ELSEVIER

Food Chemistry 77 (2002) 361–369

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

# Iron and zinc in hydrolised fractions of human milk and infant formulas using an in vitro method

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Received 1 May 2001; received in revised form 17 December 2001; accepted 17 December 2001

**Abstract**

The development of an in vitro method to simulate new-born digestion and to study iron and zinc bioavailability from human milk and cow's milk based infant formulas was carried out. Enzyme treatment was conducted in two stages involving (1) pepsin at pH 5.0 followed by (2) pancreatin at neutral pH, where the incubation times were kept short to mimic the fast transit in the infant's gastrointestinal tract. Solubility of trace elements was used to express bioavailability, and so analytes were determined in the fractions obtained after centrifugation by flame atomic absorption spectrometry (FAAS) using a high performance nebulizer. The results were compared to those obtained by performing gastric digestion at pH 2.0 for an adult, using various incubators to treat the sample and centrifugation or ultracentrifugation to separate soluble fractions. No differences in iron bioavailability from breast milk and infant formulas at different pHs could be detected due to the variability of the infant formulas analysed. However, zinc bioavailability from breast milk samples was higher than those obtained from infant formulas at the new born gastric pH. 

*Keywords:* Bioavailability; Iron; Zinc; Milk; In vitro digestion; FAAS

## 1. Introduction

Bioavailability is an important factor in the nutrition field because of its variations with different foods, foods components and gastrointestinal conditions. Bioavailability represents the integration of the various processes whereby an ingested nutrient becomes available: digestion, absorption, transport, utilisation, elimination (Favier, 1993). The major source of nutrition for the new-born infant is mother's milk, whereas formula-feeding is important in the circumstances in which breast-feeding is not possible. Human breast milk provides all the trace elements that are required by the new-born infant. Owing to the different concentrations of these nutrients, and other substances that modify their absorption, there is a great interest in the significance of micronutrient supplies, within the quest for improvement of formulations (George & De Francesca, 1989).

Among the trace elements contained in milk, iron has received the most attention. Besides anaemia, iron plays an important role in a number of physiological functions, including psychomotor development and behaviour in children and modifying immune functions (Whitehead, 1993). Zn is an essential element playing a wide variety of biochemical roles in vivo and its deficiency or excess could affect children's development (Bates & Prentice, 1994).

In vivo methods include those using stable isotopes in humans (Abrams, Wen, & Stuff, 1996; Davidsson et al., 1994; Fairweather-Tait, Fox, Wharf, & Eagles, 1995; Fomon, Serfass, Nelson, Rogers, & Frantz, 2000a, 2000b; Ziegler et al., 1989), give the best estimation of bioavailability and are the preferred methods to measure absorption because using radioisotopes is considered unethical in infant studies. In vitro methods have been employed to investigate various aspects of

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0308-8146/02/$ - see front matter 
PII: S0308-8146(02)00111-5

trace metal nutrition. In particular, these methods have been used to predict the availability from different foods including milk or infant formulas (Barberá, Farré, García, Guillén, & Lagarda, 1993; Cabrera, Lorenzo, De Mena, & Lopez, 1996; Crews, Burrell, & McWeeny, 1985a, 1985b; Diepenmaat-Wolters & Shreuder, 1993; García, Alegría, Barberá, Farré, & Lagarda, 1998; Glahn, Lai, Hsu, Thompson, Guo, & Van Campen, 1998; Jovaní, Le Masle, Alegría, Barberá, Lagarda, & Clemente, 2000; Miller, Schricker, Rasmussen, & Van Campen, 1981; Minihane, Fox, & Fairweather-Tait, 1993; Roig, Alegría, Barberá, Farré, R. & Lagarda, 1999; Shen, Luten, Robberecht, Bindels, & Deelstra, 1994; Shen, Robberecht, Bindels, & Deelstra, 1995; Shen, Van Dael, & Deelstra, 1993) as a simple, rapid, inexpensive and easy-to-control alternative to human and in vivo studies. The in vitro digestion cannot accurately reflect the complexity of natural systems but information from these experiments regarding the effects of enzymes and pH may be applicable to the in vivo situation: they permit a reasonable estimate of trace element availability. It has been developed an in vitro method simulating digestion in the new-born infant (Lönnerdal, Yuen, Glazier, & Litov, 1993; Rudloff & Lönnerdal, 1992). Several of the most important phenomena observed in vivo: inmaturity of the digestive mechanisms of the adult regarding enzymes and bile salt secretion, high postprandial pH of the stomach (5.0–6.0) and fast transit in the infant's gastrointestinal tract have to be considered (Armand et al., 1996; Chevallier, 1997; Hamosh, 1996).

A modified version of the in vitro method proposed by Lönnerdal et al. (1993) to estimate the bioavailability of Fe and Zn from human milk and infant formulas has been applied. Milk samples were subjected to a two-stage digestion procedure trying to simulate human gastric and intestinal digestive processes. Soluble fractions were separated by centrifugation and iron and zinc were determined by flame atomic absorption spectrometry (FAAS) using a high performance nebulizer Perkin Elmer (Bermejo, Domínguez, & Bermejo, 1997).

## 2. Materials and methods

### 2.1. Equipment

Gastric and intestinal digestions were carried out on a Rotabit orbital-rocking platform shaker, J.P. SELECTA S.A. (Spain) in an incubator, Boxcult, J.P. SELECTA S.A. (Spain). A SIGMA GmbH Model 2K15 laboratory centrifuge with a rotor model 12141, Osterode (Germany) and a pH-meter Crison 500, Crison Instruments, S.A. equipped with an electrode Hamilton LIQ-PLAST (Spain) were used for in vitro digestion.

The in vitro method using the incubator Boxcult for the enzymolisis procedure was compared with the one using the Stomacher® Lab Blender, Model 400, Seward Medical Limited (United Kingdom). Seward Stomacher® Lab Blender Bags were used to process the samples.

An ultracentrifuge L8-Beckmann with a rotor SW-40 was used to obtain milk whey and to compare the separate soluble fractions obtained by ultracentrifugation and centrifugation.

