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Availability of iron from milk-based formulas and fruit juices containing milk and cereals estimated by *in vitro* methods (solubility, dialysability) and uptake and transport by Caco-2 cells

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Abstract

Iron solubility, dialysability and transport and uptake (retention + transport) by Caco-2 cells as indicators of iron availability have been estimated in the *in vitro* gastrointestinal digests of infant foods (adapted, follow-up and toddler milk-based formulas and fruit juices containing milk and cereals (FMC)). Low correlation coefficients (in all cases R-squared $\leqslant$ 37.1%) were obtained between iron solubility or dialysability versus transport or uptake efficiency – a fact emphasizing the importance of incorporating Caco-2 cell cultures to *in vitro* systems in order to adapt the conditions to those found in *in vivo* assays. The highest uptake efficiency corresponded to FMC (25.6–26.1%) and toddler formulas (32.1–41.9%), the samples with the highest ascorbic acid contents and ascorbic acid/iron molar ratios. In addition, the toddler formulas contained caseinphosphopeptides with the cluster sequence SpSpSpEE, representing the binding site for minerals. In adapted formulas, greater iron uptake efficiency was obtained for the formulation containing ferrous lactate (22.7%) versus ferrous sulfate (4.7%).
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Keywords: Iron bioavailability; Infant foods; Simulated digestion; Caco-2 cells

1. Introduction

Iron deficiency is the most common nutrient deficiency in the world, and anaemia is the most prevalent nutritional health problem among children in developing and developed countries. Iron supplementation of infant foods is one way of increasing the iron content in the diet of growing infants although, in many such children, iron deficiency anaemia develops mainly as the result of inappropriate supplies of absorbable iron. It is therefore important to know whether the iron in infant foods is bioaccessible and bioavailable (Sarriá & Vaquero, 2004).

The first step toward bioavailability is represented by solubility within the intestinal tract (bioaccessibility) for subsequent absorption (Salovaara, Sandberg, & Andlid, 2002). In infant foods, iron is mainly found the form of non-heme iron, which has a strong tendency to interact with other food or meal components. On average, only about 7% of ingested non-heme iron is absorbed (Carpenter & Mahoney, 1992). The iron of the common non-heme pool is subject to the interplay of promoter factors, such as mucin, organic acids (e.g., ascorbic acid) and certain amino acids, compounds that can form soluble complexes with iron ions, and inhibitory factors, such inositol hexaphosphate and polyphenols, yielding insoluble complexes with iron and thus rendering the latter non-absorbable (Salovaara et al., 2002).

For the evaluation of iron bioavailability in infant foods, *in vitro* methods are a good alternative to *in vivo* techniques, and generally consist of simulated gastrointestinal digestion, followed by determination of how much of the iron is soluble (Bermejo et al., 2002; Sarriá &

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Vaquero, 2001) or dialyses through a membrane of a certain pore size (Bosscher et al., 2001; De Souza, Colli, & Silverio, 2005; Drago & Valencia, 2004; García, Alegría, Barberá, Farré, & Lagarda, 1998; Sarriá & Vaquero, 2001). The element solubility or dialysability can be used to establish trends in the bioavailability or relative bioavailability values of iron. These *in vitro* methods have been improved by incorporating a human colon carcinoma cell line (Caco-2) that exhibits many of the functional and morphological properties of mature human enterocytes (Pinto et al., 1983). The system is thus able to mimic and estimate the uptake and/or transport of mineral elements, and has been used to assess iron uptake from infant formulas (Etcheverry, Wissler, Wortley, & Glahn, 2004a; Glahn, Lai, Hsu, Thompson, & Guo, 1998; Jovianí, Barberá, Farré, & Martín de Aguilera, 2001; Jovianí, Viadel, Laparra, Barberá, & Farré, 2004) and fruit juices (Boato, Wortley, Liu, & Glahn, 2002; Yeung, Glahn, & Miller, 2003). To our knowledge, the transport by Caco-2 cells of iron contained in infant foods has not been investigated to date.

The aim of the present study was to evaluate the availability of iron from different infant foods, based on three parameters: solubility, dialysability and a model that combines *in vitro* digestion and iron retention and transport by Caco-2 cells.

2. Materials and methods

2.1. Samples

Totally different samples have been studied:

- Two milk-based adapted infant formulas with the same composition except for the iron salt used for enrichment: ferrous sulphate (A^s) or ferrous lactate (A^l).
- Two follow-up infant formulas, one with added *Bifidobacterium bifidum* and *Bifidobacterium longum* (FB), and the other without such addition (F).
- Two toddler formulas, one likewise containing *Bifidobacterium bifidum* and *Bifidobacterium longum* (TB), and the other without added bacteria (T).
- Three fruit juices containing different juice proportions (51–55%), of skimmed milk (6%) and cereals (1%) (FMC), intended for infants and young children (FMC^1 = pineapple and banana, FMC^2 = peach and apple, and FMC^3 = grape, orange and banana).

