracted from the infant formula were found in the low-molecular-weight region (at a retention time of 30 min, which corresponds to a molecular weight of around 10 kDa). The remaining proteins were detected at a retention time of 10 min, representing compounds with a higher molecular weight (around 400 kDa). The soy-based infant formula (NS) presented a different UV profile, with the proteins mainly distributed in the low-molecular-weight region (around 20–10 kDa).

The chromatogram obtained with ICP-MS is shown in Figure 1b. Comparing the UV and Zn (^{66}Zn) profiles, Zn is only bound to biomolecules of low molecular weight (around 10 kDa). This behavior was observed in both soy- and milk-based infant formulas.

However, the low resolution of the Superdex 200 column limits the information on Zn incorporation in biomolecules. Because Zn in the soluble protein fraction is mostly bound to low-molecular-weight compounds (around 10 kDa), a Superdex 75 column with a separation range between 3 and 70 kDa was subsequently used. The ICP-MS profile (Figure 1c) shows that Zn was associated with biomolecules in the low-molecular-mass region around 10 kDa. The results obtained by using the 2 columns, Superdex 200 and Superdex 75, may indicate that zinc is bounded to biomolecules with an estimated molecular mass around 10 kDa. However, Zn-containing peaks still appeared in the lower fractionation range given by the manufacturer, which could lead to erroneous results and conclusions. Therefore, a different column, Superdex Peptide (0.1–7 kDa), was further applied for obtaining better Zn fractionation in