A Perkin-Elmer 5500 atomic absorption spectrophotometer equipped with a deuterium lamp as a background correction system was used for iron and zinc measurements. Hollow cathode lamps operated at 30 and 15 mA were used for iron and zinc, respectively, providing resonance lines of 248.3 and 213.9 nm. The spectral bandwidth was 0.2 nm for iron and 0.7 nm for zinc. An air acetylene flame was used and the Perkin Elmer nebulizer (Germany) was a high performance nebulizer with a tantalum capillary and a ceramic impact bead.

### 2.2. Reagents

Digestive enzymes (porcine pepsin, P-7000, EC 3.4.23.1 (1:10000); porcine pancreatin, P-1750) and sodium bicarbonate ACS reagent, S-6014, were purchased from Sigma Chemicals Co. (USA). Hydrogen chloride acid (HCl) 37% as well as iron and zinc stock standard solutions (1000 g/l) were obtained from Merck, Darmstadt (Germany). All solutions were prepared using ultrapure water of 18 MΩcm specific resistivity obtained from a Milli-Q purification system, Millipore Corp., Massachussetts (USA).

All vessels were kept in 10% nitric acid for at least 48 h and washed three times with ultrapure water and preserved dried for their use.

### 2.3. Samples

Transitional breast milk samples were obtained voluntarily from women living in Galicia, North West of Spain, through the co-operation of the Hospital Clínico Universitario of Santiago de Compostela. Ten breast milk samples, eight breast milk samples from a mother obtained in different days of lactation and two pools of breast milk, were studied. They were collected in polyethylene containers by hoc trained personnel using a motorised pump. Care was paid to avoid touching the inner wall of the device or flask. The flasks were indelibly marked with an appropriate code number and samples were stored at −20 °C until treatments were performed. The 12 cow's milk-based formulas analysed are commercially available and adapted for the normal full-term newborn infants during the first 6 months of life. Infant formulas solutions were prepared by dissolving milk powder using ultrapure water, according to the manufacturer instructions. The ratio powder:water (w/v) used for the reconstitution of the sample were: 13.7% (sample 1), 13.0% (sample 2),

14.4% (sample 3), 14.3% (sample 4), 14.7% (sample 5), 13.7% (sample 6), 13.0% (sample 7), 12.7% (sample 8), 15.0% (sample 9), 14.0% (sample 10), 14.3% (sample 11), 13.0% (sample 12).

An ultracentrifuge L8-Beckmann with a rotor SW-40 was used to obtain milk whey, the run conditions were 31,000 rpm (160,000 ***g***) at 4 °C. The sample was taken out with a micropipette after fat separation (Bermejo et al., 1997).

### 2.4. In vitro gastrointestinal digestion method

This method is based on the one proposed by Lönnerdal et al. (1993), with modifications that have been made to provide optimum conditions. The samples have been subjected to a two stage pepsin (gastric) and pancreatin (intestinal) enzymolisis. The aim was to simulate digestion in the new-born infant. Furthermore pH 2.0 (corresponding to the adult) and 5.0 (newborn) were studied in the gastric step and the obtained results were compared.

Thirty millilitres of milk for infant formulas (20 ml for human milk due to the shortage of the sample, using proportion of the reactives) were used at least in triplicate. To control the possible contamination problems blanks of the reagents used in the gastric and intestinal digestion were also performed in triplicates. In the first step, the samples were pipetted to erlenmeyer flasks, pH was adjusted to 2.0 or 5.0 using HCl 2 and 5 M and 1.0 ml of 0.2 mg pepsin/ml was added (dissolved in HCl 0.1 M). Samples were incubated in the Boxcult at 37 °C during 50 min using 100 rev/min as stirring speed. The digests were placed in an ice-water bath to cool and to stop the digestion procedure. Aliquots portions of the digests (15 ml) were neutralized to pH 7.0 with $NaHCO_3$ 1.5 M and 1.0 ml of 0.15 mg pancreatin/ml (dissolved in $NaHCO_3$ 0.1 M) was added. Similarly, the samples were incubated for 30 min and then placed in an ice-water bath to cool. Finally, all the digests from all the experiments were centrifuged at 15,300 rpm (19,890 ***g***) for 30 min at 4 °C, to obtain soluble fractions.

Table 1
Effect of pH and the gastric digestion time in the amount of solubilised metal

| Time (min) | Fe (μg/ml) | | Zn (μg/ml) | |
|---|---|---|---|---|
| | Gastric | Intestinal | Gastric | Intestinal |
| *Gastric pH = 2* | | | | |
| 30 | 3.00±0.36 | 1.71±0.08 | 2.78±0.02 | 1.02±0.15 |
| 50 | 3.84±0.40 | 1.62±0.13 | 2.76±0.09 | 0.75±0.01 |
| 120 | 4.83±0.29 | 2.51±0.08 | 2.79±0.13 | 1.11±0.03 |
| 180 | 3.96±0.07 | 2.56±0.01 | 2.65±0.02 | 1.21±0.01 |
| *Gastric pH = 5* | | | | |
| 30 | 1.79±0.62 | 1.95±0.49 | 1.75±0.06 | 0.88±0.04 |
| 50 | 1.47±0.12 | 1.78±0.03 | 1.74±0.04 | 0.91±0.06 |
| 120 | 1.64±0.12 | 1.99±0.01 | 1.73±0.01 | 0.92±0.01 |
| 180 | 1.84±0.27 | 2.03±0.11 | 1.90±0.01 | 1.11±0.01 |

### 2.5. Determination of Fe and Zn by FAAS

Iron and zinc contents in milk, milk whey and soluble fractions were determined without pre-treatment of the samples by flame atomic absorption spectrometry (FAAS) with a high performance nebulizer (Bermejo et al., 1997). The most important advantage of the use of this nebuliser is the bigger sensitivity obtained without adding surfactant agents. Determinations were carried out by the addition procedure with a different dilution for each sample.

## 3. Results and discussion

### 3.1. Optimisation of the gastric digestion time

Obviously the digestive tract and the food transit in the new-born infant are different from the adult ones. To study the gastric digestion time, four different time periods were considered, 30 min, 50 min, 2 h and 3 h after the enzymolisis of an infant formula sample, element determination in the soluble fractions was carried out. The results are shown in Table 1. Two aliquots of sample were digested and determinations were performed in duplicate to obtain each result, which was calculated from four values of absorbance.