Samples were kept in their unopened vacuum commercial package (N_2/CO_2, <3% O_2, modified atmosphere) at 25 °C and protected from exposure to light until analysis. The compositions of the aforementioned samples are shown in Table 1.

2.2. Material and reagents

Enzymes and bile salts were purchased from Sigma Chemical Co. (St. Louis MO, USA): pepsin (Porcine: cat no. P-7000), pancreatin (Porcine: cat. no. P-1750) and bile extract (Porcine: cat. no. B-8631). The working dissolutions of these enzymes were prepared immediately before use.

Iron standard solutions were prepared immediately before use by dilution with distilled deionized water of a standard solution of 1000 mg/l ($FeCl_3$ in 15% HCl, Titrisol, Merck, Barcelona, Spain).

Transport buffer contained 130 mM NaCl (Merck), 10 mM KCl (Merck), 1 mM $MgSO_4$ (Sigma Chemical Co.), 50 mM HEPES (Gibco, Scotland), 5 mM glucose (Sigma Chemical Co.) and pH = 7. The transport buffer was incubated at 37 °C until starting the assay.

All reagents used were of reagent grade, and Millipore-Milli Q distilled-deionised water (Millipore Ibérica S.A.; Barcelona, Spain) was used throughout the experiments.

For iron determination, glass and polyethylene material were washed with detergent, soaked with concentrated nitric acid (sp. gr. = 1.41), and rinsed three times with distilled-deionised water before use.

For vitamin C determination, the reagents used were 2,6-dichloroindophenol, ascorbic acid, acetic acid and $NaHCO_3$ from Merck, and metaphosphoric acid from Sigma Chemical Co. To test interference of reducing ions (such as ferrous iron and cuprous copper), methylene blue (Merck) was used.

Table 1
Energy value and protein, fat, carbohydrate and iron contents of the analyzed samples, referred to 100 ml ready-to-eat sample (manufacturer supplied data)

	$A^{s,l}$ [a]	F, FB[a]	T	TB[a]	FMC^1	FMC^2	FMC^3
Energy (kcal)	68	65	65	61	60	57	56
Protein (g)	1.5	2.0	2.6	1.9	0.6	0.4	0.5
Casein (g)	0.7	1.0	1.7	1.2	0.5	0.3	0.4
Fat (g)	3.8	3.3	2.5	2.6	0.1	0.1	0.1
Carbohydrates (g)	7.1	7.0	8.0	7.7	14.3	13.7	13.5
Lactose (g)	7.1	3.5	8.0	2.5	1.3	1.2	1.2
Maltodextrins (g)	0	3.5	0	5.2	0	0	0
Fe (mg)	0.8	1.3	1.3	1.2	–	–	–

A: adapted formula (supplemented with [s]ferrous sulphate or [l]ferrous lactate), F: follow-up formula, FB: follow-up formula with bifidobacterium, FMC: juice + cereals + milk ([1]pineapple and banana, [2]peach and apple, [3]grape, orange and banana), T: toddler formula, TB: toddler formula with bifidobacterium.

[a] Powered samples were reconstituted according to the manufacturer instructions (13% w/v).

2.3. In vitro digestion

2.3.1. Method

The *in vitro* procedure was that described by Perales, Barberá, Lagarda, and Farré (2005).

2.3.2. Solubility

Different sample weights were used according to the type of sample involved (10 g adapted formula, 7 g follow-up formula, 40 g liquid toddler formula, 5 g toddler formula with bifidus, 80 g FMC). Taking into account the iron contents, different sample weights were taken with the aim of reducing the possible effects of different initial iron amounts while, in fruit juices, the highest sample weight compatible with the method to be applied was selected.

For gastric digestion the pH was adjusted to 2.0 and an amount of freshly prepared demineralized pepsin solution, sufficient to yield 0.02 g pepsin/sample, was added. The sample was incubated in a shaking water bath at 37 °C/120 strokes/min for 2 h.

To facilitate the intestinal digestion stage, the pH of the gastric digests was raised to pH 5.0. Then an amount of freshly prepared and previously demineralised pancreatin/bile solution, sufficient to provide 0.005 g of pancreatin and 0.03 g bile salts/sample, was added, and incubation was continued for an additional 2 h. To stop intestinal digestion, the sample was kept for 10 min in an ice bath. The pH was adjusted to 7.2 and the digests were centrifuged at 3500*g* for 1 h at 4 °C. The supernatants were used to determine the mineral content (solubilised fraction) and were applied in cell culture assays.

