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Addition of milk or caseinophosphopeptides to fruit beverages to improve iron bioavailability?

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ABSTRACT

A study has been made of the influence of caseinophosphopeptides (CPPs) added to a fruit beverage versus milk based fruit beverages upon iron retention, transport and uptake, using a combined simulated gastrointestinal digestion/Caco-2 cell system. Grape concentrate, orange concentrate, and apricot puree were used for sample formulation. Eight samples were assayed with/without added Fe sulphate (3 mg/100 ml fruit beverage) and/or added Zn sulphate (1.6 mg/100 ml fruit beverage), with/without skimmed milk (11% v/v). The addition of milk to fruit beverages exerted a positive effect on iron retention, transport and uptake versus fruit beverages, and this effect was greater than that of CPPs added to soluble fractions of fruit beverages. The addition of CPPs to soluble fractions of fruit beverages improved iron transport. Iron supplementation increased Fe retention, transport and uptake – the effect being more notable in samples with milk. Zinc supplementation did not affect Fe retention, transport or uptake.

1. Introduction

Iron deficiency is one of the most important nutrient deficiencies, and its more severe stages are associated with anemia. In this way, it was considered that Fe fortification is a good strategy for decreasing its deficiency – fruit beverages being a good vehicle, since they offer good solubility of nutrients and contain a low concentration of mineral absorption inhibitors.

The caseinophosphopeptides (CPPs) can be released from casein (CN) by enzymatic hydrolysis, gastrointestinal digestion or during food processing, and present a cluster (SpSpSpEE) that represents the binding sites for minerals such as Fe. Thus, CPPs may play an important role in mineral bioavailability (Meisel & Fitzgerald, 2003). CPPs are suitable supplements for fortifying foods, since Fe bound to CPPs presents good bioavailability, with increased solubility and the prevention of interactions with other minerals (Amaro & Cámara, 2004). In rats, an improvement has been reported in most blood parameters (hemoglobin and hematocrit) relating to Fe status, with increased Fe liver storage, in iron-deficient rats fed with Fe bound to hydrolysed β-CN or to β-CN(1–25) (Aït-Oukhatar et al., 1997, 1999). In addition, an increase in serum Fe levels was observed from Fe bound to peptides (hydrolysed CN) (Chaud et al., 2002).

In those studies where evaluations have been made of the influence of casein fractions, β-CN(1-25) has demonstrated a positive effect upon Fe bioavailability in rats (Ani-Kibangou et al., 2005; Aït-Oukhatar et al., 2002; Pèrés et al., 1999a,b). This effect is greater than in the case of Fe bound to whole CPPs, Fe bound to β-CN (Bouhallab et al., 2002), or Fe bound to α_s-CN (a mixture of α_{s1}-CN(59–79) and α_{s2}-CN(2–21)) (Kinbagou et al., 2005).

In humans, the effect of CPPs is controversial. In this sense, Hurrell, Lynch, Trinidad, Dassenko, and Cook (1989) showed an increase in Fe absorption after the ingestion of products of CN hydrolysis, whereas Aït-Oukhatar et al. (2002) did not observe effects on Fe absorption in the case of Fe bound to β-CN(1–25). In Caco-2 cells, a significant increase in total Fe uptake has been observed from Fe bound to β-CN(1–25) versus Fe bound to a mixture of α_s-CN (Kinbagou et al., 2005). The addition of CPPs (90% as phosphoserine clustered peptides) to Fe citrate solution not subjected or subjected to simulated gastrointestinal digestion decreased Fe uptake (Yeung, Glahn, & Miller, 2001) or ferritin synthesis (Yeung, Glahn, & Miller, 2002). Etcheverry, Wallingford, Miller, and Glahn (2004) did not record effects or observe a decrease in ferritin synthesis according to the commercial source and/or the degree of hydrolysis (16–20%) of different CPP preparations added to human milk. In a recent study, Kibangou et al. (2008) reported greater Fe transport when Fe was bound to β-CN(1–25) versus Fe sulphate or pyrophosphate.

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An increase in ferritin synthesis in Caco-2 cells has been attributed to the presence of milk (Wortley, Leusner, Good, Gugger, & Glahn, 2005) or peptidic fractions obtained from milk (Argyri, Miller, Glahn, Zhu, & Kapsokefalou, 2007), subjected to simulated gastrointestinal digestion. In fruit beverages fortified with Fe, we have observed an increase in ferritin synthesis in samples with milk versus samples without milk (Cilla, Perales, Lagarda, Barberá, & Farré, 2008).

Based on the above considerations, it would be interesting to compare the influence of CPPs added to fruit beverage versus milk based fruit beverages in relation to Fe retention (cell monolayer content), transport (iron transport across the monolayer) and uptake (sum of retention and transport) in an *in vitro* model (Caco-2 cells). Since Fe and Zn supplementation is common practice in beverages of this kind, an evaluation is made of the influence of Zn supplementation upon Fe bioavailability in beverages with or without added Fe.

2. Materials and methods

2.1. Material and reagents

Enzymes and bile salts were purchased from Sigma Chemical Co. (St. Louis, MO, USA), including 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.

Water of cellular grade was used for the preparation of reagents and throughout the *in vitro* digestion assay (Aqua B Braun, Braun Medical, Barcelona, Spain).

Transport buffer contained 130 mM NaCl (Merck), 10 mM KCl (Merck), 1 mM $MgSO_4$ (Sigma Chemical Co.), 5 mM glucose (Sigma Chemical Co.), and 50 mM HEPES (Gibco, Scotland) at pH 7.4.

