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Influence of Components of Infant Formulas on in Vitro Iron, Zinc, and Calcium Availability

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The effect of casein content and Ca concentration on Fe, Zn, and Ca dialyzability was assessed using a response surface design. Tested casein levels were 5.31−13.75 g/L (34.8−90.2% of total protein). Whey protein was added to complete 15.25 g/L total protein. Calcium levels were adjusted with calcium citrate within a range between 417.4 and 804.9 mg/L. Through the experimental design utilized, we found that of both assessed factors, only the casein content significantly influenced Fe and Zn dialyzability. Protein composition did not influence calcium dialyzability, and calcium concentration did not affect either Fe or Zn dialyzability. No effect of casein−Ca on iron, zinc, and calcium dialyzability was found. According to these results, whey-dominant formulas are less prone to hamper mineral availability, and are therefore suitable in order to improve iron and zinc availability.

KEYWORDS: Infant formulas; casein; iron; zinc; calcium; mineral dialyzability

INTRODUCTION

Generally, neonates receive all essential nutrients from human milk, but when breast feeding is not possible, the nutritional needs of infants can be met through infant formulas. Human milk is the standard for deciding on the appropriate level of a nutrient to be used in infant formulas. Although human milk has a protein content of 9−11 g/L, most term starting formulas have approximately 15−17 g/L protein to compensate for essential amino acid differences (*1*). Cow's milk proteins α-lactalbumin, β-lactoglobulin, and casein are the most commonly used substitutes for human milk proteins in the production of infant formulas (*2*). Since human milk has been considered to have a 40:60 casein-to-whey protein ratio, most infant formula manufacturers have attempted to make the formulas resemble human milk by adapting the original 82:18 casein-to-whey protein ratio in cow's milk (*3*). However, some of them have 80:20 casein-to-whey protein ratios, and the adequacy of the ratio is controversial (*4*).

Mineral bioavailability from cow's milk infant formulas is lower than that from human milk. The composition and concentration of casein and whey proteins in cow's milk, structural properties of specific phosphopeptides formed during digestion, and even calcium concentration, among other factors, have been ascribed to be responsible for this lower bioavailability (*5*−*8*). For this reason, the iron content of fortified term infant formulas is much higher than that of human milk. In Europe, term infant formulas contain approximately 5−8 mg/L iron, while in the United States the iron content is approximately 12−14 mg/L (*9*, *10*). The increase in the use of iron-fortified infant formulas in developed countries has led to a decrease in the prevalence of iron-deficiency anemia. The zinc concentration in cow's milk formulas is also higher than that in breast milk. Due to the low bioavailability of zinc from cow's milk infant formulas, the Committee on Nutrition of the American Academy of Pediatrics recommends zinc supplementation of infant formulas (*11*).

To properly formulate mineral concentration in infant formulas to ensure that the amount of absorbed nutrient is similar to that from human milk, bioavailability studies are needed (*9*). Ideally, bioavailability should be evaluated through human studies. However, complexity and cost limit their applicability (*12*). In vitro methods have been used to estimate potential availability. Dialyzability after simulated gastrointestinal digestion is one of the more widespread methods used to assess availability, since the formation of mineral low-molecular-weight soluble complexes is a prerequisite for bioavailability. The results of this in vitro test evaluate only the fraction of the element available for absorption. However, conditions prevailing in the intestinal tract are major determinants for iron and zinc absorption (*13*). Therefore, dialyzability may be a useful indicator of availability for absorption, as was first demonstrated by Schricker et al. (*14*) for iron. Comparison of Fe and Zn dialyzability results with data from human studies shows good agreement (*3*, *5*, *7*, *11*, *15*−*18*).The method has also been used to measure Ca availability (*13*, *19*, *20*). Although in this case complex homeostatic mechanisms regulate not only absorption but also retention, information on mineral dialyzability is useful to know the amount of soluble and potentially absorbable Ca.