2.3.3. Dialysis

Dialysis comprised a gastric step, common to that of the solubility method, followed by an intestinal step where dialysis is included (dialysis bag: molecular weight 10–12,000 Da; Visking 3-20/322, Medicell, London, UK). Dialysate was collected to measure its iron content.

2.4. Caco-2 method

2.4.1. Cell culture

Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC 86010202, Salisbury, UK), and were used between passages 70 and 80. Stock cultures were maintained in minimum essential medium (MEM; Gibco BRL Life Technologies, Scotland, UK) supplemented with 10% v/v foetal bovine serum (FBS), 1% v/v non-essential amino acids (Gibco), 1% v/v L-glutamine (Gibco), 1% v/v antibiotic solution (penicillin–streptomycin) (Gibco) and 0.1% v/v fungizone (Gibco) at pH 7.2–7.4. The cells were maintained at 37 °C in an incubator (Nuaire, NU-4500, Minnesota, USA) under a 5% CO_2, 95% air atmosphere at constant humidity. Culture medium was changed every two days.

2.4.2. Sample preparation

The gastrointestinal digests from the solubility assay were heated for 4 min at 100 °C to inhibit sample proteases, and then cooled by immersion in an ice bath. Aliquots of 20 g of the inactivated digests were transferred to polypropylene centrifuge tubes and centrifuged at 3500*g* for 1 h at 4 °C. Glucose (5 mM final concentration) and HEPES (50 mM final concentration) were added to the supernatant fraction to make it similar to the culture medium and facilitate cell viability, while water was added to adjust the osmolarity to 310 ± 10 mOsm/kg (Freezing point osmometer, Osmomat 030, Berlin, Germany).

In Caco-2 retention and transport assays, the soluble fraction obtained with the solubility method was used instead of the dialysate, because the soluble fraction is more similar to the digest obtained under *in vivo* conditions. In addition, the use of dialysate dramatically reduced the amount of iron added to cells, because only the soluble forms of iron with a molecular weight lower than the cut-off value of the dialysis membrane are able to dialyze – resulting in very low iron contents that increased variability in the uptake assays.

2.4.3. Iron retention and transport

To evaluate mineral retention and transport, the cells were seeded onto polyester membrane chamber inserts (24-mm diameter, 0.4-μm pore size; Transwell®, Costar Corp., NY, USA) at a density of 35×10^4 cells/filter, with 2.5 ml of medium in the basal chamber and 1.5 ml of suspended cells in the apical chamber. The transwell filters were placed in 6-well plates dividing an apical or a donor-like compartment from a basal or acceptor compartment.

Nineteen to 21 days after initial seeding, spent culture medium was aspirated from the apical and basolateral chambers and apical and basolateral cell surfaces of the monolayer were washed three times with phosphate buffer saline at 37 °C. Then, 2.5 ml of transport buffer (130 mM NaCl, 10 mM KCl, 1 mM $MgSO_4$, 5 mM glucose, 50 mM HEPES) were added to the basal chamber, and 1.5 ml of heated soluble mineral fraction were added to the apical chamber. Cell cultures were incubated at 37 °C under 5% CO_2 with 95% relative humidity for 2 h. Cell viability after 2 h of exposure to the soluble mineral fraction was assessed by trypan blue exclusion, and was typically 80–95%.

After incubation for the retention study, the apical compartment was aspirated, the insert was removed, and the monolayer was washed three times with buffer solution [150 mM NaCl (Merck) + 1 mM EDTA (Sigma) + 10 mM HEPES (Biowhittaker), pH = 7.4] at 4 °C to remove non-specifically bound mineral and residual medium. The cells were lysed by adding 1 ml of 2% sodium dodecyl sulfate (SDS).

The basal chamber solution was pipetted off for the determination of iron transport across the monolayer.

2.5. Iron determination

Total, soluble or dialysate iron contents and iron of cell monolayer, transport buffer (transport blank) and the basal chamber contents were measured by atomic absorption spectrophotometry (AAS, Perkin–Elmer, model 2380, Boston, USA). All samples, with the exception of the dialysate, were previously subjected to dry digestion at 450 °C.

Solubility percentages were calculated as follows: solubility $\% = 100 \times S/C$, where S = soluble iron content (μg Fe/g sample), and C = total iron content of the sample (μg Fe/g sample).

Dialysis percentages were calculated as follows: dialysis $\% = 100 \times D/C$, where D = iron content of the dialysate (μg Fe/g sample), and C = total iron content of the sample (μg Fe/g sample).