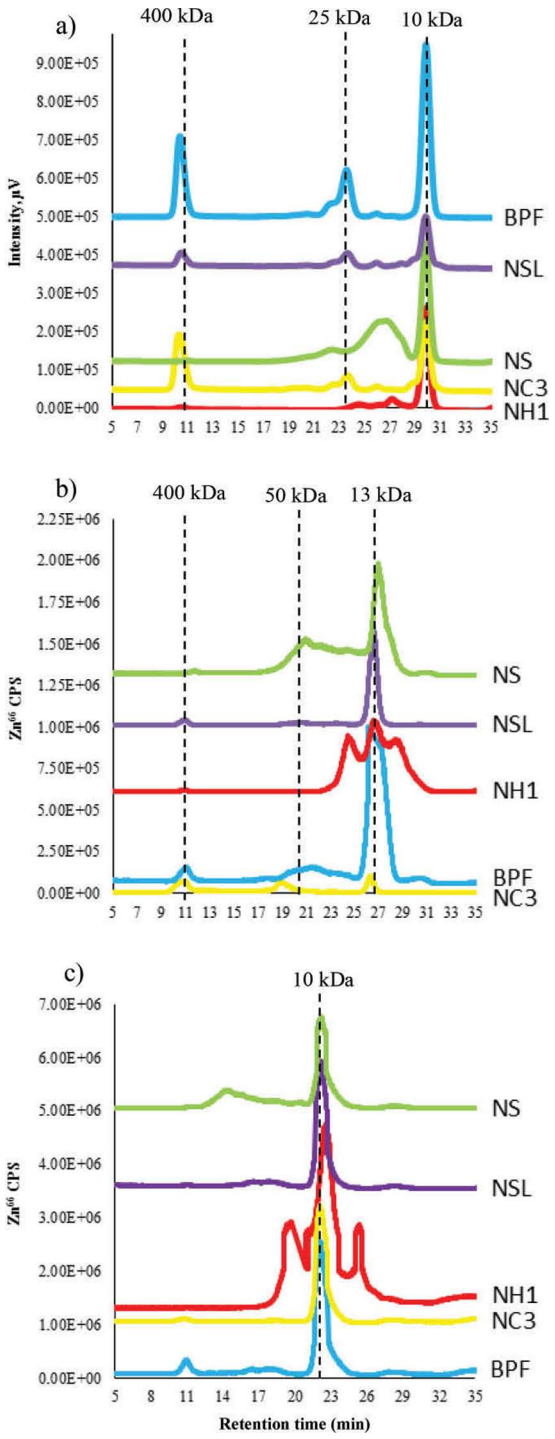

Figure 1. (a) Chromatograms on Superdex 200 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) obtained by size-exclusion chromatography (SEC)-UV-visible spectrometry corresponding to the Tris-HCl soluble protein fraction from infant formulas (NC3, BPF, NH1, NSL, and NS). (b) Chromatograms on Superdex 200 obtained by SEC-inductively coupled plasma-mass spectrometry (ICP-MS) at 64 m/z corresponding to the Tris-HCl soluble protein fraction from infant formulas (NC3, BPF, NH1, NSL, and NS). CPS = counts per second. (c) Chromatograms on Superdex 75 at 64 m/z corresponding to the Tris-HCl soluble protein fraction from infant formulas (NC3, BPF, NH1, NSL, and NS). Color version available online.

the soluble protein fraction. Figure 2a shows the ICP-MS profile of the protein soluble fraction. The chromatographic profiles obtained are highly dependent on the type of infant formula tested, with estimated molecular weights ranged from 0.2 to 7 kDa. Given the small size of the biomolecules, the presence of Zn-free form was evaluated by passing a 2 mg/L Zn^{2+} solution through the column and further analysis by ICP-MS. Results (not shown) indicated that Zn^{2+} standard appeared at a retention time of 35 min, representing a retention time value higher than those found for the Zn-containing peaks in the infant formula tested; this confirmed that Zn is mainly associated with biomolecules of low molecular weight rather than Zn-free form. The results obtained from our fractionation study suggest that Zn is mainly present in the cow milk-based infant formula in the soluble protein fraction, whereas only 7 and 24% of total Zn was detected in the soluble protein fraction of the soy-based and cow milk lactose-free infant formulas. The low percentage of Zn in the soluble protein fraction found in these 2 types of infant formula could have influence in their bioavailability and, therefore, nutritional value in relation to Zn.

Availability of Zn in Infant Formulas by In Vitro Methods

Results from in vitro simulated gastrointestinal digestion of infant formula are shown in Table 3. Soluble Zn content after applying a simulated gastric and gastrointestinal digestion in infant formula ranged from 30 to 90% and from 30 to 70%, respectively. The Zn solubility values obtained are higher than those in the literature (Singh et al., 2016) for raw cow milk (the starting material for preparing cow-milk infant formula), with values ranged from 15 to 20%. This suggests that both Zn supplementation and the treatment used to improve digestibility favor Zn solubility

No significant ($P > 0.05$) differences were observed between the percentages of Zn found in gastric and gastrointestinal extracts in all the infant formula tested, except for a follow-up formula (NC3) composed mainly of standard cow milk. The remaining infant formula used in our study was formulated for infants from 1 d old and, therefore, were all treated to include hydrolyzed proteins to improve digestibility. One-day-old infants have a gastrointestinal tract that does not produce the same digestive enzymes as a child or adult. Babies can digest breast milk more easily than infant formula because breast milk contains enzymes (amylase and lipase) that aid digestion. The treatment used to improve digestibility might explain why we found no effect of digestive enzymes in releasing Zn. In general, no significant ($P > 0.05$) differences were observed be-

Table 3. Zinc percentages in gastric and gastrointestinal extracts (mean ± SD; n = 10)

Sample	Gastric digestion[1] (%)	Gastrointestinal digestion (solubility)[1] (%)
NH1[2]	73 ± 2	62 ± 5
NC3[2]	29 ± 4	64 ± 9
NSL[2] (lactose-free infant formula	71 ± 4	68 ± 4
NS[3] (soy-based infant formula)	35 ± 6	25 ± 6
BPF[2]	86 ± 6	63 ± 9

[1]Gastric digestion or solubility = 100 × gastric or soluble content/total zinc content.
[2]Cow milk-based infant formulas.
[3]Formula derived from soy protein-based infant formula.