Mineral bioavailability has been analyzed in commercial formulas in which different composition, mineral sources, and/or processing conditions may influence mineral bioavailability (*7*, *11*). Minerals may be added to infant formulas before or after heat processing. Addition of minerals before heat processing may induce interactions that affect nutrient bioavailability

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Table 1. Protein and Mineral Content of Fluid Skim Milk, Casein, and Whey Protein Concentrate

component	fluid skim milk	casein	whey protein concentrate
proteins (g%)	3.00 ± 0.06	88.68 ± 0.08	70.47 ± 0.27
iron (mg%)	0.05 ± 0.01	0.49 ± 0.02	0.95 ± 0.1
zinc (mg%)	0.37 ± 0.01	4.57 ± 0.12	0.89 ± 0.02
calcium (mg%)	107.4 ± 2.2	40.11 ± 0.92	928.98 ± 24.22

Table 2. Treatment Schedule for Two-Factor Central Composite Design Showing the Levels of the Two Variables, Casein and Calcium Content

run	casein (g/L)	casein:whey	calcium (g/L)
1	9.53 (0)[a]	62.5:37.5	611.0 (0)
2	5.85 (–1)	38.4:61.6	442.0 (–1)
3	13.21 (1)	86.6:13.4	442.0 (–1)
4	5.85 (–1)	38.4:61.6	780.0 (1)
5	13.21 (1)	86.6:13.4	780.0 (1)
6	9.53 (0)	62.5:37.5	611.0 (0)
7	5.31 (–1.414)	34.8:65.2	611.0 (0)
8	13.75 (1.414)	90.2:9.8	611.0 (0)
9	9.53 (0)	62.5:37.5	417.1 (–1.414)
10	9.53 (0)	62.5:37.5	804.9 (1.414)
11	9.53 (0)	62.5:37.5	611.0 (0)

[a] Numbers in parentheses are the coded values of the independent variables in the experimental design.

(*21*). Therefore, the purpose of this study was to examine the influence of casein-to-whey ratio, the Ca concentration, and the interaction Ca−casein on Fe, Zn, and Ca dialyzability on experimental formulas where these were the only variables. A response surface methodology with a central composite orthogonal design was used to study the influence of infant formula composition on mineral dialyzability as an indicator of mineral availability.

MATERIALS AND METHODS

Materials. L-Ascorbic acid, PIPES buffer, dithiothreitol, sodium dodecyl sulfate, *o*-phthaldialdehyde, digestive enzymes, and bile salts were purchased from Sigma Chemical Co. (St. Louis, MO). Metaphosphoric acid was purchased from J.T. Baker (Phillipsburg, NJ). Spectra/Pore I dialysis tubing (cutoff 6000−8000) was purchased from Fischer Scientific (Fairlawn, NJ). Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). All other chemicals were reagent grade.

Fluid pasteurized skim cow's milk and corn oil were purchased from the market. Casein, lactose, vitamin mix (A, D, E, K1, B1, B2, B6, B12, niacin, folic acid, pantotenic acid, biotin, coline, inositol, and ascorbic acid), and calcium citrate (Ca, 19.14 ± 0.13 g%; Fe, 5.69 ± 0.33 mg%; Zn 0.64 ± 0.05 mg%) were a gift from Nestlé Argentina SA. Ultrafiltered whey concentrate and lecithin were a gift from SANCOR C.U.L., Argentina. $FeSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 7H_2O$ were analytical grade. The protein, iron, zinc, and calcium contents of commercial skim milk, whey concentrate, and casein are shown in **Table 1**.