Standard Fe solutions were prepared immediately before use by dilution with distilled deionized water of a standard solution of 1000 mg/l (Titrisol, Merck, Barcelona, Spain).

2.2. Samples

Grape concentrate, orange concentrate, and apricot puree were used for sample formulation. Eight samples were assayed with/without added Fe (Fe (sulphate) – 3 mg/100 ml fruit beverage) and/or added Zn (Zn (sulphate) – 1.6 mg/100 ml fruit beverage), with/without skimmed milk (FbM) (11%, v/v): Fb (fruit beverage), FbFe, FbZn, FbFeZn, FbM (milk based fruit beverage), FbMFe, FbMZn, and FbMFeZn. The composition of samples is shown in Table 1. All fruit beverages were subjected to pasteurization at 95 °C for 45 s. The beverages were packed hot and then cooled in a water bath. Their pH was 3.79 ± 0.17.

Table 1
Composition of fruit beverages analysed.

Component (g/100 g)	Fb/FbFe/FbZn/FbFeZn	FbM/FbMFe/FbMZn/FbMFeZn
Osmosis water	58.7	57.7
Apricot puree	24.5	24.5
Grape concentrate	7.2	7.2
Orange concentrate	4.2	4.2
Sugar	5.1	5.1
Pectin	0.354	0.354
L-Ascorbic acid	0.054	0.054

Fb = fruit beverage (grape + orange + apricot).
Fe: supplemented with Fe sulphate (3 mg/100 ml of fruit beverage).
Zn: supplemented with Zn sulphate (1.6 mg/100 ml of fruit beverage).
M: skimmed milk 11% (v/v).

2.3. Simulated gastrointestinal digestion

Samples were subjected to the same process of gastrointestinal digestion according to the method of Perales, Barberá, Lagarda, and Farré (2005). Eighty grams of sample were digested by simulated gastrointestinal digestion using two demineralized enzymatic solutions (pepsin solution at pH 2 for 2 h at 37 °C, and pancreatin–bile solution at pH 6.5 for 2 h at 37 °C). The pH was adjusted to 7.2, and the digests were then heated for 4 min at 100 °C to inhibit proteolytic activity and preserve cell viability. Finally, the inactivated digests were centrifuged (3500*g*, 60 min at 4 °C) to obtain the soluble fraction.

2.4. CPPs added to fruit beverages

The pool of CPPs added to the soluble fraction of fruit beverage was prepared by simulated gastrointestinal digestion as mentioned above, from whole CN, following the method of Miquel et al. (2006). Briefly, the pasteurized skimmed milk was subjected to isoelectric precipitation at pH 4.6 and centrifuged (4500*g*, 20 min at 10 °C) to yield CN, which was freeze-dried. CN (1.2 g) was digested as mentioned above and then centrifuged (3500*g*, 60 min at 4 °C). The CN not digested was precipitated at pH 4.6, centrifuged (4500*g*, 20 min at 10 °C), and then the supernatants were selectively precipitated with $CaCl_2$ and an equal amount of absolute ethanol at pH 8. The preparation was then centrifuged at 12,000*g* for 10 min at 10 °C. The precipitate was washed with 50% (v/v) ethanol, lyophilized, and stored at −20 °C.

The amount of CPPs (57 mg) added to the soluble fraction of fruit beverages (Fb + CPPs, FbFe + CPPs, FbZn + CPPs and FbFeZn + CPPs) (20 g) was the same as the amount of CPPs proceeding from FbM, taking into account that the latter contains 11% (v/v) of skimmed milk. Besides, in our laboratory the yield in obtaining CPPs (simulated gastrointestinal digestion and selective precipitation) was 15.49% (w/w) from CN. This CN was obtained from skimmed milk, which is the same raw material used for the elaboration of fruit beverages with milk (FbM, FbMFe, FbMZn and FbMFeZn).

2.5. Caco-2 method

2.5.1. Cell culture

Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC 86010202, Salisbury, UK). Cultures were maintained and grown as previously described (Perales et al., 2005).

2.5.2. Sample preparation

Glucose (5 mM final concentration) and HEPES (50 mM final concentration) were added to soluble fraction, and water of cellular grade was added to adjust the osmolarity to 310 ± 10 mOsm/kg (freezing-point osmometer, Osmomat 030, Berlin, Germany).

2.5.3. Assays of retention, transport and uptake in Caco-2 cells

To evaluate Fe retention, transport and uptake, the method of Perales et al. (2005) was used, with a modification (in this study 2 ml was used in the basolateral chamber).

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 50,000 cells/cm^2, with 2 ml of medium in the basolateral chamber and 1.5 ml of suspended cells in the apical chamber.

At 14–21 days from initial seeding, spent culture medium was aspired from the apical and basolateral chambers, and washed three times with phosphate-buffered saline (PBS) at 37 °C. Then 2 ml of transport buffer was added to the basolateral chamber, and 1.5 ml of soluble fraction of samples to the apical chamber. Cell

cultures were incubated at 37 °C under 5% CO_2 with 95% relative humidity for 2 h. After incubation, the apical compartment was aspirated, the insert was removed, and the monolayer was washed three times with buffer solution at 4 °C to remove non-specifically bound mineral and residual medium. The cells were lysed by adding 1 ml of 2% sodium dodecyl sulphate. The basolateral chamber solution was pipetted off for the determination of Fe transport across the monolayer.