After statistical comparison of the standard deviations by applying Cochran's, Bartlett's and Hartley's tests (Miller & Miller, 1993), a multiple range test using the Student–Newman–Keuls method was performed to compare the means. That was because of ANOVA test must only be applied if the standard deviations are statistically similar (all standard deviations were found statistically different). After this, most of the means were statistically different and 50 min was selected as the digestion time because it is approximately the staying time in the stomach for human milk and milk formulas (Chevallier, 1997).

### 3.2. Comparison between ultracentrifugation and centrifugation to obtain soluble fractions

Element determination in the soluble fractions of an infant formula and human milk was performed after centrifugation (the Sigma 2K15 operating at 19,890 ***g***, 31,000 rpm, during 30, 60 or 120 min, at 4 °C, using 10 ml as sample volume) or ultracentrifugation (the L8-M Beckman ultracentrifuge operating at 160,000 ***g***, 31,000 rpm, during 10 min, at 4 °C, using 12 ml as sample volume).

The results showed that there was a slight difference between the different time periods (Table 2). Three aliquots of the sample were digested, and determinations

Table 2
Comparison between ultracentrifugation and centrifugation to obtain soluble fractions

| | Ultracentrifugation | Cent. 30 min | Cent. 60 min | Cent. 120 min |
|---|---|---|---|---|
| *(1) From a breast milk sample [Fe] (μg/ml)* | | | | |
| Gastric digest pH 2 | 0.09±0.01 | 0.09±0.01 | 0.09±0.01 | 0.09±0.01 |
| Gastrointest.dig. pH2 | 0.08±0.01 | 0.08±0.01 | 0.08±0.01 | 0.09±0.01 |
| Gastric digest pH 5 | 0.16±0.01 | 0.15±0.01 | 0.14±0.01 | 0.16±0.01 |
| Gastrointest.dig. pH5 | 0.17±0.01 | 0.18±0.01 | 0.16±0.01 | 0.17±0.01 |
| *(2) From a breast milk sample [Zn] (μg/ml)* | | | | |
| Gastric digest pH 2 | 0.37±0.03 | 0.40±0.06 | 0.46±0.03 | 0.42±0.01 |
| Gastrointest.dig. pH2 | 0.38±0.01 | 0.28±0.03 | 0.25±0.03 | 0.23±0.02 |
| Gastric digest pH 5 | 0.64±0.06 | 0.63±0.06 | 0.61±0.02 | 0.65±0.03 |
| Gastrointest.dig. pH5 | 0.35±0.01 | 0.37±0.05 | 0.35±0.02 | 0.32±0.03 |
| *(3) From an infant formula [Fe] (μg/ml)* | | | | |
| Gastric digest pH 2 | 4.68±0.32 | 4.42±0.25 | 4.40±0.10 | 5.76±0.22 |
| Gastrointest.dig. pH2 | 3.03±0.10 | 4.20±0.12 | 3.94±0.41 | 2.90±0.33 |
| Gastric digest pH 5 | 1.83±0.24 | 1.31±0.15 | 1.29±0.09 | 1.23±0.09 |
| Gastrointest.dig. pH5 | 2.28±0.08 | 1.98±0.15 | 1.84±0.14 | 1.98±0.10 |
| *(4) From an infant formula [Zn] (μg/ml)* | | | | |
| Gastric digest pH 2 | 2.33±0.05 | 2.09±0.08 | 1.78±0.16 | 2.63±0.02 |
| Gastrointest.dig. pH2 | 1.21±0.07 | 1.01±0.03 | 0.83±0.11 | 0.80±0.09 |
| Gastric digest pH 5 | 1.35±0.12 | 1.64±0.18 | 1.60±0.01 | 1.61±0.15 |
| Gastrointest.dig. pH5 | 0.47±0.09 | 0.43±0.09 | 0.34±0.04 | 0.36±0.02 |

were performed in duplicate to obtain each result, which was calculated from six values of absorbance.

As above the standard deviations were statistically compared, and ANOVA and multiple range tests were applied to compare the means (Miller & Miller, 1993). For the iron determination in hydrolysed fractions of human milk at gastric pH 2, there were not found statistical differences for all ultracentrifugation and centrifugation times. For all other iron and zinc determination in hydrolysed fractions of human milk and infant formula at any gastric conditions, the differences were statistically significant for all ultracentrifugation and centrifugation times. Therefore, 30 min as centrifugation time has been chosen as compromise time in order to carry out the overall process (gastric and intestinal digestion at two pH tested) for both human milk and infant formula and for iron and zinc determinations.

### 3.3. Comparison between Stomacher and Incubation Camera Boxcult

The in vitro method using the Boxcult incubation camera for the enzymolisis procedure was compared with the one using the Stomacher® Lab Blender, Model 400, Seward Medical Limited. The Stomacher is an instrument for homogenizing with two paddles acting on a plastic bag with the sample inside making a similar manner to the action of the stomach.

Iron and zinc were determined in the soluble fractions of an infant formula after in vitro digestion using the Stomacher and the Boxcult incubation camera. The obtained results are presented in Table 3. Three aliquots of the sample were digested, and determinations were performed in duplicate to obtain each result, which was calculated from six values of absorbance.

Results indicated that no significant difference between the two systems could be shown, even if some values differed slightly from each other (Miller & Miller,

Table 3
Comparison between Stomacher and Boxcult at different gastric pHs

| | Fe ( μg /ml) | | Zn (μg/ml) | |
|---|---|---|---|---|
| | Boxcult | Stomacher | Boxcult | Stomacher |
| 2G | 6.84±0.37 | 8.79±0.13 | 4.16±0.43 | 5.24±0.43 |
| 2I | 5.99±0.06 | 5.07±0.09 | 2.40±0.15 | 1.58±0.10 |
| 5G | 1.35±0.04 | 1.43±0.12 | 3.70±0.01 | 4.12±0.26 |
| 5I | 6.18±0.15 | 4.65±0.17 | 2.41±0.22 | 0.84±0.08 |