Experimental Design. A Central Composite Orthogonal Design (CCOD) (2^2 + star) was used to study the simultaneous effect of two variables on mineral dialyzability. The CCOD is typically used for quantitative factors and designed to estimate all the main effects plus the desired quadratics and two-way interactions. Orthogonality makes it possible to measure the desired effects independently of each other. The experiments were based on a five-level, two-factor factorial design with three replicates in the central point. The variables were casein concentration (5.31−13.75 g/L, corresponding to 34.8−90.2% of total protein) and calcium concentration (417.1−804.9 mg/L), each at five levels, such as −1.414, −1, 0, 1, and 1.414. The CCOD is shown in **Table 2**, where casein-to-whey protein ratios are included in each case. Experiments were randomized. Casein levels were chosen to evaluate a wide range of casein-to-whey protein ratios including those present in infant formulas. Calcium levels were selected within the limit values stated by the LSRO report (*22*).

Preparation of Experimental Infant Formula. Experimental infant formulas were prepared to 40% total solids. Casein, whey, lactose, vitamin mix containing 28.37 ± 0.31 g% ascorbic acid, and minerals were weighed and dispersed in fluid nonfat milk. After that, corn oil with soy lecithin (final concentration 0.02 g%) was added, and the mixture was homogenized during 2 min utilizing a 500-W Braun food processor. The homogenized mixture was diluted to 13% solids with deionized water and refrigerated at 4 °C. The final composition was protein 15.25 g/L, carbohydrates 76.3 g/L, lipids 34.10 g/L, Fe 10.34 ± 0.71 mg/L, Zn 8.16 ± 0.33 mg/L, and ascorbic acid (AA) 135.7 ± 28.5 mg/L. The level of total protein corresponds to a term starting infant formula. Iron, zinc, and calcium levels were adjusted with ferrous sulfate, zinc sulfate, and calcium citrate, respectively. An AA:Fe molar ratio of 4:1 was chosen considering the usual levels of addition of ascorbic acid to cow's infant formulas. A 10% excess was added, since preliminary trials showed that, after 22 h of refrigerated storage, losses of AA occur in iron-fortified milk.

Determination of Iron, Zinc, and Calcium Dialyzability (DFe%, DZn%, and DCa%). A modification of the widespread in vitro Miller's method (*23*) according to Wolfgor et al. (*24*) was followed. Aliquots (50 g) of homogenized samples were adjusted to pH 2.0 with 6 N HCl and, after addition of 1.6 mL of pepsin digestion mixture (16% pepsin solution in 0.1 N HCl), were incubated at 37 °C during 2 h in a shaking water bath. At the end of pepsin digestion, two aliquots of digest (15 g) were weighed into wide-necked 100-mL flasks. Dialysis bags containing 18.75 mL of PIPES buffer were placed in each flask. The buffer molarity used for each particular formula was calculated in order to obtain a final pH of digest−dialysate 6.5 ± 0.2. The main factors taken into account to calculate buffer molarity were buffer capacity of food matrix (HCl needed to reach pH 2), HCl milliequivalents (mEq) incorporated with pepsin solution, and acid mEq generated through enzymatic hydrolysis during in vitro digestion (*15*). Since the intrinsic pH of food was 6.7, there was no need to consider it. To calculate the buffer molarity, the following equations were utilized:

$$M = [\text{total acid mEq} + (f \times \text{total acid mEq})]/(fV)$$

where

$$f = [10^{(\text{PIPES } pK_a - \text{desired final pH})}] = 10^{(6.8 - 6.5)} = 1.995$$

and V is the volume of buffer in the dialysis bag.

Total acid mEq resulted from adding (a) HCl mEq needed to adjust each food matrix to pH 2; (b) HCl mEq from pepsin solution in the aliquot of pepsin digest (0.048); and (c) acid mEq generated by hydrolysis, which was calculated as follows: Each pepsin digest plus bile−pancreatin solution was adjusted to pH 6.5 and incubated during 120 min at 37 °C. Acid mEq generated by hydrolysis was calculated by subsequent titration to pH 6.5 with 0.1 N NaOH (0.312 mEq). PIPES solutions of 0.1065 ± 0.009 mol/L were used for formulas.