2.6. Fe determination

The Fe of the soluble fraction added to cells, cell monolayer, transport buffer (transport blank) and basolateral chamber contents was measured by atomic absorption spectrophotometry (Perkin–Elmer, model 2380, Boston, USA). All samples were previously subjected to dry digestion at 450 °C.

Differences were estimated between Fe content of the cell monolayer incubated with added soluble fraction, and the Fe content of non-exposed cell monolayer (retention blank) yielded an estimation of the cellular retention of Fe. Cellular transport was calculated by the difference between Fe present in the basolateral chamber and in transport buffer. Cellular uptake was evaluated as the sum of Fe retention and transport.

Retention percentages were calculated as follows: retention (%) = Fe retention (μg/well)/soluble Fe added to well.

Transport percentages were calculated as follows: transport (%) = Fe transport (μg/well)/soluble Fe added to well.

The results obtained were likewise expressed as μg Fe/mg cell protein. To this effect, cellular protein was determined according to the method of Lowry, Rosebrough, Farr, and Randall (1951).

2.7. Statistical analysis

For evaluating the possible influence of CPPs added to fruit beverages, Fe and Zn content of samples upon Fe retention, transport and uptake, the results obtained were analysed by factorial ANOVA and the Fisher test to determine significant differences ($p < 0.05$). To this effect we used a factorial design with three factors: (a) matrix with three levels: samples without CPPs, M1 (Fb, FbFe, FbZn, and FbFeZn); samples with CPPs, M2 (Fb + CPPs, FbFe + CPPs, FbZn + CPPs, and FbFeZn + CPPs); and samples with milk, M3 (FbM, FbMFe, FbMZn, and FbMFeZn); (b) Fe content with two levels: high level (1) (samples supplemented with Fe) and low level (0) (samples not supplemented with Fe); and (c) Zn content with two levels: high level (1) (samples supplemented with Zn) and low level (0) (samples not supplemented with Zn). The factorial design was composed of 12 treatments or different combinations of treatments, hereafter referred to as "assay". The different assays were performed at random. Table 2 shows the factorial design used.

The results obtained corresponding to the different types of addition made, and were used to study the main effect and the second-order interactions.

The main effect of Fe and Zn content was calculated by the difference between the means of Fe retention, transport and uptake expressed as μg/mg cell protein corresponding to the highest (1) and lowest (0) levels of these factors. For the matrix, the main effect was calculated as above, but considering the three possibilities (M1M2, M2M3 and M1M3), where the lowest level was M1 (samples without CPPs), the highest level was M3 (samples with milk), and M2 (samples with CPPs) was considered to be high or low level

Table 2
The levels of factorial design.

Factors	Factorial design levels		
Matrix	M1	M2	M3
Fe content	1	0	
Zn content	1	0	
Factorial plan assays	Fe	Zn	Matrix
-1-	0	0	M1
-2-	1	0	M1
-3-	0	1	M1
-4-	1	1	M1
-5-	0	0	M2
-6-	1	0	M2
-7-	0	1	M2
-8-	1	1	M2
-9-	0	0	M3
-10-	1	0	M3
-11-	0	1	M3
-12-	1	1	M3

M1: samples without CPPs (Fb, FbFe, FbZn, and FbFeZn).
M2: samples with CPPs (Fb + CPPs, FbFe + CPPs, FbZn + CPPs, and FbFeZn + CPPs).
M3: samples with milk (FbM, FbMFe, FbMZn, and FbMFeZn).
1: High level of Fe or Zn content factors (samples supplemented).
0: Low level of Fe or Zn content factors (samples not supplemented).

Table 3
Caco-2 cell Fe retention, transport and uptake (mean values ± standard deviation ($n = 8$)).

Sample[a]	Fe soluble	Retention			Transport			Uptake		
	Added (μg)	(μg)	(%)	(μg/mg Cell protein)	(μg)	(%)	(μg/mg Cell protein)	(μg)	(%)	(μg/mg Cell protein)
Fb	1.22 ± 0.27	0.61 ± 0.02	49.70 ± 1.89	0.56 ± 0.02	0.23 ± 0.13	19.03 ± 10.68	0.21 ± 0.12	0.84±0.15	68.45 ± 12.17	0.76 ± 0.14
FbFe	11.75 ± 0.32	0.59 ± 0.25	5.05 ± 2.28	0.54 ± 0.23	0.25 ± 0.08	2.10 ± 0.66	0.26 ± 0.08	0.84 ± 0.17	7.15 ± 1.44	0.76 ± 0.16
FbZn	0.82 ± 0.05	0.18 ± 0.06	21.89 ± 5.06	0.17 ± 0.05	0.18 ± 0.06	21.23 ± 6.69	0.16 ± 0.06	0.36 ± 0.12	43.30 ± 14.66	0.32 ± 0.11
FbFeZn	9.29 ± 0.40	0.48 ± 0.01	4.94 ± 0.05	0.44 ± 0.01	0.81 ± 0.19	8.70 ± 2.44	0.73 ± 0.17	1.28 ± 0.20	13.78 ± 2.13	1.17 ± 0.18
Fb + CPPs	1.22 ± 0.03	0.45 ± 0.17	46.06 ± 0.47	0.41 ± 0.15	0.79 ± 0.09	80.49 ± 20.44	0.68 ± 0.08	1.20 ± 0.26	97.95 ± 21.44	1.08 ± 0.23
FbFe + CPPs	11.06 ± 0.08	0.83 ± 0.47	7.50 ± 4.19	0.76 ± 0.43	0.83 ± 0.56	7.49 ± 0.56	0.76 ± 0.05	1.66 ± 0.41	15.01 ± 3.71	1.51 ± 0.38
FbZn + CPPs	0.97 ± 0.01	0.38 ± 0.21	39.24 ± 22.04	0.35 ± 0.19	0.53 ± 0.04	54.73 ± 4.50	0.48 ± 0.04	0.91 ± 0.18	93.81 ± 16.09	0.83 ± 0.16
FbFeZn + CPPs	7.96 ± 0.77	0.46 ± 0.33	5.39 ± 3.84	0.42 ± 0.30	0.76 ± 0.11	8.92 ± 1.28	0.69 ± 0.10	1.22 ± 0.73	15.33 ± 5.76	1.11 ± 0.61
FbM	1.56 ± 0.34	0.76 ± 0.18	48.42 ± 11.56	1.16 ± 0.28	0.26 ± 0.06	16.45 ± 3.64	0.40 ± 0.09	1.02 ± 0.12	65.06 ± 7.69	1.56 ± 0.18
FbMFe	16.39 ± 0.98	2.93 ± 1.28	17.91 ± 7.78	1.68 ± 0.73	5.37 ± 0.16	32.79 ± 0.98	3.08 ± 0.09	8.30 ± 0.73	50.64 ± 5.76	4.76 ± 0.61
FbMZn	0.73 ± 0.18	0.10 ± 0.03	13.53 ± 4.01	0.10 ± 0.03	0.15 ± 0.09	20.42 ± 12.18	0.15 ± 0.09	0.25 ± 0.18	34.25 ± 16.09	0.25 ± 0.16
FbMFeZn	13.46 ± 0.52	3.54 ± 0.86	26.31 ± 6.40	3.34 ± 0.81	2.31 ± 0.98	17.17 ± 7.27	2.18 ± 0.92	5.85 ± 1.84	44.95 ± 15.76	5.53 ± 1.73