Table 4
Calibration curves

| Gastric pH | | Fe (μg/ml) | Zn (μg/ml) |
|---|---|---|---|
| *Breast milk: addition curves* | | | |
| 2 | Gastric | $A = 0.102[Fe] + 0.026$ | $A = 0.972[Zn] + 0.028$ |
| | Intestinal | $A = 0.099[Fe] + 0.017$ | $A = 0.941[Zn] + 0.019$ |
| 5 | Gastric | $A = 0.112[Fe] + 0.014$ | $A = 0.980[Zn] + 0.028$ |
| | Intestinal | $A = 0.109[Fe] + 0.016$ | $A = 0.942[Zn] + 0.015$ |
| *Infant formulas: addition curves* | | | |
| 2 | Gastric | $A = 0.092[Fe] + 0.052$ | $A = 0.661[Zn] + 0.130$ |
| | Intestinal | $A = 0.103[Fe] + 0.023$ | $A = 0.670[Zn] + 0.017$ |
| 5 | Gastric | $A = 0.095[Fe] + 0.021$ | $A = 0.650[Zn] + 0.103$ |
| | Intestinal | $A = 0.103[Fe] + 0.023$ | $A = 0.679[Zn] + 0.011$ |

Table 5
Precision of the in vitro method ($n=5$)

| | Gastric pH | | Breast milk | | | Infant formulas | | |
|---|---|---|---|---|---|---|---|---|
| | | | $X$ | SD | RSD(%) | $X$ | SD | RSD(%) |
| Fe (μg/ml) | 2 | Gastric | 0.16 | 0.01 | 6.3 | 7.71 | 0.34 | 4.4 |
| | | Intestinal | 0.15 | 0.01 | 6.7 | 7.45 | 0.25 | 3.4 |
| | 5 | Gastric | 0.12 | 0.01 | 8.3 | 0.90 | 0.07 | 7.8 |
| | | Intestinal | 0.14 | 0.01 | 7.1 | 7.90 | 0.22 | 2.8 |
| Zn (μg/ml) | 2 | Gastric | 4.31 | 0.09 | 2.1 | 4.90 | 0.12 | 2.4 |
| | | Intestinal | 2.93 | 0.16 | 5.5 | 3.05 | 0.11 | 3.6 |
| | 5 | Gastric | 4.49 | 0.09 | 2.0 | 3.97 | 0.11 | 2.8 |
| | | Intestinal | 2.80 | 0.04 | 1.4 | 3.14 | 0.13 | 4.1 |

Table 6
Interday precision of the in vitro method ($n=2$)

| | Fe ( μg/ml) | | | Zn (μg/ml) | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | RSD (%) | 1 | 2 | RSD (%) |
| 2G | 4.54±0.90 | 5.20±0.29 | 9.6 | 1.07±0.03 | 1.12±0.03 | 3.2 |
| 2I | 1.65±0.16 | 2.11±0.07 | 17.3 | 0.91±0.04 | 1.19±0.16 | 18.9 |
| 5G | 1.78±0.06 | 1.98±0.04 | 7.5 | 1.26±0.04 | 1.30±0.04 | 2.2 |
| 5I | 1.40±0.08 | 1.49±0.12 | 6.8 | 0.68±0.03 | 0.77±0.03 | 8.8 |

1993). The digestion in the incubation camera using erlenmeyer flasks was the preferred method because it makes the control of the temperature possible throughout the process and the contamination risk seems to be less than with bags. Bags might break during digestion in the Stomacher and the method is time-consuming because of the difficulties to handle the bags and to measure pH.

### 3.4. Element determination in soluble fractions

#### 3.4.1. Calibration curves

Standard addition graphs (Table 4) for both elements and the different soluble fractions (gastric and intestinal) were compared by means of the $t$-test and statistically obtaining no significant difference. A is the absorbance (peak area) and Fe and Zn are the concentrations in μg/ml. Standard additions using any of the different soluble fractions of the milk sample were used for all the measurements.

#### 3.4.2. Precision of the in vitro method

The within precision of the method was tested by determining iron and zinc in the soluble fractions after enzymolisis of five replicates of an infant formula milk sample and a breast milk sample. Results are shown in Table 5. The values of relative standard deviations are considered acceptable because they include the errors throughout the whole procedure: in vitro digestion, centrifugation and element determination.

A sample was analysed two different days as an aproximation to evaluate the interday precision of the method. Results are reported in Table 6. RSDs obtained were smaller than 20% in all cases.

#### 3.4.3. Sensitivity

The detectability was studied for both metals and expressed through the limit of detection (LOD) and the limit of quantification (LOQ) (Table 7). LOD and LOQ are defined as 3 and 10 SD/$m$, respectively, where SD is the standard deviation of 10 replicate measurements of a blank (mean absorbance value $A$) and $m$ is the slope of the addition graph. On the other hand, sensitivity of a method is defined in FAAS as the concentration of analyte that produces an absorbance signal of 0.0044

Table 7
Sensitivity

| | $A_{blank}$ | SD | $m$ | LOD | LOQ | Sensitivity |
|---|---|---|---|---|---|---|
| *Breast milk* | | | | | | |
| Fe (μg/ml) | 0.002 | $6.8\ 10^{-4}$ | 0.078 | 0.026 | 0.086 | 0.06 |
| Zn (μg/ml) | 0.004 | $6.8\ 10^{-4}$ | 0.764 | 0.003 | 0.009 | 0.01 |
| *Infant formula* | | | | | | |
| Fe (μg/ml) | 0.002 | $8.4\ 10^{-4}$ | 0.103 | 0.024 | 0.081 | 0.04 |
| Zn (μg/ml) | 0.004 | $6.8\ 10^{-4}$ | 0.661 | 0.003 | 0.010 | 0.06 |

(Table 7). In all cases, the sensitivity obtained was adequate.

### 3.4.4. Applications

The in vitro method was applied to 12 infant formula milk samples, eight transitional breast milk samples (2–5, 7–10) from a mother obtained in different days of lactation and three pools of breast milks samples (1, 6). The cow's milk-based formulas analysed are commercially available and adapted for normal full-term newborn infants approximately during the 6 first months of life. Due to the limited amounts of breast milk sample avaliable to perform the study, the two different pH values were applied to different human milk samples: pH 2.0 (samples 1–5) and pH 5.0 (samples 6–10).