Samples were incubated for 50 min in a shaking water bath at 37 °C. Pancreatin−bile mixture (3.75 mL of 2.5% bile, 0.4% pancreatin solution in 0.1 N $NaHCO_3$) was then added to each flask, and the incubation continued for another 2 h. At the end of pancreatin−bile incubation, the dialysis bags were removed and rinsed with water. The bag contents were transferred to weighed flasks, weighed, and analyzed for mineral content by flame atomic absorption spectroscopy (AAS). Assessment of minerals in pepsin digests was made by AAS after wet ashing with HNO_3−$HClO_4$ (50:50). Lanthanum was added to all samples, and standards were analyzed for Ca to a 0.5% final concentration to correct for possible phosphate interference.

Mineral dialyzability was calculated from the amount of each dialyzed mineral, expressed as a percentage of the total amount present in each pepsin digest:

$$\text{dialyzable mineral (\%)} = [D/(WA)] \times 100$$

where D is the total amount of dialyzed mineral (μg), W is the weight of pepsin digest (g), and A is the concentration of each mineral in the pepsin digest (μg/g).

Dialyzability was performed 24 h after formula elaboration. In cow's milk, intrinsic iron is mostly bound to casein. Immediately after

Table 3. Central Composite Design Responses for Iron, Zinc, and Calcium Dialyzability (DFe%, DZn%, DCa%)[a]

no.	DFe%	DZn%	DCa%	no.	DFe%	DZn%	DCa%
1	18.20	22.29	28.96	7	24.78	31.59	30.27
2	23.42	29.96	26.58	8	16.51	18.00	29.14
3	15.36	17.33	26.03	9	18.51	21.39	27.80
4	23.78	27.71	32.93	10	19.65	21.74	34.06
5	16.47	18.93	32.00	11	18.96	22.00	29.60
6	18.62	20.27	29.19				

[a] Treatment schedule as in **Table 2**.

fortification, extrinsic iron is evenly bound to casein and whey proteins. After 24 h, iron distribution stabilizes, resembling that of intrinsic iron, and remains almost constant (*25*). As iron distribution among different milk fractions may influence iron availability results, experiments were made 24 h after formula preparation.

Each experiment was carried out at least twice, and all analyses were performed in duplicate. Average values were reported.

Ascorbic Acid (AA) Content. Assessment was made on vitamin premix, preliminary trials, and final experimental formulas according to Behrens and Madére (*26*) but using dithiothreitol to reduce dehydroascorbic acid (*27*).

Protein Content. Measurement was made on raw ingredients and final experimental formulas by a micro-Kjeldahl method (*28*).

Dialyzable Free Amino Groups. Dialyzed free amino groups released from the hydrolysis of milk proteins, considered as an indirect indicator of digestibility, were determined using the *o*-phthaldialdehyde method (*29*). The percentage of dialyzed free amines (DFA) mEq with respect to the total amount of mEq of peptide bonds potentially hydrolyzed was calculated using the factors 8.8 mEq/g and 8.2 mEq/g for whey and casein, respectively (*30*):

$$\text{DFA\%} = 100 \times \text{dialyzed free amines (mEq)}/[\text{whey protein (g)} \times 8.8\ \text{mEq/g} + \text{casein protein (g)} \times 8.2\ \text{mEq/g}]$$

Reference Materials. Triplicate samples of SRM Infant formula 1846 (NIST, Gaithersburg, MD) were run with each set of unknown samples, to validate each analytical batch for iron, zinc, calcium, and ascorbic acid. Agreement of the triplicate means for the reference material within 5% of the certified value, and less than 5% relative standard deviation for the triplicates, was required for acceptance of the data.

Statistical Analyses. A second-order polynomial equation was used to fit the experimental data given in **Table 3**. The model proposed for the responses was

$$\text{D\%} = b_0 + [b_1\ \text{casein (g/L)}] + [b_2\ \text{calcium (mg/L)}] + [b_3\ \text{casein (g/L)}^2] + [b_4\ \text{casein (g/L)} \times \text{calcium (mg/L)}] + [b_5\ \text{calcium (mg/L)}^2]$$

where D% is the response for mineral dialyzability, and b_{0-5} are constant coefficients.