% Retention, transport or uptake = 100 × (μg Fe retention, transport or uptake)/μg Fe soluble added.
Protein range: between (0.65–1.74) mg cell protein/well.
[a] Fb: fruit beverage; FbFe: fruit beverage with Fe; FbZn: fruit beverage with Zn; FbFeZn: fruit beverage with Fe and Zn; Fb + CPPs: fruit beverage with CPPs; FbFe + CPPs: fruit beverage with Fe and CPPs; FbZn + CPPs: fruit beverage with Zn and CPPs; FbFeZn + CPPs: fruit beverage with Fe, Zn and CPPs; FbM: fruit beverage with milk; FbMFe: fruit beverage with Fe and milk; FbMZn: fruit beverage with Zn and milk; FbMFeZn: fruit beverage with Fe, Zn and milk.

according to whether comparison was made with M1 or M3, respectively.

Second-order interactions (Box, Hunter, & Hunter, 1978) between the factors Fe and Zn content, which presents two levels, was calculated as $C = (A - B)/2$, where A is the difference in Fe retention or transport or uptake between the high level (1) and the low level (0) of a factor, in the high level of the other factor. B is the same difference, though in the low level of the latter.

Interactions between two factors, one with two levels (Fe or Zn content) and the other with three (matrix) were calculated in the same way, but taking into account the three differences (M1M2, M2M3 and M1M3) between the high and low levels of the Fe or Zn content factor – the high and low levels for the matrix factor being the same as when studying the main effect.

3. Results and discussion

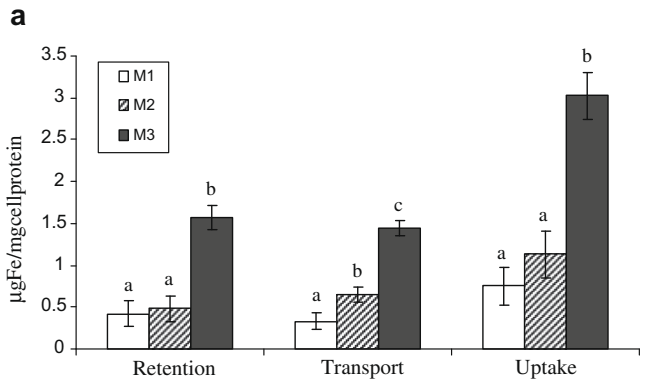
3.1. Influence of CPPs or milk on Fe bioavailability

The results obtained in Fe retention, transport and uptake are shown in Table 3. Fig. 1a presents the results of ANOVA for Fe retention, transport and uptake taking into account the matrix factor expressed as μg/mg cell protein, independently of the Fe and Zn content factors. The figure shows significantly higher ($p < 0.05$) Fe retention and uptake in samples with milk (M3) than in samples without (M1) or with CPPs (M2) (uptake: 4 and 2.7 times higher; retention: 3.7 and 3.3 times higher, respectively). There were statistically significant differences in Fe transport for three matrixes, following the order: M3 > M2 > M1.