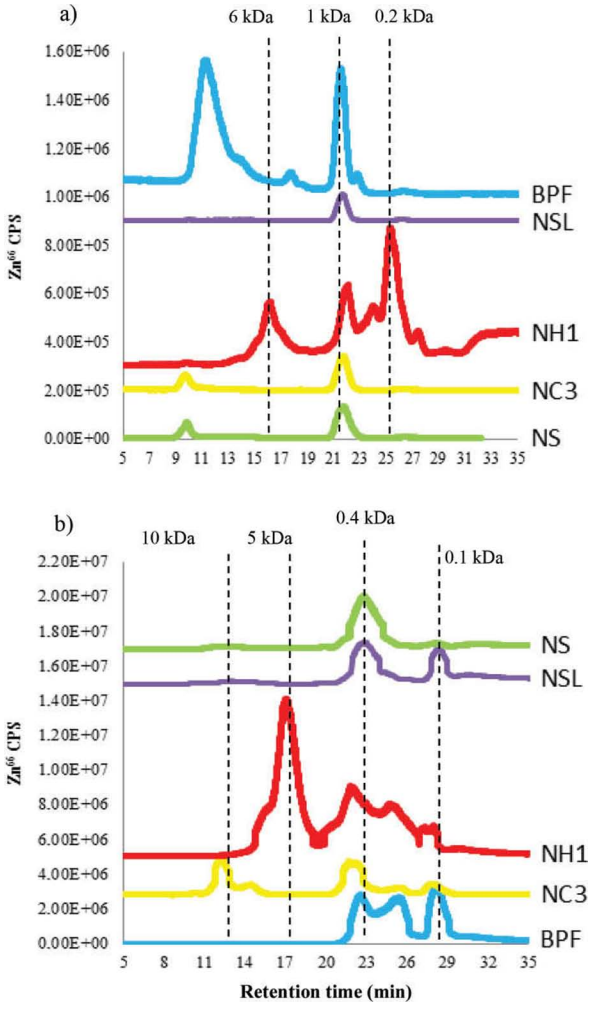

Figure 2. (a) Chromatograms on Superdex (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) Peptide obtained by size-exclusion chromatography–inductively coupled plasma-mass spectrometry (SEC-ICP-MS) at 64 m/z corresponding to the Tris-HCl soluble protein fraction from infant formulas (NC3, BPF, NH1, NSL, and NS). CPS = counts per second. (b) Chromatograms on Superdex Peptide obtained by SEC-ICP-MS at 64 m/z corresponding to gastrointestinal extracts from infant formulas (NC3, BPF, NH1, NSL, and NS). Color version available online.

tween the percentages of Zn found in gastrointestinal extracts from all the formulas derived from cow milk. However, a low percentage of Zn (30%) was found in the gastrointestinal extract from soy-based infant formula. This result is in agreement with the low percentage of Zn detected in the low-soluble protein fraction of the soy-based infant formula (7%). It is well known that Zn bioaccessibility is highly dependent on the food matrix. Isolated soy protein-based formulas currently on the market are all free of cow milk protein and lactose and have been employed as a milk substitute for infants unable to tolerate a cow milk protein-based formula. Until 1980, mineral absorption from soy formulas was erratic because of the poor stability of the suspensions and the presence of excessive soy phytates. Phytates are organic compounds derived from phytic acid or inositol polyphosphate ($C_6H_{18}O_{24}P_6$) that are widely distributed in all seeds and, possibly, all cells of plants. A high content of phytate in food has been considered as a limiting factor for the mineral bioaccessibility and bioavailability. Soy protein isolate formulas still contain 1.5% phytates able to bind zinc, therefore decreasing its bioaccessibility. As with other essential elements, the bioavailability of zinc from foods is dependent on the presence of dietary components in the intestinal lumen; for example, calcium and iron influence Zn bioaccessibility according to a number of studies that included integrated approaches based on the interaction of these 3 minerals (Krebs, 2000; Perales et al., 2006; Olivares et al., 2012). The presence of phytate and nucleic acids (phosphorus-containing compounds) decreases zinc absorption, and calcium might have a potential inhibitory effect on zinc that is only evident when phytate is present in the food (Bosscher et al., 2002; Liang et al., 2010). The amount and type of protein in the diet also affects zinc absorption. In, general, animal proteins present in foods, such as beef, eggs, and cheese, have been shown to have a positive effect on zinc absorption, with the exception of casein (Drago and Valencia, 2004). It has been also reported that the use of hydrolysates of milk

proteins benefits Zn absorption. For instance, Wang et al. (2011) demonstrated the highest Zn-release activity of yak casein hydrolysate (prepared with alcalase and trypsin) compared with the intact yak casein, suggesting the use of yak casein hydrolysate as a vehicle for delivering Zn. However, the Zn compound selected for supplementation affects Zn bioaccessibility in infant formula. In agreement with this, Guillem et al. (2000) fortified infant formula with different Zn compounds. Data obtained from in vitro dialyzability assays revealed that Zn availability in milk-based formulas decreases in the order of oxide > gluconate = chloride = lactate = citrate > acetate, and from soy-based infant formulas in the order of gluconate > oxide > lactate = chloride = acetate > sulfate > citrate.