Responses were monitored by evaluating an experimental formula prepared to have 7.625 g/L casein (casein-to-whey protein ratio 50:50) and 520 mg/L calcium. Experimental results were compared with values generated using the fitted model. To visualize the relationship between independent variables, casein and calcium levels, and the response in terms of iron, zinc, and calcium dialyzability, the fitted polynomial equations were expressed as surface, using STATGRAPHICS plus 3 (Manugistics Inc., Rockville, MD).

RESULTS

The values of iron, zinc, and calcium dialyzability for different combinations of casein and calcium concentration are shown in **Table 3**.

Analysis of variance (ANOVA) for responses is shown in **Table 4**. Of both assessed factors, only the casein content significantly influenced iron and zinc dialyzability, and the first- and second-order terms were found to be significant. Calcium dialyzability related only with calcium content and only first-order term was significant. The high values of the coefficient of determination (r^2) suggest that the model is a good fit.

Table 4. Analysis of Variance for the Overall Effect of the Two Variables on Iron, Zinc, and Calcium Dialyzability (DFe%, DZn%, DCa%)

source	degrees of freedom	*F* ratio DFe%	*F* ratio DZn%	*F* ratio DCa%
A (casein)	1	446.49[a]	303.65[a]	2.04
B (calcium)	1	5.58	0.01	100.89[a]
AA	1	24.27[b]	25.39[b]	0.69
AB	1	0.67	5.45	0.06
BB	1	0.01	0.25	2.16
r^2 [c]		0.979254	0.970567	0.909786

[a] $p < 0.001$. [b] $p < 0.01$. [c] Adjusted for degrees of freedom.

Table 5. Regression Coefficients of the Second-Order Polynomials for Iron, Zinc, and Calcium Dialyzability (DFe%, DZn%, DCa%)

coefficient[a]	DFe%	DZn%	DCa%
b_0	37.0479	57.283	24.6257
b_1	−2.90345[b]	−5.60197[b]	0.451463
b_2	−0.00124355	−0.00547667	−0.00660975[b]
b_3	0.0892367[c]	0.164768[c]	−0.0247575
b_4	3.01486×10^{-4}	0.00154763	-1.52753×10^{-4}
b_5	6.9441×10^{-7}	-7.76906×10^{-6}	2.08372×10^{-5}

[a] b_0 represents the intercept and b_1, b_2, b_3, b_4, and b_5 are the coefficients for casein concentration, calcium concentration, casein concentration quadratic term, casein–calcium concentration interaction, and calcium concentration quadratic term, respectively. [b] $p < 0.001$. [c] $p < 0.01$.

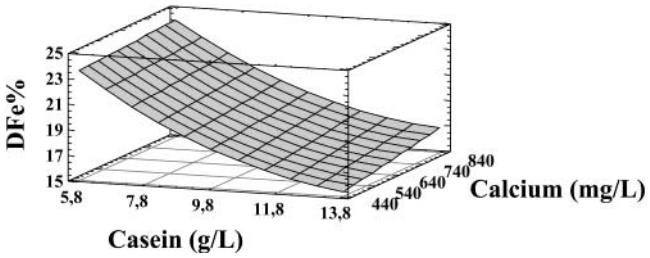

Figure 1. Response surface showing the effect of casein content and Ca concentration on iron dialyzability (DFe%) from experimental infant formulas.

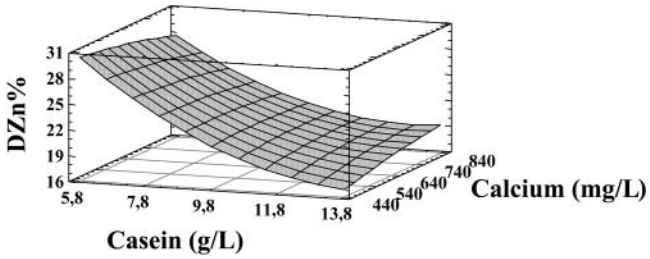

Figure 2. Response surface showing the effect of casein content and Ca concentration on zinc dialyzability (DZn%) from experimental infant formulas.