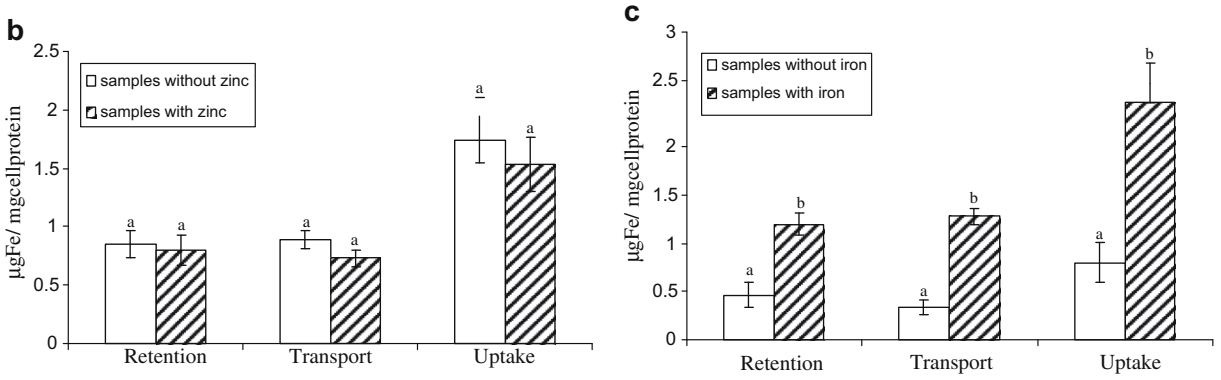
In vitro studies (Caco-2 cells) that evaluated the influence of CPPs on Fe bioavailability are contradictory. The addition to human milk of CPP preparations with variable degrees of hydrolysis (16–20%) and corresponding to different commercial sources exerted no effect, or reduced the synthesis of ferritin (Etcheverry et al., 2004).

Yeung et al. (2001 and 2002) found that the addition of CPPs (90% as phosphoserine clustered peptides) to Fe citrate chelate solution not subjected or subjected to simulated gastrointestinal digestion decreased the Fe uptake percentage or ferritin synthesis, respectively. This result was attributed to the greater affinity of CPPs for Fe versus citrate, where the binding of Fe to CPPs inhibited Fe uptake. However, we did not observe a decrease in Fe uptake. This could be attributed to various factors: the Fe salt used, i.e., sulphate versus citrate; the use of a soluble fraction of fruit beverages, where other components present could modify uptake, versus a model solution, and the different CPPs:Fe ratios used (367:1 or 3958:1 in Fe samples supplemented or not, respectively, versus 125:1 or 1250:1 in above mentioned studies).

Yeung et al. (2002), when using a CPP preparation with a lesser number of phosphoserine clustered peptides (20%) added to Fe sulphate solution recorded no significant differences ($p < 0.05$) in ferritin synthesis. This result agrees with our study, where the same Fe salt was used, and the CPPs: Fe ratio in samples not supplemented with Fe (3958:1) was similar to that used by these authors (4412:1).



M1 = samples without CPPs; M2 = samples with CPPs; M3 = samples with milk.



Different letter (a, b, c) in the same group in the same graphic indicates statistically significant differences (p<0.05).

Fig. 1. Representation of ANOVA mean values and standard deviation for the iron retention, transport and uptake expressed as μg/mg cell protein taking into account the following factors: (a) the matrix, (b) Zn content, and (c) Fe content.

It also has been demonstrated that the different fractions of CPPs present different effects on Fe bioavailability. Kinbagou et al. (2005) showed in Caco-2 cells that Fe bound to β-CN(1–25) presented a higher total uptake and retention of Fe than Fe gluconate or Fe bound to a mixture of α_{s1}-CN(59–79) and α_{s2}-CN(2–21), thus indicating that structure and conformation are important. This fact was confirmed by Bouhallab et al. (2002), who in a perfused rat duodenal loop system observed a higher uptake and net absorption of Fe from fractions β-CN versus α_s-CN. In a previous study by our group, the CPPs of the pool used in this work were identified, obtaining a greater percentage of CPPs proceeding from α_s-CN versus β-CN fractions (data not shown). This could explain the lack of an enhancer effect of CPPs added to fruit beverages upon Fe uptake.

In samples with added CPPs (M2), Fe transport was higher than in samples without CPPs (M1) (see Fig. 1a) – no modifications in retention and uptake being observed. These results agree the observations of Kibangou et al. (2008), who in Caco-2 cells observed that Fe transport of Fe bound to β-CN(1–25) was higher than in the case of Fe sulphate (the same salt used by us) or Fe pyrophosphate, though they reported lesser Fe retention and uptake. The differences with respect to this study could be due to the fact that we have worked with fruit beverages subjected to simulated gastrointestinal digestion with/without added CPPs, while the above authors worked with Fe solutions without food matrix and a specific CPP (β-CN(1–25)).

In the opposite sense, the above mentioned authors in an earlier study (Kinbagou et al., 2005) did not observe improvement in Fe transport (net absorption) in Caco-2 cells or rat duodenal loop in the presence of β-CN(1–25) versus Fe gluconate.

The presence of milk in fruit beverages had a positive effect on fruit beverages Fe retention, transport and uptake. This effect moreover was greater than the effect of CPPs added to soluble fractions of fruit beverages (see Fig. 1a). This positive effect of milk has been reported by others authors, who observed that the addition of milk increased ferritin synthesis from cereal fortified with Fe sulphate, the same Fe compound used in this study (Wortley et al., 2005), and from a milk based fruit beverage (Cilla et al., 2008), using an *in vitro* digestion and Caco-2 cell model. Whereas Cilla et al. (2008) attributed this effect to CPPs, Wortley et al. (2005) indicated that perhaps the milk proteins prevent some of the binding of Fe to inhibitors such as phytates and phenolics, thus increasing its bioavailability. The main inhibitors of Fe bioavailability in the samples used in our study (fruit beverages) are polyphenols, which could interact with milk proteins.