An additional factor that could alter bioaccessibility is the fat content. In the current study, in vitro-simulated gastrointestinal digestion of infant formula was performed in defatted and nondefatted samples. No significant differences ($P > 0.05$) were observed between the values provided for both type of samples, which is in agreement with the low percentage of Zn bound to the lipid fraction.

The gastrointestinal extracts from each infant formula were further analyzed by SEC-ICP-MS using the Superdex Peptide (0.1–7 kDa) column. The ^{66}Zn chromatographic profiles in Figure 2 suggest that Zn-containing peaks are associated with the low-molecular-weight region <10 kDa, which could imply that Zn-bound compounds in the gastrointestinal extracts were small enough to be absorbed by the intestine. To determine the Zn concentration in the 0.1- to 7-kDa fraction, the Zn-containing peaks were collected and subsequently analyzed by ICP-MS following the experimental conditions given in Table 1. Around 60 and 30% of total Zn content was found in the 0.1- to 7-kDa fraction of cow milk- and soy protein-based infant formulas, respectively. These results are in agreement with those obtained when determining Zn in the soluble fraction, suggesting the presence of Zn in the soluble fraction of the infant formula as Zn bound to molecules of low molecular weight and, therefore, small enough to be used in physiological functions.

To verify, an in vitro dialyzability technique was applied by using the procedure described in the Material and Methods section. The in vitro dialyzability assay is considered the most appropriate method to study Zn bioaccessibility; thus far, it is the only method that has been validated against human studies (Hotz, 2005). As shown in Table 2, the percentage of Zn in the dialyzed fractions were very low (11% or less) and similar to those reported by other authors for the same kinds of samples (Guillem et al., 2000; Perales et al., 2006). The highest Zn dialysis percentage was found in a cow milk-based infant formula composed of hydrolyzed proteins and prebiotics (NH1 sample). The addition of prebiotics (galacto-oligosaccharides, fructo-oligosaccharide, polydextrose, and mixtures of these) in infant formula has benefits, as prebiotics are known to alter the gastrointestinal microbiota resembling that of breastfed infants. Infants supplemented with this type of formula have a lower stool pH, a better stool consistency and frequency, and a higher concentration of bifidobacteria in their intestine compared with infants on a nonsupplemented standard formula (Vandenplas et al., 2015). The combined presence of prebiotics and hydrolyzed milk proteins may have influence on Zn availability, thus leading to the highest dialysis percentage value compared with the other infant formulas tested. The lowest Zn dialysis percentage corresponded to a follow-up cow milk-based infant formula (NC3 sample); the presence of nonhydrolyzed proteins in samples of this kind could decrease their dialyzability.

According to the data obtained by SEC-ICP-MS analysis of the gastrointestinal extracts (Figure 2b), the soluble Zn found in the supernatant after applying the gastrointestinal in vitro digestion method is available for absorption. However, the concentration of Zn in dialysates was lower than expected according to the molecular weight of the Zn compounds found in the gastrointestinal extracts with smaller size (from 0.1 to 7 kDa) than the pore size of the dialysis membrane used (molecular weight cutoff = 10–12 kDa). Therefore, although all soluble Zn seems to be available for absorption, the amount of Zn absorbed according to the in vitro dialyzability technique is much lower. This means that some of the soluble Zn does not dialyze in the in vitro assay conditions applied. Although in vitro dialyzability technique is recommended as the best in vitro method for estimating the absorption of Zn in the small intestine, the procedure is dependent on several factors that may influence the results, such as a rigorous control of pH and characteristics and performance of the selected cutoff of the dialysis membrane. Commercial dialysis membranes have broad pore size distributions and are over 1,000 times thicker than the molecules they are designed to separate, leading to poor size cutoff properties, filtrate loss within the membranes, and low transport rates; these factors could lead to differences when correlating in vivo and in vitro data. In vitro solubility or dialyzability methods correlate in most cases with human absorption studies in ranking iron and zinc availability from different meals; however, the effect of the food matrix (milk and tea) as well as certain proteins and organic acids can lead to unpredicted results. For instance, it has been observed that dialyzability methods exclude iron bound to large molecules, which in some cases is available,