The regression coefficients for the equation which has been fitted to the data are given in **Table 5**. The response surfaces based on this function for iron, zinc, and calcium dialyzability are shown in **Figures 1**, **2**, and **3**, respectively.

The suitability of the model equations for predicting mineral dialyzability values was tested using an experimental formula prepared with a casein-to-whey protein ratio of 50:50, and 520 mg/L calcium. As shown in **Table 6**, the experimental values obtained were found to be in agreement with the predicted ones,

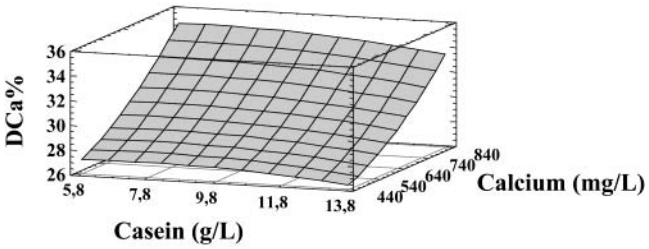

Figure 3. Response surface showing the effect of casein content and Ca concentration on calcium dialyzability (DCa%) from experimental infant formulas.

Table 6. Model Predicted and Experimental Values of Response for Iron, Zinc, and Calcium Dialyzability (DFe%, DZn%, DCa%) for an Experimental Formula Having 7.625 g/L Casein (Casein:Whey Protein 50:50) and 520 mg/L Calcium

responses	predicted value	range	experimental values[a]
DFe%	20.83	20.01–21.66	20.50 ± 0.57
DZn%	25.34	22.96–27.71	23.60 ± 0.53
DCa%	28.22	27.52–28.92	27.72 ± 0.89

[a] Mean ± SD ($n = 4$).

obtained using the second-order polynomial equation and the corresponding regression coefficients.

The percentages of dialyzed free amines mEq, with respect to the total amount of mEq of peptide bonds potentially hydrolyzed, did not present statistically significant differences, and the mean value for all experimental formulas was 13.66 ± 0.99%.

DISCUSSION

Effect of Composition on Iron Dialyzability. *Influence of W:C Ratio.* The values of iron dialyzability (15.36−24.78%) from experimental infant formulas (**Table 3**) were similar to those found previously from commercial fluid UHT-sterilized term starting infant formulas (18.89−22.98%) and determined using the same methodology (*31*). The lowest value for iron dialyzability was found in formulas with the highest casein content, demonstrating the inhibitory effect of casein on iron dialyzability. The inhibitory effect of milk proteins was observed by Hurrell et al. (*32*). They measured dialyzable iron in semisynthetic meals and demonstrated that substituting casein for egg white proteins reduced dialyzable iron by approximately 83%. The inhibitory effect of casein on iron dialyzability was stronger than that of whey proteins. These authors also measured human iron absorption and suggested that the iron-binding properties of the pepsin−pancreatin digestion products of both casein and whey play a major role in the reduction of iron dialyzability and in the inhibition of iron absorption in humans. Kane and Miller (*33*) postulated that the influence of proteins on Fe bioavailability might be related to the properties of undigested or partially digested proteins. Thus, the affinity of these products for iron and the size of the compounds formed could determine both the dialyzability of Fe in vitro and its availability for in vivo absorption.