In a recent study, Argyri et al. (2007) demonstrated in Caco-2 cells a positive effect on ferritin synthesis from milk digest and different peptidic fractions. The most pronounced effect was observed for two peptidic fractions of molecular weights between 1000 and 5000, and these authors speculated that active peptides in these peptidic fractions were CPPs. In this sense, in the soluble fraction of milk based fruit beverage (FbM) used in our study, 10 CPPs of molecular weights between 880 and 2455 proceeding from α_{s1}-, α_{s2}- and β-CN were identified (data not showed). This and the previously commented interaction between polyphenols and milk proteins could explain the increase in Fe retention, transport and uptake of samples with milk (M3).

3.2. Influence of Fe and Zn content on Fe bioavailability. Interactions

The main effects of Zn and Fe content factors and the values obtained for second-order interactions are shown in Table 4. Zinc supplementation did not affect Fe retention, transport or uptake (Fig. 1b, and Table 4), independently of the matrix and Fe content factors. Iron supplementation, as expected, increased Fe retention, transport and uptake (Fig. 1c, and Table 4), independently of the matrix and Zn content factors.

With respect to the interaction between the factors matrix and Zn content (Table 4, and Fig. 2a–c), the only significant interaction ($p < 0.05$) corresponds to M1M3 × Zn for Fe transport. However, in the interactions M1M3 × Zn and M2M3 × Zn for retention, M1M2 × Zn and M2M3 × Zn for transport, and M1M2 × Zn and M1M3 × Zn for uptake, prevalence applies to the differences obtained – despite the fact that significance was not reached ($p < 0.05$), given the magnitude of the interaction (see Table 4).

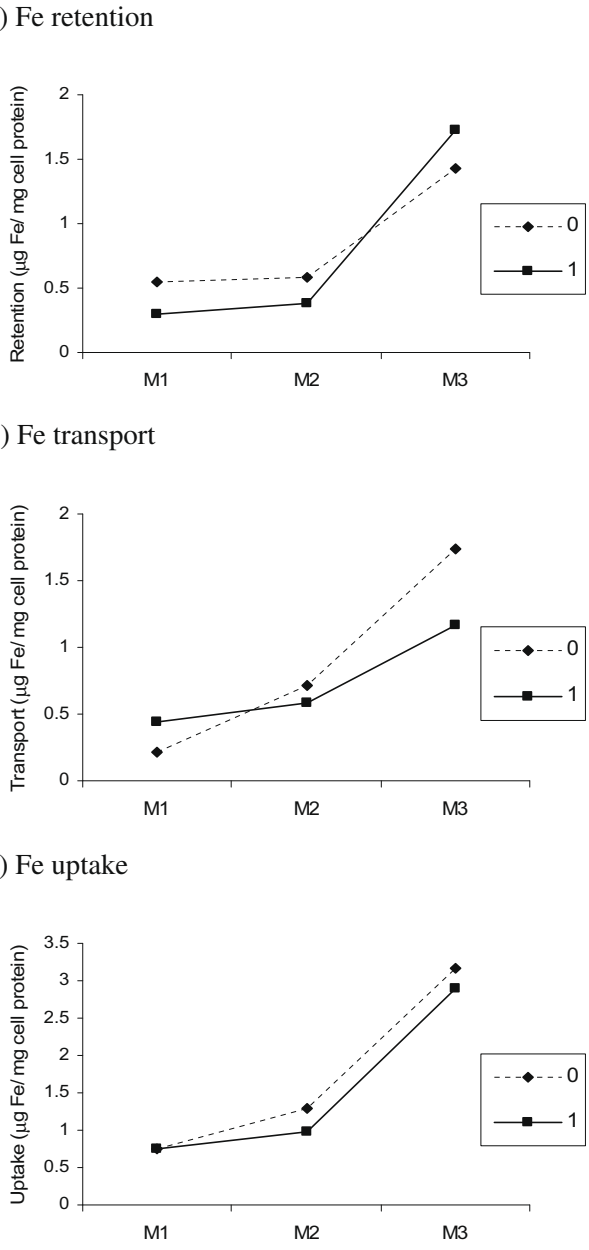
Zinc supplementation of samples with CPPs (M2) and milk (M3) reduces Fe transport and uptake. In the case of retention, Zn supplementation in M2 reduces retention, while an increase is noted in M3. In contrast, Zn supplementation in samples without CPPs (M1) does not affect uptake, increases transport, and reduces retention. It must be noted that the highest Fe retention, transport and uptake values correspond to the samples with milk (M3), regardless of whether they are supplemented with Zn or not (see Fig. 2a–c). As a result, interaction between the two factors is conditioned by the effect of Zn supplementation in the different matrices.

Table 4
Main effect and second-order interactions obtained.

Main Effect		Mean ± standard deviation (μg Fe/mg cell protein)		
		Retention	Transport	Uptake
Matrix addition	M1M2	0.058 ± 0.007	0.320 ± 0.001[a]	0.381 ± 0.051
	M1M3	1.151 ± 0.013[a]	1.121 ± 0.004[a]	2.275 ± 0.051[a]
	M2M3	1.093 ± 0.006[a]	0.801 ± 0.004[a]	1.894 ± 0.001[a]
Fe addition		0.740 ± 0.003[a]	0.931 ± 0.002[a]	1.670 ± 0.001[a]
Zn addition		−0.048 ± 0.003	−0.156 ± 0.002	−0.203 ± 0.027
Second-order interactions				
Matrix × Zn content	M1M2 × Zn	0.023 ± 0.010	−0.180 ± 0.001	−0.154 ± 0.066
	M1M3 × Zn	0.272 ± 0.018	−0.399 ± 0.006[a]	−0.124 ± 0.001
	M2M3 × Zn	0.248 ± 0.028	−0.219 ± 0.006	0.029 ± 0.066
Matrix × Fe content	M1M2 × Fe	0.044 ± 0.010	−0.075 ± 0.001	−0.034 ± 0.001
	M1M3 × Fe	0.879 ± 0.018[a]	1.029 ± 0.006[a]	1.905 ± 0.001[a]
	M2M3 × Fe	0.834 ± 0.028[a]	1.104 ± 0.006[a]	1.939 ± 0.001[a]
Fe × Zn content		0.457 ± 0.015[a]	0.004 ± 0.003	0.460 ± 0.038