Differences between the iron content of the cell monolayer incubated with soluble mineral fraction and the basal iron content of non-exposed cell monolayer (retention blank) yielded an estimation of the cellular retention (μg) of iron. Cellular transport was evaluated by the difference between the iron amount in the basal chamber and in transport buffer (transport blank).

Retention percentages were calculated as follows: retention $\% = 100 \times R/C$, where R = iron retention (μg Fe/well), and C = total iron content of the sample added (μg Fe).

Transport percentages were calculated as follows: transport $\% = 100 \times T/C$, where T = cellular transport (μg Fe/well), and C = total iron content of the sample added (μg Fe).

Due to the differences among samples in terms of the solubility of iron after *in vitro* digestion, iron transport and uptake (retention + transport) efficiencies were expressed as follows: % transport efficiency = (% solubility × % transport)/100, and % uptake efficiency = (% solubility × % uptake)/100.

2.6. Ascorbic acid determination

The AOAC 967.21 ascorbic acid in vitamin preparations and juices, 2,6-dichlorophenolindophenol titrimetric method was used to determine the ascorbic acid content in samples (Horwitz, 2000).

2.7. Statistical analysis

Eight aliquots were used in the solubility and dialysis assays, and 5 in the Caco-2 cell assays. To achieve compliance with the variance analysis hypothesis (data normality and variance homogeneity within the considered groups) prior to one-way analysis of variance (ANOVA), a logarithmic transformation was applied to all the variables used. Selected pairs of means were compared by Tukey's test. Values of $p < 0.05$ were considered statistically significant.

Simple regression analysis was also applied to the results, with the aim of estimating the possible relationship between iron content of the samples and iron solubility, iron dialysis or Caco-2 cell transport and uptake efficiency, and also the possible relationship between ascorbic acid, soluble or dialyzable iron, and Caco- 2 cell transport and uptake efficiency.

The Statgraphics Plus version 5.0 statistical package (Rockville, Maryland, USA) was used throughout.

3. Results and discussion

Ascorbic acid and iron (total, soluble and dialysate) contents are listed in Table 2, while the values corresponding to iron retention, transport and uptake (retention + transport) are summarized in Table 3. The percentages of iron dialysate taken up by Caco-2 cells relative to soluble iron are represented in Fig. 1.

In infants (children aged 0–12 months), iron is provided by human milk and/or infant formulas. According to European legislation (Directive 91/321/CEE), adapted formulas and follow-up formulas should afford iron contents in the range of 0.5–1.5 mg/100 kcal and 1–2 mg/100 kcal, respectively. The analyzed samples had iron contents (see Table 2) in agreement with the EU Directive.

As could be expected, in the analyzed infant foods (adapted, follow-up and toddler formulas and fruit juices), soluble fractions had higher iron contents (0.3–73.6 μg/g) than had dialysates (0.2–12.2 μg/g) (see Table 2). This means that, under the applied *in vitro* assay conditions, only iron compounds of sizes smaller than the pore size of the dialysis membrane are dialysed (cut-off molecular weight 10–12,000 Da).

Iron bioavailability can be affected by the matrix composition of the samples. Components of milk, such as casein, and of fruit juices, such as ascorbic, citric and malic acids, and the iron salt used for enrichment could explain differences among samples.

The most potent promoter of iron absorption is ascorbic acid, that first converts Fe(III) to the more soluble Fe(II), which maintains its solubility in the alkaline environment, and prevents the formation of polynuclear complexes, and the reduced iron is the transport form mediated by the apical transporter DMT1/NRAMP2 in intestinal cells (Mackenzie & Hediger, 2004). Secondly, in the acid environment, ascorbic acid forms a chelate with ferric chloride that remains stable at alkaline pH values (Fairweather-Tait, Fox, Wharf, & Eagles, 1995).

The highest iron dialysis percentages (16.4–17.0% and 8.8–14.9%, respectively) corresponded to fruit juices and the toddler formula without *bifidus* (see Table 2). In fruit juices, this could be due to the high ascorbic acid content (about 3.5- to 8-fold greater than in infant formulas), together with organic acids, such as citric and malic and low molecular weight soluble iron complexes (Hazell & Johnson, 1987); this could explain the high iron dialysis percentage recorded despite the low solubility percentage.