In order to calculate the percentage of an element that is transformed into absorbable forms in the digestive tract, the results of the studies can be expressed as the soluble fraction of the element under experimental conditions (Tables 8 and 9). Figs. 1 and 2 show total and whey metal content in the samples. Moreover, iron and zinc in the gastric and gastrointestinal soluble fractions are shown. All values were corrected by subtracting the blanks.

pH is an important factor in digestion, especially for infants, because the lower concentration of acid precludes a meaningful contribution of pepsin to protein digestion. Element concentration values in the gastric digestion stage at pH 2 are similar to the same as the total metal concentration in infant formulas samples. In the case of breast milk samples Fe concentration of all digestion stages are approximately the same as the whey milk concentration. Solubility of elements was found to increase in the gastric digestions whereas in the gastrointestinal ones (corresponding to the intestinal tract), where the absorption should be greater they were seen to decrease. The reduction in solubility of analytes was more notable for zinc whilst the effect on iron was notably less, being only observed when digesting infant formula samples at pH 2 (adults). The different protein composition of both milk types (George & De Francesca, 1989; Rudloff & Kunz, 1997) can produce differences in

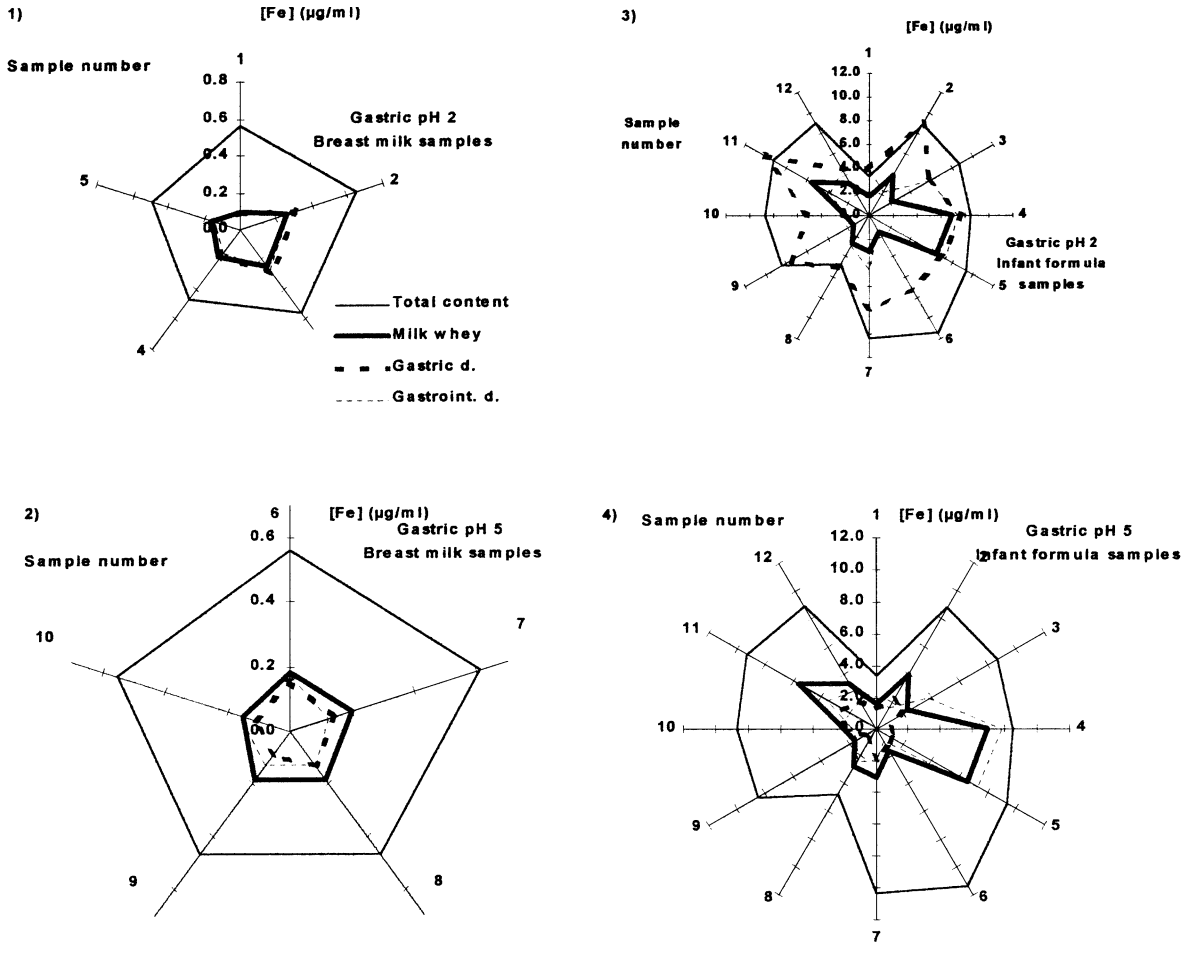


Fig. 1. Fe concentration (µg/ml) in milk, milk whey, gastric digests and gastrointestinal digests of: (1) breast milk, gastric pH 2 (solubilibity in the gastrointestinal digest 33.3±13.0%), (2) breast milk, pH 5 ( 26.9±4.2%), (3) infant formulas, pH 2 (51.7±29.5%), (4) infant formulas, pH 5 (39.1±25.3%).

the metals availability. The higher zinc availability at pH 2.0 has been explained by a lower Zn-binding capacity of casein at the lower pH of adult (Shen et al., 1993). Zn in cow's milk is highly bound to casein micelles, 1/3 of the zinc is loosely bound to casein phosfoserine residues whereas two-thirds are more tightly bound to colloidal calcium phosphate (Shen et al., 1994). Thus, the zinc solubilised by phosphate or some other anion during gastric digestion may precipitate after intestinal digestion due to the neutral pH of intestinal digestion (Cabrera et al., 1996).