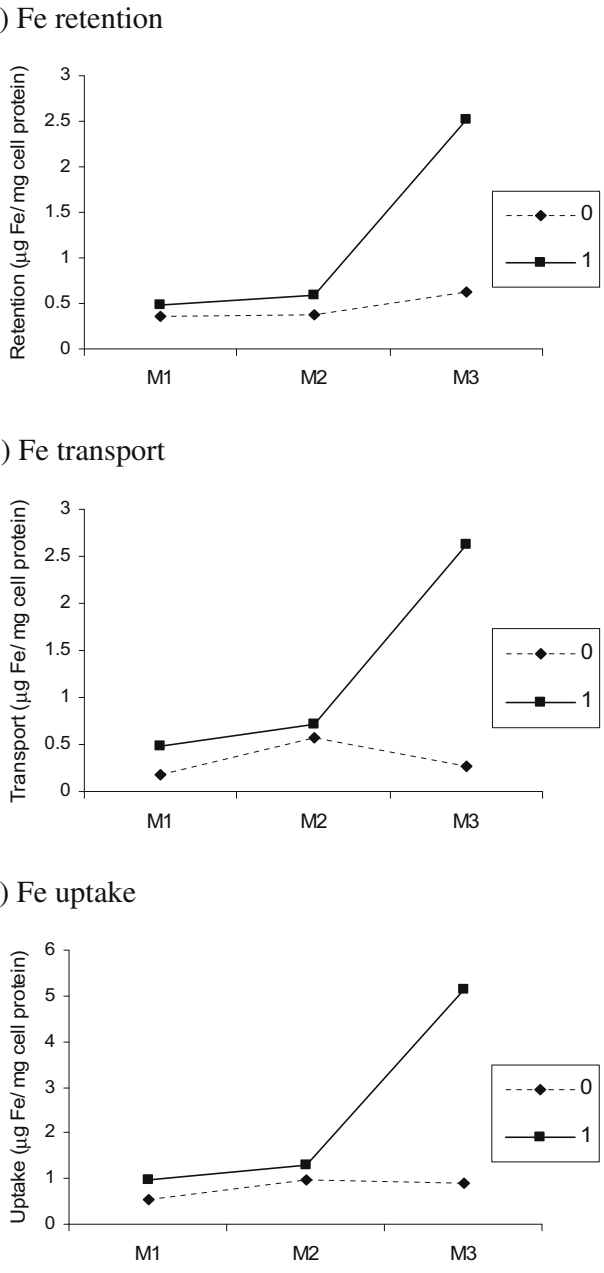
M1: samples without CPPs (Fb, FbFe, FbZn, and FbFeZn); M2: samples with CPPs (Fb + CPPs, FbFe + CPPs, FbZn + CPPs, and FbFeZn + CPPs); M3: samples with milk (FbM, FbMFe, FbMZn, and FbMFeZn).

[a] Indicates significant differences ($p < 0.05$).



M1: samples without CPPs; M2: samples with CPPs; M3: samples with milk; 0: samples not supplemented with Zn; 1: samples supplemented with Zn.

Fig. 2. Interactions graphics between the factors matrix and Zn content for retention, transport and uptake of Fe.



M1: samples without CPPs; M2: samples with CPPs; M3: samples with milk; 0:samples not supplemented with Fe; 1: samples supplemented with Fe.

Fig. 3. Interactions graphics between the factors matrix and Fe content for retention, transport and uptake of Fe.

With respect to interaction between the factors matrix and Fe content (Table 4, and Fig. 3a–c), all the interactions for these two factors are significant ($p < 0.05$) for Fe retention, transport and uptake, except those corresponding to M1M2 × Fe (see Table 4). This corroborates the above mentioned positive effect of the presence of milk upon Fe bioavailability.

Iron supplementation increases Fe retention, transport and uptake in all the samples analysed. This effect in turn is more pronounced for the samples with milk (M3) (see Fig. 3a–c).

With respect to interaction between the factors Zn and Fe content (Table 4, and Fig. 4a and b), Fe × Zn interaction is only statistically significant and positive for Fe retention. However, while not statistically significant, in the Fe × Zn interaction in relation to Fe uptake, prevalence applies to the difference obtained, in view of the magnitude of the interaction (see Table 4).

In relation to Fe retention and uptake, the behaviour differs according to supplementation of the samples with the two mineral elements. In samples supplemented with Fe, the addition of Zn increases Fe retention and uptake, independently of the matrix factor – thus indicating that the interaction between the two factors is positive (see Fig. 4a and b).