The positive effect of ascorbic acid was also observed in iron uptake by Caco-2 assays, where high percentages were

Table 2
Ascorbic acid (AA) and total, soluble and dialysate iron contents (mean values ± standard deviation, $n = 8$)

Sample	AA (mg/100 ml)	Iron					
		Total content (μg/g)	Total content (mg/100 kcal)	Soluble fraction (μg/g)	Solubility (%)	Dialysate fraction (μg/g)	Dialysis (%)
A^{s}	8.6 ± 0.1	45.9 ± 4.5	0.9	44.1 ± 6.4	97.5 ± 1.8^{a}	2.5 ± 0.8	5.2 ± 0.3^{a}
A^{l}	8.6 ± 0.1	47.5 ± 4.3	0.9	47.1 ± 7.9	99.1 ± 2.4^{a}	2.3 ± 0.6	5.0 ± 0.6^{a}
F	8.3 ± 0.2	90.1 ± 3.7	1.8	73.5 ± 3.5	81.7 ± 3.8^{b}	6.4 ± 1.1	7.1 ± 1.3^{b}
FB	8.3 ± 0.1	90.1 ± 5.5	1.8	73.6 ± 2.2	81.8 ± 2.5^{b}	6.3 ± 0.9	7.0 ± 1.1^{b}
T[a]	6.4 ± 0.2	78.0 ± 2.8	1.6	57.3 ± 2.1	73.8 ± 2.3^{c}	12.2 ± 1.4	14.9 ± 1.5^{c}
TB	15.4 ± 0.4	73.0 ± 1.9	1.5	60.4 ± 1.4	82.7 ± 1.9^{b}	6.7 ± 0.8	8.8 ± 0.8^{b}
FMC^{1}	52.4 ± 1.2	0.9 ± 0.03	0.2	0.3 ± 0.03	31.3 ± 3.7^{d}	0.2 ± 0.03	16.8 ± 3.3^{c}
FMC^{2}	53.1 ± 1.4	0.9 ± 0.05	0.2	0.3 ± 0.05	31.9 ± 5.6^{d}	0.2 ± 0.03	17.0 ± 3.4^{c}
FMC^{3}	53.7 ± 1.5	0.9 ± 0.1	0.2	0.3 ± 0.07	31.9 ± 8.0^{d}	0.2 ± 0.02	16.4 ± 2.4^{c}

A: adapted formula (supplemented with ^{s}ferrous sulphate or ^{l}ferrous lactate), F: follow-up formula, FB: follow-up formula with bifidobacterium, FMC: juice + cereals + milk (^{1}pineapple and banana, ^{2}peach and apple, ^{3}grape, orange and banana), T: toddler formula, TB: toddler formula with bifidobacterium.
ANOVA (Statgraphics Plus version 5.0). Non-coincidence of superscripts (a, b, c, d) denotes statistically significant differences ($p < 0.05$) between values in the same column.
[a] Liquid sample (contents are referred to dry product).

Table 3
Caco-2 cell iron uptake assay (mean values ± standard deviation, $n = 5$)

Sample	Soluble iron added[a] (μg)	Retention (μg)	Retention (%)	Transport (μg)	Transport (%)	Transport efficiency (%)	Uptake (μg)	Uptake (%)	Uptake efficiency (%)
A^{s}	8.1	0.3 ± 0.05	3.7 ± 0.06^{a}	0.1 ± 0.09	1.2 ± 0.1^{a}	1.2 ± 0.1^{a}	0.4 ± 0.09	4.9 ± 0.1^{a}	4.7 ± 0.4^{a}
A^{l}	8.5	0.3 ± 0.09	3.5 ± 0.1^{b}	1.6 ± 0.09	18.9 ± 1.1^{b}	18.8 ± 1.1^{b}	1.9 ± 0.1	22.4 ± 2.3^{b}	22.7 ± 1.3^{b}
F	7.6	0.5 ± 0.1	6.6 ± 1.3^{c}	0.8 ± 0.1	10.5 ± 1.4^{c}	8.6 ± 0.8^{c}	1.3 ± 0.1	17.1 ± 0.9^{c}	13.8 ± 0.6^{c}
FB	6.6	0.5 ± 0.1	7.6 ± 1.6^{c}	0.2 ± 0.03	3.0 ± 0.6^{d}	2.5 ± 0.2^{d}	0.7 ± 0.06	10.6 ± 1.0^{d}	8.2 ± 0.9^{d}
T[b]	2.2	0.4 ± 0.08	18.2 ± 4.1^{d}	0.5 ± 0.1	22.7 ± 1.1^{e}	16.8 ± 6.7^{b}	0.9 ± 0.08	40.9 ± 3.6^{e}	32.1 ± 4.2^{e}
TB	4.6	0.2 ± 0.09	4.3 ± 1.9^{c}	2.1 ± 0.2	45.6 ± 5.0^{f}	37.7 ± 4.2^{e}	2.3 ± 0.2	49.9 ± 5.1^{e}	41.9 ± 5.7^{f}
FMC^{1}	1.1	0.3 ± 0.03	27.3 ± 1.5^{e}	0.6 ± 0.03	54.5 ± 7.1^{f}	17.1 ± 5.6^{b}	0.9 ± 0.1	81.8 ± 3.3^{f}	25.6 ± 1.2^{e}
FMC^{2}	1.1	0.3 ± 0.02	27.3 ± 3.1^{e}	0.6 ± 0.03	54.5 ± 5.2^{f}	17.4 ± 4.5^{b}	0.9 ± 0.09	81.8 ± 4.6^{f}	26.1 ± 2.6^{e}
FMC^{3}	1.1	0.3 ± 0.06	27.3 ± 1.9^{e}	0.6 ± 0.02	54.5 ± 5.5^{f}	17.4 ± 2.0^{b}	0.9 ± 0.05	81.8 ± 2.5^{f}	26.1 ± 1.9^{e}