Finally, considering the variabilility between the samples studied, especially regarding to the infant formulas, it cannot be stated that there are statistically significant differences between iron bioavailability (expressed as solubility in the gastrointestinal digests) from breast milk (pH 2.0 33.3±13.0%, pH 5.0 26.9±4.2%) and infant formulas (pH 2.0 51.7±29.5%, pH 5.0 39.1±25.3%) at the different pHs. However, zinc bioavailability from breast milk samples is higher (pH 5.0 50.9±15.7%) than the values obtained from infant formulas (pH 5.0 20.2±15.6%) working at the newborn

Table 8
Solubility in the gastrointestinal digest of breast milk samples (%)

| Sample number | Gastric pH 2 | | Sample number | Gastric pH 5 | |
|---|---|---|---|---|---|
| | Fe (%) | Zn (%) | | Fe (%) | Zn (%) |
| 1 | 14.3±1.8 | 47.5±5.1 | 6 | 30.4±1.8 | 49.3±6.7 |
| 2 | 41.5±1.5 | 44.6±4.0 | 7 | 19.7±1.6 | 39.1±4.3 |
| 3 | 48.2±5.4 | 40.3±6.0 | 8 | 27.7±2.1 | 78.1±4.7 |
| 4 | 34.0±2.1 | 37.7±1.6 | 9 | 27.7±2.1 | 44.1±3.4 |
| 5 | 28.6±2.0 | 56.0±2.2 | 10 | 29.1±1.8 | 43.7±3.4 |
| *X* | 33.3 | 45.2 | | 26.9 | 50.9 |
| SD | 13.0 | 7.1 | | 4.2 | 15.7 |

gastric pH ($t_{calculated} = 3.6 > t_c = 2.1$, $P = 0.05$). As iron and zinc concentration are smaller in breast milk samples than in infant formula samples, this fact has to be considered too, to be able to evaluate total metal content given to the new-born infant. Thus, some authors have considered the possibility of lowering the level of iron fortification in these products (Dalton, Sargent, O'Connor, Olmstead, & Klein, 1997; Hemminki,

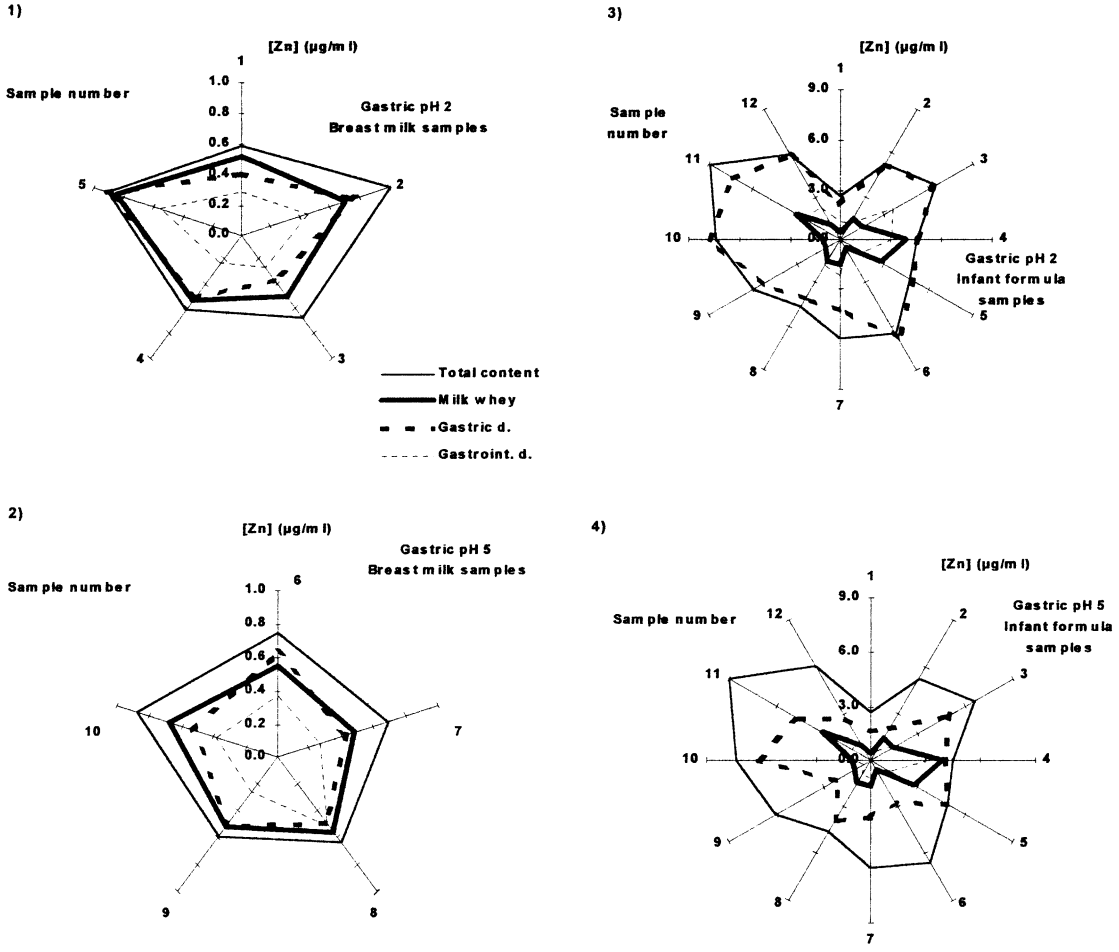


Fig. 2. Zn concentration (µg/ml) in milk, milk whey, gastric digests and gastrointestinal digests of: (1) breast milk, gastric pH 2 (solubility in the gastrointestinal digest 45.2±7.1%), (2) breast milk, pH 5 (50.9±15.7%), (3) infant formulas, pH 2 (33.4±16.4%), (4) infant formulas, pH 5 (20.2±15.6%).

Table 9
Solubility in the gastrointestinal digest of infant formula samples (%)

| Sample number | Gastric pH 2 | | Gastric pH 5 | |
|---|---|---|---|---|
| | Fe (%) | Zn (%) | Fe (%) | Zn (%) |
| 1 | 113.2±3.4 | 38.8±1.2 | 60.7±4.6 | 16.5±3.5 |
| 2 | 27.6±1.8 | 24.0±1.4 | 17.5±2.0 | 12.6±1.4 |
| 3 | 67.0±1.1 | 55.1±4.8 | 43.9±3.0 | 23.5±2.1 |
| 4 | 87.0±2.9 | 67.0±2.4 | 91.4±3.9 | 68.8±2.9 |
| 5 | 77.4±2.3 | 30.4±0.8 | 77.2±1.6 | 25.3±0.4 |
| 6 | 12.0±2.5 | 8.6±0.5 | 8.1±1.0 | 7.1±0.5 |
| 7 | 44.7±1.6 | 36.7±3.3 | 19.1±0.7 | 16.4±0.8 |
| 8 | 63.0±0.9 | 38.4±0.7 | 48.9±2.4 | 18.6±1.8 |
| 9 | 15.6±0.4 | 15.6±1.0 | 16.2±1.7 | 8.5±0.3 |
| 10 | 24.1±0.8 | 19.2±0.5 | 17.0±1.4 | 12.2±1.1 |
| 11 | 41.0±3.4 | 28.5±1.3 | 37.9±2.9 | 13.4±1.2 |
| 12 | 48.0±2.5 | 38.8±2.3 | 30.9±1.1 | 19.4±1.2 |
| *X* | 51.7 | 33.4 | 39.1 | 20.2 |
| SD | 29.5 | 16.4 | 25.3 | 15.6 |

Nemet, Horvath, Malin, Schuler, & Hollan, 1995; Hertrampf et al., 1998).