and includes iron bound to small molecules, which is not always available. Roig et al. (1999) found a better agreement between the in vivo percentages of calcium absorption and in vitro soluble calcium percentages than between the percentage of calcium that is able to pass through the intestinal walls when performing in vivo studies and the dialyzed calcium percentages. Despite these limitations, in vitro experiments based on solubility or dialyzability are very valuable tools to help understand factors that may affect subsequent mineral absorption. The current study revealed that in vitro dialysis results must be interpreted with some caution. Dialysis values should be validated by applying either in vitro assays with Caco-2 cells or by performing speciation studies. The application of speciation analysis (SEC-ICP-MS) is a powerful and essential tool for establishing the performance of in vitro bioaccessibility studies, for obtaining a better agreement between in vivo and in vitro methods, and for gaining a deeper insight into the mechanisms affecting the absorption of essential elements such as Zn.

CONCLUSIONS

More studies are needed to validate the in vitro methods for measuring zinc bioavailability. Studies of zinc availability in infant formula are of vital importance due to the role of zinc in the normal growth and development of infants and children. In this study, the percentages of Zn solubility obtained by applying the in vitro digestion method to infant formula ranged from 60 to 70% when formulations were manufactured from cow milk; however, values as low as 23% were obtained when soy-based infant formula were evaluated. Analysis by SEC-ICP-MS of gastrointestinal extracts revealed that Zn is associated with the low-molecular-weight region (0.1–7 kDa), which are small enough to be absorbed by the intestine and, therefore, to be use in physiological functions. However, after applying an in vitro dialyzability technique, the values of percentage of Zn in the dialysates were lower than expected (11% or less). Although the in vitro dialyzability technique is recommended as the best in vitro method for estimating the absorption of Zn in the small intestine, several factors may affect the performance of the technique. The use of speciation tools have been demonstrated as a complementary analytical tool for validating in vitro methods for measuring bioaccessibility.

ACKNOWLEDGMENTS

The authors thank the Spanish Commission of Science and Technology (CTQ2014-54801-C2-1-R) and the Community of Madrid/FEDER (Fondo Europeo de Desarrollo Regional)/ERDF (European Regional Development Fund, Madrid, Spain) programme (S2013/ABI-3028, AVANSECAL-CM; Estrategias avanzadas para la mejora y control de la calidad y la seguridad de los alimentos/Advanced strategies for assessing quality and food safety, Madrid, Spain) for funding.

REFERENCES

Alzate, A., M. C. Pérez-Conde, A. M. Gutiérrez, and C. Cámara. 2010. Selenium-enriched fermented milk: A suitable dairy product to improve. Int. Dairy J. 20:761–769.

Bosscher, D., R. Van Cauwenberg, J. C. V. an der Auwera, H. Robberecht, and H. Deelstra. 2002. Calcium, iron and zinc availability from weaning meals. Acta Paediatr. 91:761–768.

Brätter, P., I. Navarro, V. E. Negretti, and A. Raab. 1998. Speciation as an analytical aid in trace element research in infant nutrition. Analyst 123:821–826.

Drago, S. R., and M. E. Valencia. 2004. Influence of components of infant formulas on *in vitro* iron, zinc and calcium availability. J. Agric. Food Chem. 52:3202–3207.

European Commission. 1991. Commission Directive 91/321/EEC of 14 May 1991 on infant formulae and follow-on formulae. No. L175.

Etcheverry, P., M. A. Grusak, and L. E. Fleige. 2012. Application of in vitro bioaccessibility and bioavailability methods for calcium, carotenoids, folate, iron, magnesium, polyphenols, zinc, and vitamins B6, B12, D, and E. Front Physiol. 3:317.