A: adapted formula (supplemented with ^{s}ferrous sulphate or ^{l}ferrous lactate), F: follow-up formula, FB: follow-up formula with bifidobacterium, FMC: juice + cereals + milk (^{1}pineapple and banana, ^{2}peach and apple, ^{3}grape, orange and banana), T: toddler formula, TB: toddler formula with bifidobacterium.
ANOVA (Statgraphics Plus version 5.0). Non-coincidence of superscripts (a, b, c, d, e, f) denotes statistically significant differences ($p < 0.05$) between values in the same column.
[a] Soluble iron content obtained after *in vitro* digestion method applied to the samples.
[b] Liquid sample (contents are referred to dry product).

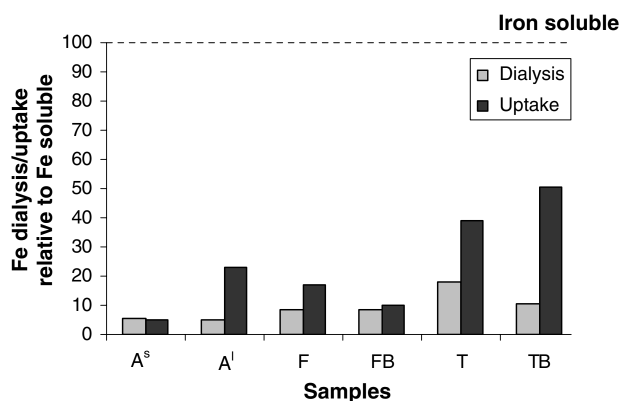

Fig. 1. Percentages of iron dialysate/taken up by Caco-2 cells relative to soluble iron (assigned as 100%).

obtained in FMC samples (81.8 %) (see Table 3). This is in agreement with the observations of Han, Failla, Hill, Morris, and Smith (1995a), Gangloff, Glahn, Miller, and Van (1996) and Salovaara et al. (2002), who reported an increase in iron uptake by Caco-2 cells when ascorbic acid was present. Han, Failla, Hill, Morris, and Smith (1995b) concluded that the increase in iron uptake by ascorbic acid seemed to be primarily due to reduction rather than to chelation of Fe(III), and the amino acids present may have retarded the reoxidation of ascorbate-generated Fe(II) by forming a stable complex with Fe(II) – suggesting that Fe(II) is the chemical species efficiently transported across the brush border of the cell membrane. In the analyzed infant formulas, the highest ascorbic acid content corresponded to TB (15.4 mg/100 ml of sample), which was also

the formula yielding the highest iron uptake and transport efficiency percentages, though other factors that will be discussed below, such as, for instance casein, can also affect iron uptake. The ascorbic acid/iron molar ratio has to be taken into account, in addition to the absolute iron content. In studies carried out in children (Fairweather-Tait et al., 1995; Stekel et al., 1986), an increase in iron absorption, when ascorbic acid is added, has been reported. Accordingly, ascorbic acid/iron molar ratios of about 1.5 can result in 2- to 3-fold increases in iron absorption from iron-fortified cereals (Fairweather-Tait et al., 1995), reaching a maximum value at 4:1 ascorbic acid/ iron molar ratio (Stekel et al., 1986).

In the present study, the samples yielding the highest iron uptake percentage efficiencies were those having the highest ascorbic acid/iron ratios: fruit juices (186–192) and the toddler formula with *bifidus* (ratio 4.1).

While a beneficial effect of malic acid upon iron uptake by Caco-2 cells has been reported (Salovaara et al., 2002), agreement is lacking in the case of the effect of citric acid. Glahn et al. (1998) reported a decrease in Caco-2 cell iron uptake from infant formulas with added citric acid, while Salovaara et al. (2002) failed to observe this negative effect.