Influence of Calcium Content. In our study, calcium content did not affect iron dialyzability (**Table 3**). While it is frequently stated that calcium impairs iron absorption, the inconsistent results across human studies suggest that calcium−iron interactions are complex and the mechanisms involved are not fully understood (*34*, *35*). Hallberg et al. (*5*) stated that, although calcium content in infant formulas is often 2 or 3 times higher than that in human milk, a moderate excess of calcium is not considered to have any negative effects on iron bioavailability. Hallberg et al. (*36*) pointed out that a minimal concentration of calcium is needed to achieve an effect; the main inhibition of the iron absorption is obtained with the first 150−200 mg of calcium, and little further inhibition will occur by further increasing the calcium intake. However, in a previous study, we evaluated commercial infant formulas supplemented with different calcium levels (312−1724 mg/L) and sources and found a negative correlation between calcium content and iron dialyzability (*31*). In that study, in addition to different calcium content, different calcium sources used in commercial formulations could have influenced iron dialyzability. Some in vivo studies showed that the calcium source influences iron absorption. Monsen and Cook (*37*) observed a negative effect of a calcium phosphate salt on iron absorption from a semisynthetic meal but found no effect of calcium chloride. Dawson-Hughes et al. (*38*) observed in postmenopausal women that calcium carbonate and hydroxyapatite (calcium monohydroxy-orthophosphate) decreased iron whole-body retention. In a study using the double-radioisotope technique, Cook et al. (*39*) observed that 600 mg of calcium from either calcium carbonate or calcium phosphate inhibited absorption of ferrous sulfate when taken with food. However, the degree of inhibition was not statistically significant when calcium citrate was used.

In our experimental formulas, the source of calcium was calcium citrate. It is possible that this source does not form insoluble polymineral−ligand complexes with fortification iron, and this could explain the lack of an inhibitory effect of the different calcium contents on iron dialyzability at high or low casein levels.

Therefore, calcium citrate might prove to be an adequate source of calcium for infant formulas, since it did not show a negative physicochemical interaction with iron at the evaluated levels. As this and other sources of calcium or salt mixes are used in infant formulas, it would be interesting to study the effect of other sources of calcium on iron dialyzability.

Effect of Composition on Zinc Dialyzability. *Influence of W:C Ratio.* The value of zinc dialyzability (18.0−31.59%) from experimental infant formulas was similar to that found previously from commercial fluid UHT-sterilized term starting infant formulas (23.39−28.56%) and determined using the same methodology (*31*). Using the selected experimental design, we found that zinc dialyzability was adversely affected by casein content; the lowest values were found in formulas with the highest casein-to-whey protein ratio. This is in agreement with results of Zn absorption from human studies. Lönnerdal et al. (*11*) observed that in cow's milk formula, adjusted in its casein-to-whey protein ratio to resemble human milk, zinc absorption was significantly higher than that from nonadjusted cow's milk. In cow's milk infant formulas, casein is the principal fraction that binds zinc, while whey and fat fraction bind minor amounts of this mineral (*7*). Singh et al. (*40*) observed that casein micelles incorporated considerable amounts of zinc added to skim milk as $ZnCl_2$. Binding of a large proportion of Zn to casein may result in the entrapment of Zn in casein curds formed in the stomach, which may be incompletely digested in the small intestine, thus rendering a significant proportion of Zn unavailable for absorption (*41*).

Influence of Calcium Content. In our study, calcium content did not affect zinc dialyzability. While there is not much evidence about calcium−zinc interaction, Wood and Zheng (*42*) observed that high-calcium diets impaired zinc balance in humans, but this may have depended on increased endogenous fecal zinc losses, since true zinc absorption was apparently not affected. On the other hand, Dawson-Hughes et al. (*38*) observed

that calcium carbonate and hydroxyapatite did not have an effect on zinc retention in postmenopausal women.