In order to improve infant formulas composition, it would be necessary to develop and to perfect digestion methods using infant gastrointestinal conditions, because these methods are scarcely avaliable in literature. The variation between the different formula milks and the interaction with dietary components (Favier, 1993; Hallberg & Rossander-Hultén, 1993; Jackson & Lee, 1992; Whitehead, 1993), technologies to produce infant formulas and the extent of the heat treatments affecting to lipid–protein and protein–protein interactions at the different pHs will have to be considered.

## 4. Conclusions

Breast milk samples and cow's milk based formulas were subjected to a two stage digestion procedure developed to mimic new-born gastric and intestinal digestive processes. Digestions were performed using an Incubation Camera Boxcult, 50 min was selected as an adequate gastric digestion time and centrifugation during 50 min at 19,890 ***g*** was used to obtain soluble fractions.

The solubility of iron and zinc was measured directly by diluting the sample on completion of treatments by FAAS using a high performance nebulizer. The results were compared to those obtained by performing gastric digestion at pH 2.0 for the adult. Election of gastric pH had an important effect due to the different results obtained for human and infant formula milk samples. Due to the variability between the infant formula samples studied, it couldn't be stated significant global differences between iron bioavailability from breast milk and infant formulas at the different pHs. However, zinc bioavailability from breast milk samples was higher than the values obtained from infant formulas working at the newborn gastric pH.

## Acknowledgements

We thank the collaboration of Jani Madetoja (Erasmus student from Helsinki University, Finland) in this work. The support of the research projects 1997, CEO12 Laboratorios Ordesa (Barcelona, Spain), and R-PETRI PTR-1995/0384-OP, is gratefully acknowledged.

## References

Abrams, S. A., Wen, J., & Stuff, J. E. (1996). Absorption of calcium, zinc, and iron from breast milk by five-to seven-month-old infants. *Pediatric Research, 39 (3)*, 384–390.

Armand, M., Hamosh, M., Mehta, N. R., Angelus, A. A., Philpott, J. R., Henderson, T. R., Dwyer, N. K., Lairon, D., & Hamosh, P. (1996). Effect of human milk or formula on gastric function and fat digestion in the premature infant. *Pediatric Research*, *40*, 429–437.

Barberá, R., Farré, R., García, A., Guillén, A., & Lagarda, M. J. (1993). Estimate of bioavailability of iron, copper and zinc in infant formula—"studies in vitro". In U. Schlemmer (Ed.), *Bioavailability '93; nutritional, chemical and food processing implications of nutrient availability. Proceedings, Part 1: Ettlingen* (pp. 243–247). Karlsruhe: Bundesfoschunganstalt für Ernáhrung.

Bates, J. B., & Prentice, A. (1994). Breast milk as a source of vitamins, essential minerals and trace elements. *Pharmacology and Therapeutics, 62 (1–2)*, 193–220.

Bermejo, P., Domínguez, R., & Bermejo, A. (1997). Direct determination of Fe and Zn in different components of cow milk by FAAS with a high performance nebulizer. *Talanta*, *45*, 325–330.

Cabrera, C., Lorenzo, M. L., De Mena, C., & Lopez, M. C. (1996). Chromium, copper, iron, manganese, selenium and zinc levels in dairy products: in vitro study of absorbable fractions. *International Journal of Food Sciences and Nutrition*, *47*, 331–339.

Chevallier, B. (1997). *Nutrición Infantil*. Barcelona: Ed. Masson D. L.

Crews, H. M., Burrell, J. A., & Mc Weeny, D. J. (1985a). Trace element solubility from food following enzymolisis. *Zeitschrift für Lebensmitteluntersuchung und Forschung*, *180*, 221–226.

Crews, H. M., Burrell, J. A., & Mc Weeny, D. J. (1985b). Comparison of trace element solubility from food items treated separately and in combination. *Zeitschrift für Lebensmitteluntersuchung und Forschung*, *180*, 405–410.

Dalton, M. A., Sargent, J. D., O'Connor, G. T., Olmstead, E. M., & Klein, R. Z. (1997). Calcium and phosphorus supplementation of iron-fortified infant formula: no effect on iron status of healthy full-term infants. *American Journal of Clinical Nutrition*, *65*, 921–926.

Davidsson, L., Galán, P., Kastenmayer, P., Cherouvrier, F., Juillerat, M. A., Hercberg, S., & Hurrell, R. F. (1994). Iron bioavailability studied in infants: the influence of phytic acid and ascorbic acid in infant formulas based on soy isolate. *Pediatric Research*, *36*, 816–822.

Diepenmaat-Wolters, M. G. E., & Schreuder, H. A. W. (1993). A continuous in vitro method for estimation of the bioavailability of minerals and trace elements in food. In U. Schlemmer (Ed.), *Bioavailability '93; nutritional, chemical and food processing implications of nutrient availability. Proceedings, Part 2: Ettlingen* (pp. 43–47). Karlsruhe: Bundesfoschunganstalt für Ernährung.

Fairweather-Tait, S., Fox, T., Wharf, S. G., & Eagles, J. (1995). The bioavailability of iron in different weaning foods and the enhancing effect of a fruit drink containing ascorbic acid. *Pediatric Research*, *37*(4), 389–394.

Favier, A. E. (1993). Nutritional and clinical factors affecting the bioavailability of trace elements in humans. In U. Schlemmer (Ed.), *Bioavailability '93; nutritional, chemical and food processing implications of nutrient availability. Proceedings, Part 1: Ettlingen* (pp. 202–212). Karlsruhe: Bundesfoschunganstalt für Ernährung.