It is well known that fibre and polyphenols, that may be present in fruit juices, are able to exert an inhibitory effect on iron absorption (Salovaara et al., 2002). However, the fibre contents of the analyzed fruit juices were very low (0.2%, data provided by the manufacturer), and they were light-coloured. Light-coloured fruit juices promoted iron uptake by Caco-2 cells from infant cereals, while dark-coloured fruit juices, such as prune and red grape juices, exert a marked inhibitory effect, due to their high contents of phenolic compounds, which appear to outweigh the promotional effect of ascorbic acid (Boato et al., 2002).

Casein can also affect iron bioavailability, though there is a lack of agreement on the role of casein and casein phosphopeptides (CPP) in relation to iron availability.

Fe(II) binds to the serine phosphate residues of the caseins, though the influence of such binding upon Fe(II) bioavailability remains uncertain (West, 1986). In dialysis assays, casein seems to exert an important inhibitory effect on iron dialysis, the dialysis percentage of iron being lower in infant formulas having casein as the main protein fraction, as against other protein sources. In addition, the dialysis percentage increases with the hydrolysis of casein (Drago & Valencia, 2004; García et al., 1998).

Studies of the Caco-2 cell uptake of iron from cow and human milks show the negative effect of casein, which could be explained in terms of very slow digestion by the alkaline phosphatase of the brush border of the cell membrane, which releases the phosphorus–iron complex from casein, or/and by the very strong affinity of casein for iron – which prevents the latter from interacting with the intestinal cell receptor (Etcheverry, Miller, & Glahn, 2004b). During the manufacturing process, or during the digestion of infant formulas, casein can release peptides and among them CPP, which can bind iron and affect its absorption. One third of the casein content of bovine milk is composed of β-casein which, via hydrolysis, releases β-CN(1–25). Iron bound to β-CN(1–25) remains soluble in the digestive tract, is not altered by ionic strength, because of the presence of coordination links, preventing iron from insolubilization or from interactions with other minerals or trace elements, and maintaining iron in a protected stable state until it reaches the enterocyte receptor – thereby favouring iron absorption (Aït-Oukhatar et al., 2002; Chaud et al., 2002).

The CPPs released in the *in vitro* gastrointestinal digestion applied in this study (Miquel et al., 2005), and their effect on iron solubility, could justify the observed greater solubility of iron from infant formulas (73.8–99.1%)than from fruit juices (31.3–31.9%) (see Table 2), which have a lower casein content (see Table 1).

In the studied formulas, the highest iron uptake efficiency corresponded to toddler formulas (32.1–41.9%), whose digests had a larger number of CPPs with the cluster sequence SpSpSpEE (representing the binding site for minerals) than had the adapted and follow-up formulas, as has been previously reported (Miquel et al., 2005). An increased iron uptake by Caco-2 cells in the presence of CPPs containing the phosphoserine cluster, as against sodium caseinate, has been reported by Yeung, Glahn, and Miller (2002). These could contribute to explain the higher iron uptake efficiency obtained with toddler formulas when compared to the adapted and follow-up formulations.

The type of iron salt used (lactate or sulphate) did not affect ($p > 0.05$) solubility or dialysis percentages of iron from adapted formulas (see Table 2), in agreement with the lack of significant differences in the dialysabilities of iron from infant formulas supplemented with different iron salts (among them ferrous lactate and sulphate) reported by Domínguez et al. (2004). However, it should be noted that a greater iron uptake efficiency was recorded for the formula supplemented with ferrous lactate (22.7 ± 1.3) than that with ferrous sulphate (4.7 ± 0.4). On the same lines, an increased absorption of iron from ferrous lactate as against ferrous sulphate has been reported in rats, though the effect was dose-dependent (Yun-Ji, 1999).

In follow-up formulas (F and FB), differing only in the presence or absence of *Bifidobacterium,* no differences ($p < 0.05$) in iron solubility or dialysis were found, transport (2.5% vs 8.6%) and uptake efficiency (8.2% vs 13.8%) being lower ($p < 0.05$) in the formulas with *Bifidobacterium* added vs those without *Bifidobacterium* added. Toddler formula with *Bifidobacterium* (TB) (powdered form) yielded higher percentages of iron solubility (82.7 vs 73.8), transport (37.7 vs 16.8) and uptake efficiency (41.9 vs 32.1) than did the toddler formulas without *Bifidobacterium* (T) (liquid form), though the iron dialysis percentage was lower (8.8 vs 14.9) (see Tables 2 and 3). Therefore, the effect cannot be ascribed to the *Bifidobacterium* addition but to differences in the manufacturing

processing of the two toddler formulas - the liquid formulation suffering stronger thermal treatment than the powdered one. In this sense, Sarriá and Vaquero (2001, 2004) reported that liquid formula sterilization induces protein changes, such as hydrolysis, denaturation and the formation of greater molecular weight compounds – including Maillard reaction products. The decrease in high molecular weight proteins in liquid formula may reduce the number of ligands formed with iron, and could be responsible for the increase in iron dialysis observed in liquid infant formulas as against the powdered formulation. This is in agreement with the results obtained in the present study, where a greater dialysis percentage of iron from liquid toddler formula (TB) was recorded versus powdered toddler formula (T). In a previous study, a greater dialysis percentage of iron from protein-hydrolyzed formulas as against non-hydrolyzed protein formulas has been reported (García et al., 1998).