European Food Safety Authority (EFSA). 2009. Scientific opinion on the appropriate age for introduction of complementary feeding of infants. EFSA J. 10.2903/j.efsa.2009.1423.

European Food Safety Authority (EFSA). 2014. Scientific opinion on dietary reference values for zinc. EFSA J. 10.2903/j.efsa.2014.3844.

Fernández-García, E., I. Carvajal-Lérida, and A. Pérez-Gálvez. 2009. In vitro bioaccessibility assessment as a prediction tool of nutritional efficiency. Nutr. Res. 29:751–760.

Guillem, A., A. Alegría, R. Barberá, R. Farré, M. J. Lagarda, and G. Clemente. 2000. In vitro Dialyzability of zinc from different salts used in the supplementation of infant formulas. Biol. Trace Elem. Res. 75:11–19.

Harmaza, Y. H., and E. I. Slobozhanina. 2014. Zinc essentiality and toxicity. Biophysical aspects. Biophysics (Oxf.) 59:264–275.

Hotz, C. 2005. Evidence of the usefulness of in vitro dialyzability, Caco-2 cell models animal models and algorithms to predict zinc bioavailability in humans. Int. J. Vitam. Nutr. Res. 75:423–435.

Krebs, N. F. 2000. Overview of zinc absorption and excretion in the human gastrointestinal tract. J. Nutr. 130:1374–1377.

Liang, J., B. Z. Han, M. J. Nout, and R. J. Hamer. 2010. In vitro solubility of calcium, iron and zinc in relation to phytic acid levels in rice-based consumer products in China. Int. J. Food Sci. Nutr. 61:40–51.

Lönnerdal, B. 2014. Infant formula and infant nutrition: Bioactive proteins of human milk and implications for composition of infant formulas. Am. J. Clin. Nutr. 99:712S–717S.

Moreda-Piñeiro, J., E. Alonso-Rodriguez, V. Romarís-Hortas, A. Moreda-Piñeiro, P. López-Mahía, S. Muniategui-Lorenzo, D. Prada-Rodríguez, and P. Bermejo-Barrera. 2012. Assessment of the bioavailability of toxic and non-toxic arsenic species in seafood samples. Food Chem. 130:552–560.

Olivares, M., F. Pizarro, M. Ruz, and D. López de Romaña. 2012. Acute inhibition of iron bioavailability by zinc: Studies in human. Biometals 25:657–664.

Palomo, M., A. M. Gutiérrez, M. C. Pérez-Conde, C. Cámara, and Y. Madrid. 2014. Se metallomics during lactic fermentation of Se-enriched yogurt. Food Chem. 164:371–379.

Perales, S., R. Barberá, M. J. Lagarda, and R. Farré. 2006. Bioavailability of zinc from infant foods by *in vitro* methods (solubility,

dialyzability and uptake and transport by Caco-2 cells). J. Sci. Food Agric. 86:971–978.
Prasad, A. S. 1998. Zinc in human health: An update. J. Trace Elem. Exp. Med. 11:63–87.
Roig, M. J., A. Alegrõ, R. Barbera, R. Farre, and M. J. Lagarda. 1999. Calcium bioavailability in human milk, cow milk and infant formulas. Comparison between dialysis and solubility methods. Food Chem. 65:353–357.
Singh, P., P. Surendra, and W. Aalbersberg. 2016. Bioavailability of Fe and Zn in selected legumes, cereals, meat and milk products consumed in Fiji. Food Chem. 207:125–131.
Vandenplas, Y., I. Zakharova, and Y. Dmitrieva. 2015. Oligosaccharides in infant formula: More evidence to validate the role of prebiotics. Br. J. Nutr. 113:1339–1344.
Wang, X., J. Zhou, P. S. Tong, and X. Y. Mao. 2011. Zinc-binding capacity of yak casein hydrolysate and the zinc-releasing characteristics of casein hydrolysate-zinc complexes. J. Dairy Sci. 94:2731–2740.
WHO. 2009. Infant and young child feeding. Model chapter for textbooks for medical students and allied health professionals. World Health Organization (WHO), Geneva, Switzerland.