In samples not supplemented with Fe (13 µM, with a molar ratio Fe:Zn in soluble fraction added to cell monolayer of 1:0.7 (samples without Zn supplementation) and 1:3.5 (samples with Zn

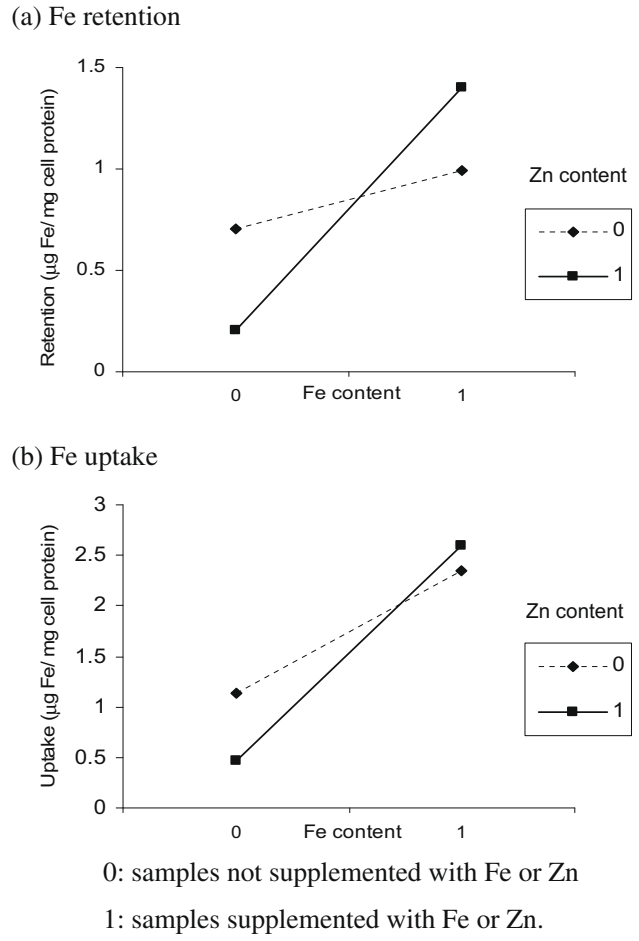


Fig. 4. Interactions graphics between the factors Fe and Zn content for retention and uptake of Fe.

supplementation)), the uptake and retention of Fe decreased when the Zn concentration was higher than the Fe concentration (see Table 3, and Fig. 4a and b). These results agree with the interaction reported by Arredondo, Martínez, Núñez, Ruz, and Olivares (2006) in Caco-2 cells, showing a decrease in Fe uptake with a Fe:Zn molar ratio of up to 1:1 using a Fe concentration of 10 µM. These authors used standard solutions of Fe and Zn, whereas in our study these elements have been added to a food matrix (fruit beverage) and subjected to simulated gastrointestinal digestion, which implies increased similarity to the *in vivo* situation. In a previous study by our group, a negative correlation was observed between Fe retained and the amount of Zn present in the soluble fraction from school meals obtained by the same simulated gastrointestinal digestion as used in this study (Cámara, Barberá, Amaro, & Farré, 2007), at a range of Fe soluble concentrations added to Caco-2 cells of between 12 and 49 µM, and a molar ratio Fe:Zn of between 1:0.69 and 1:2. The addition of 25 or 50 µM of Zn to a food matrix digest with 50 µM of Fe decreased ferritin synthesis by 58% and 82%, respectively, in Caco-2 cells (Glahn, Wortley, South, & Miller, 2002).

However, Tandy et al. (2000) and Yamaji et al. (2001) observed no significant decrease in Fe uptake when Caco-2 cells were incubated with a standard solution of 1 µM ^{55}Fe in the presence of 100-fold excess Zn. It is difficult to compare these results with those obtained in the present study, since the Fe and Zn concentrations used are very different, and the authors moreover used standard solutions – not a food matrix subjected to simulated gastrointestinal digestion, as in our case.

In our samples supplemented with Fe (139 µM, with a molar ratio Fe:Zn in the soluble fraction added to the cell monolayer of 1:0.06 (samples without Zn supplementation) and 1:0.3 (samples with Zn supplementation)), the Zn content of samples did not decrease Fe uptake, but increased Fe retention (see Table 3, and Fig. 4a and b). Using the perfused rat loop model, Pèrés et al. (1999a) reported a decrease in the net absorption of Fe as gluconate (100 µM), in the presence of Zn sulphate at a Fe:Zn molar ratio of 1:1.5.

Recently, in humans, it has been shown that at equimolar concentrations of Zn and Fe (1:1), no significant effect of Zn on Fe bioavailability is observed at lower doses (0.5 mg of Fe and 0.59 mg of Zn). However, higher doses (10 mg of Fe and 11.71 mg of Zn) exert a 56% inhibitory effect on Fe bioavailability (Olivares, Pizarro, & Ruz, 2007). These observations could explain the different behaviour of the effect of Zn on Fe uptake according to whether the samples are supplemented or not with this element.

4. Conclusion

The addition of milk to fruit beverages exerted a positive effect on iron retention, transport and uptake versus fruit beverages, and this effect was greater than in the case of CPPs added to soluble fractions of fruit beverages. The addition of CPPs to soluble fractions of fruit beverages improved iron transport. Although Zn supplementing of the fruit beverages exerted no statistically significant influence ($p < 0.05$) on iron retention, transport and uptake, we consider it to be interesting that a decrease in iron retention, transport and uptake was observed when the molar ratio Fe:Zn was 1:3.5 (samples supplemented with Zn and not with Fe).

More studies are clearly needed to confirm the results, especially in humans, to determine which concrete caseinophosphopeptides favour iron bioavailability. In addition, studies are required on the addition of functional ingredients to fruit beverages with the purpose of favouring iron bioavailability.

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