An increase in thermal treatment reduces free/available lysine and leads to the formation of insoluble melanoidins that bind iron and reduce its solubility (Delgado-Andrade, Seiquer, Nieto, & Navarro, 2004). In addition, the contents of Maillard reaction products and high molecular protein aggregates that can bind iron to yield insoluble complexes is greater in liquid than in powdered infant formulas (Sarriá & Vaquero, 2001). These observations could partially explain the lower solubility percentage of iron from liquid toddler formula (T) (73.8 ± 2.3) when compared with powdered toddler formula (TB) (82.7 ± 1.9).

The effects of Maillard reaction products on iron bioavailability have been demonstrated in rats. In effect, increased iron absorption has been reported in suckling rats fed powdered as against liquid infant formulas (the latter having a greater presence of Maillard reaction products, altered proteins and lactulose) (Sarriá & Vaquero, 2001, 2004). The increased presence of Maillard reaction products in the liquid toddler formula (T) when compared to the powdered (TB) could explain the greater iron transport and uptake efficiency percentages found in TB (see Table 3).

It is difficult to compare the values of iron solubility or dialysability obtained in this study with those reported by others authors (Table 4), not only because samples can differ in their composition, but mainly because of the differences in the methodology applied – particularly as regards the amount and activity degree of the enzymes used, the pH values and incubation times during the gastric and intestinal steps, and even centrifugation speed in the case of solubility. The enzyme demineralization applied in this study could also have contributed to the observed differences.

Solubility and dialysis percentages provide information on the amount of iron that can be present in the intestinal lumen and available for absorption (bioaccessibility). The correlation coefficients obtained in this study, plotting iron solubility or dialyzability against transport or uptake efficiency, were very low, in all cases R-squared $\leqslant 37.1\%$ – thus stressing the importance of incorporating Caco-2 cell cultures into *in vitro* systems in order to more closely resemble the conditions found in *in vivo* assays. According to the results presented in Fig. 1, percentages of uptake by Caco-2 cells of iron from the soluble fraction were higher than dialysis percentages, except for the adapted formula supplemented with ferrous sulphate (A^s). In general, less than 50% of the soluble iron was taken up by Caco-2 cells, but less than 20% of soluble iron was dialyzed.

In summary, the results obtained in this study, involving complex foods, such as infant formulas and fruit juices containing milk and cereals, show that food composition and processing affect iron bioavailability. The effects are complex and can differ from those expected when only the effects of individual components are considered – thus underscoring the importance of conducting studies on whole foods.

Although no differences in bioaccessibility (solubility and dialysis) were found between ferrous sulphate and lactate, percentages of iron uptake efficiency were higher with the latter, a fact that would recommend the addition of the second. The enhancer/promoter effect of ascorbic acid and of the molar ratio, ascorbic acid to iron stood out,

Table 4
Iron solubility and dialysability percentages in infant (adapted or follow-up) milk-based formulas (data from different authors and the present study)

Infant formula	Iron	Present study[a] (powdered infant formula)
Solubility		
Powdered infant formula	97.5 (Sarriá & Vaquero, 2001)	Adapted: 98.3 Follow-up: 81.8
Liquid infant formula	96.1 (Sarriá & Vaquero, 2001)	
Adapted gastric pH 2	51.7 ± 29.5 (Bermejo et al., 2002)	
Dialysis		
Adapted	4.2 (García et al., 1998)	Adapted: 5.1 Follow-up: 7.1
Follow-up	4.9 (García et al., 1998)	
Infant formula gastric pH 2	4.1 ± 0.7 (Bosscher et al., 2001)	
Powdered infant formula	23.1 ± 1.5 (Sarriá & Vaquero, 2001)	
Liquid infant formula	28.9 ± 2.3 (Sarriá & Vaquero, 2001)	
Infant formula	15.4–24.8 (Drago & Valencia, 2004)	
Infant formula	3.6–32.2 (De Souza et al., 2005)	

[a] Mean values.

although the practical application of this observation is limited by the ranges of these nutrient contents established by the European Legislation (Directive 91/321/CEE).

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