Effect of Composition on Calcium Dialyzability. *Influence of W:C Ratio.* The values of calcium dialyzability for experimental infant formulas (26.03−34.06%) were higher than those reported for commercial fluid term starting infant formulas (15.17−25.9%), determined using the same methodology (*31*), probably because a soluble calcium complex was utilized in the present study. Calcium dialyzability did not increase with casein content. The possible influence of casein on calcium dialyzability has been reported (*43*). The positive effect on calcium absorption has been ascribed to casein phosphopeptides formed by enzymatic hydrolysis in the intestinal lumen which maintain calcium in a soluble and absorbable form (*44*). However, Rudloff and Lönnerdal (*45*) observed that bovine whey protein, or a hydrolysate thereof, and casein have similar effects on calcium absorption measured in weaning rhesus monkeys.

Influence of Calcium Content. In our study, DCa% increased with calcium concentration. The soluble source of calcium and the calcium levels used in experimental infant formulas may determine a larger amount of free calcium able to dialyze when its content increases, despite the capacity of casein to form complexes. This observation agrees with previous findings reported by Roig et al. (*43*), who found that calcium content is the factor that most influences calcium dialyzability from infant formulas.

Influence of Whey Protein:Casein Ratio on Free Amine Dialyzability. As protein digestibility could be a factor influencing mineral dialyzability results, the percentage of dialyzed free amines (DFA) mEq with respect to the total amount of mEq of peptide bonds potentially hydrolyzed was determined in experimental formulas with different whey protein:casein ratios, as an indirect indicator of digestibility. DFA are terminal free amines from peptides capable of dialyzing or free amino acids produced by enzymatic digestion. These ligands with chelating ability could form low-molecular-weight soluble complexes with minerals influencing their availability. In our study, free amines dialyzed to the same extent in all the experimental formulas tested, regardless of casein content. Jakobsson et al. (*2*) observed that, in term starting infant formulas, hydrolysis of whey proteins occurred at a considerably slower rate than that of casein in an in vitro assay with duodenal juices. Likewise, it was observed in preterm formulas (*46*) and in bovine milk (*47*) that casein is hydrolyzed faster than α-lactalbumin and *β*-lactoglobulin, utilizing the same in vitro technique. However, Miller et al. (*48*) considered that these observations are due to the fact that the hydrolysis was carried out only with duodenal juices. Casein incorporated into milk micelles, or into a curd or precipitate produced by acidic conditions of gastric digestion, would have reduced digestibility. This is likely due to the limited access of proteases to specific bonds when the proteins are complexed or precipitated as in a curd.

On the other hand, infant formulas are iron fortified, and it has been reported that iron impairs casein hydrolysis. This effect could occur through the inhibition of digestive enzymes or the decrease of iron−casein digestibility with respect to native casein (*49*). Naz et al. (*50*) observed that both Fe^{2+} and Fe^{3+} slowed trypsin proteolysis of casein, but zinc did not affect the proteolytic activity. As previously stated, we observed, working with a single iron level and with gastric−intestinal digestion, that experimental formulas did not present significant differences in dialyzed free amines.

As already mentioned, heat processing after mineral addition may induce interactions affecting mineral availability. However, mineral dialyzability values from previously assessed commercial fluid UHT-sterilized infant formulas and from the experimental formulas used in this study were in the same range, suggesting that UHT treatment did not greatly influence mineral availability, as would be the case in conventional in-bottle sterilization (*21*). Besides, many powdered formulas are prepared by dry blending of components, thereby avoiding nutrient interactions.

Our results confirm the inhibitory effect of casein on iron and zinc dialyzability and a lack of effect of casein−Ca interaction on iron, zinc, and calcium dialyzability. No influence of protein composition on calcium dialyzability was found. According to this results, whey-dominant formulas are less prone to hamper mineral availability, and are therefore desirable in order to improve iron and zinc availability.

As new formulas with lower protein content and increased concentrations of α-lactalbumin are being developed (*1*), mineral bioavailability from these formulas will have to be re-evaluated in order to adjust mineral addition. Even though bioavailability would have to be ultimately assessed in human studies, data from in vitro studies, although not quantitatively predictive of iron bioavailability to humans, could provide useful information regarding the influence of changes in composition on potential mineral availability.

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