Fomon, S. J., Serfass, R. E., Nelson, S. E., Rogers, R. R., & Frantz, J. A. (2000a). Less than 80% of absorbed iron is promptly incorporated into erythrocytes of infants. *The Journal of Nutrition, 130 (1)*, 45–52.

Fomon, S. J., Serfass, R. E., Nelson, S. E., Rogers, R. R., & Frantz, J. A. (2000b). Time course and time effect of dietary iron level on iron incorporation into erytrocytes by infants. *The Journal of Nutrition*, *130*(3), 541–545.

García, R., Alegría, A., Barberá, R., Farré, R., & Lagarda, M. J. (1998). Dializability of iron, zinc and copper of different types of infant formulas marketed in Spain. *Biological Trace Element Research*, *65*, 7–17.

George, D. E., & De Francesca, B. A. (1989). Human milk in comparison to cow milk. In E. Lebenthal (Ed.), *Textbook of gastroenterology and nutrition in infancy* (pp. 239–260). New York: Raven Press Ltd.

Glahn, R. P., Lai, C., Hsu, J., Thompson, J. F., Guo, M., & Van Campen, D. R. (1998). Decreased citrato improves iron availability from infant formula: application of an in vitro digestion/Caco-2 Cell Culture Model. *The Journal of Nutrition*, *128*, 257–264.

Hallberg, L., & Rossander-Hultén, L. (1993). Factors influencing the bioavailability of dietary iron in man. In U. Schlemmer (Ed.), *Bioavailability '93; nutritional, chemical and food processing implications of nutrient availability. Proceedings, Part 2: Ettlingen* (pp. 23–32). Karlsruhe: Bundesfoschunganstalt für Ernährung.

Hamosh, M. (1996). Digestion in the newborn. *Clinical Perinatology*, *23*, 191–209.

Hemminki, E., Nemet, K., Horvath, M., Malin, M., Schuler, D., & Hollan, S. (1995). Impact of iron fortification of milk formulas on infants growth and health. *Nutrition Research*, *15*, 491–503.

Hertrampf, E., Olivares, M., Pizarro, F., & Walter, T. (1998). High absorption of fortification iron from current infant formulas. *The Journal of Pediatric Gastroenterology and Nutrition*, *27*, 425–430.

Jackson, L. S., & Lee, K. (1992). The effect of dairy products on iron bioavailability. *Critical Reviews in Food Science and Nutrition*, *31*(4), 259–270.

Jovaní, M., Le Masle, M., Alegría, A., Barberá, R., Lagarda, M. J., & Clemente, G. (2000). In vitro interactions between calcium, zinc, copper and iron in milk- and soy-based infant formulas. *Food Science and Tecnology International*, *6*(1), 25–31.

Lönnerdal, B., Yuen, M., Glazier, C., & Litov, R. (1993). Magnesium bioavailability from human milk, cow milk and infant formula in suckling rat pups. *The American Journal of Clinical Nutrition*, *58*, 392–397.

Miller, J. C., & Miller, J. N. (1993). *Estadística para Química Analítica*. Argentina: Ed. Addison-Wesley Iberoamericana.

Miller, D. D., Schricker, B. R., Rasmussen, R. R., & Van Campen, D. (1981). An in vitro estimation of iron availability from meals. *The American Journal of Clinical Nutrition*, *34*, 2248–2256.

Minihane, A. M., Fox, T. E., & Fairweather-Tait, S. J. (1993). A continuous in vitro method to predict bioavailability of Fe from foods. In U. Schlemmer (Ed.), *Bioavailability '93; nutritional, chemical and food processing implications of nutrient availability. Proceedings, Part 2: Ettlingen* (pp. 175–179). Karlsruhe: Bundesfoschunganstalt für Ernährung.

Roig, M. J., Alegría, A., Barberá, R., Farré, R., & Lagarda, M. J. (1999). Calcium bioavailability in human milk, cow milk and infant formulas—comparison between dialysis and solubility methods. *Food Chemistry*, *65*, 353–357.

Rudloff, S., & Kunz, C. (1997). Protein and nonprotein nitrogen components in human milk, bovine milk, and infant formula: quantitative and qualitative aspects in infant nutrition. *Journal of Pediatric Gastroenterology and Nutrition, 24 (3)*, 328–344.

Rudloff, S., & Lönnerdal, B. (1992). Solubility and digestibility of milk proteins in infant formulas exposed to different heat treatments. *Journal of Pediatric Gastroenterology and Nutrition*, *15*(1), 25–33.

Shen, L., Luten, J., Robberecht, H., Bindels, J., & Deelstra, H. (1994). Modification of an in vitro method for estimating the bioavailability of zinc anc calcium from foods. *Zeitschrift für Lebensmitteluntersuchung und Forschung*, *199*, 442–445.

Shen, L., Robberecht, H., Van Dael, P., & Deelstra, H. (1995). Estimation of the bioavailability of zinc and calcium from human, cow's, goat and sheep milk by an in vitro method. *Biological Trace Element Research*, *49*, 107–118.

Shen, L. H., Van Dael, P., & Deelstra, H. (1993). Comparison of the in vitro availability of selenium, zinc anc calcium from human, cow's, goat and sheep milk. In U. Schlemmer (Ed.), *Bioavailability '93; Nutritional, Chemical and Food processing implications of nutrient availability. Proceedings, Part 1: Ettlingen* (pp. 309–313). Karlsruhe: Bundesfoschunganstalt für Ernährung.

Whitehead, R. G. (1993). Significance of changing lifestyle, nutritional status and reduced food consumption for the bioavailability of nutrients. In U. Schlemmer (Ed.), *Bioavailability '93; Nutritional, Chemical and Food processing implications of nutrient availability. Proceedings, Part 1: Ettlingen* (pp. 3–12). Kalsruhe: Bundesfoschunganstalt für Ernährung.

Ziegler, E. E., Serfass, R. E., Nelson, S. E., Figueroa-Colon, R., Edwards, B. B., Houk, R. S., & Thompson, J. J. (1989). Effect of low zinc intake on absorption and excretion of zinc by infants studied with Zn70 as extrinsic tag. *The Journal of Nutrition*, *119*, 1647–1653.