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Iron Bioavailability in Fortified Fruit Beverages Using Ferritin Synthesis by Caco-2 Cells

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The bioavailability of iron from fortified fruit beverages was estimated by an in vitro system including enzymatic digestion, iron uptake by Caco-2 cells, and ferritin formation determined via an enzyme immunoassay (ELISA). Thus, the aim of the present study was to assess iron bioavailability as influenced by the presence of known dietary promoter and inhibitory factors in fortified fruit beverages containing iron and/or zinc and/or skimmed milk. No negative effect ($p > 0.05$) derived from micronutrient interaction can be ascribed to zinc supplementation on iron availability. Besides, the presence of caseinophosphopeptides derived from casein hydrolysis during digestion may confer enhancing effects on iron absorption in samples with milk added with respect to nonadded samples ($p < 0.05$). Therefore, from a nutritional point of view, individuals in need of optimal iron absorption may choose dairy samples to ensure optimal iron bioavailability.

KEYWORDS: In vitro digestion; iron bioavailability; Caco-2 cells; ferritin; fruit beverages

INTRODUCTION

Iron deficiency anemia is one of the most common nutritional problems worldwide. This lack of iron appears when iron demand by the body is not met by iron provided by or absorbed from the diet. Of the two types of iron in the diet, heme and nonheme iron, the latter is very abundant in vegetable foods and is used for the fortification of foodstuffs (*1*). However, it is assumed that poor bioavailability of dietary nonheme iron is a major factor contributing to the problem of anemia. On the other hand, zinc deficiency can result in the deterioration of many metabolic functions, since this mineral is a cofactor for over 200 biologically important enzymes involved in protein synthesis (*2*). Moreover, micronutrient interactions between iron and zinc must be taken into account in the mineral supplementation of fruit beverages (Fbs), due to the fact that mineral elements having similar electronic configurations can share absorption pathways, resulting in competition for uptake by the mucosal cells (*3*). Altogether, the efficacy of iron fortificants is influenced by the interplay of enhancer/promoter (ascorbic acid, citric acid, meat, and β-casein hydrolysis products, for example, caseinophosphopeptides) and inhibitory factors (phytic acid, polyphenols, casein, and other minerals such as calcium and zinc) present in the food matrix and/or diet (*4*).

To improve nutritional value and provide bioactive components, Fbs are often commercially supplemented with milk, vitamins, and/or minerals. In this context, Haro-Vicente et al. (*5*) have reported that citric fruit juices are suitable for iron fortification from a nutritional standpoint, since these beverages lack certain inhibitors such as phytates or oxalates and present reduced problems of iron fortificant stability and discoloration due to added iron remaining in the ferrous state under the low pH conditions of these acid beverages, together with quicker mineral absorption from liquid rather than solid foods.

Because nonheme iron needs to be in ionic and soluble form to be available for absorption, the first step toward bioavailability is represented by solubility within the intestinal tract (bioaccessibility), to allow subsequent absorption (*6*). In vitro solubility or dialysability criteria to estimate iron bioavailability have been used in studies of fortified Fbs using dialysis in citric fruit juices (*5*) or solubility, dialysis and uptake, and transport by Caco-2 cells in fruit juices containing milk and cereals (*4*). On the other hand, in vivo iron bioavailability has been reported in studies of Fbs accompanying a meal to ensure the possible enhancing effect of these Fbs upon iron absorption (*7*−*9*). In the latter studies, iron absorption was measured from red blood cell incorporation of iron stable isotopes after the administration of radiolabeled meals.

The use of a coupled in vitro Caco-2 cell/ferritin synthesis model that closely mimics the physiological situation may allow us to assess iron availability from foods through highly sensitive and accurate measures—eliminating the need for extrinsic or intrinsic labeling of food iron in availability assays (*10*). This method has been used in fruit juices (*11*) measuring ferritin formation with an immunoradiometric assay. In turn, ferritin formation determined via enzyme immunoassay (enzyme-linked immunosorbent assay, ELISA) has only been applied to model systems (*12*) or milk-based infant formulas (*13*).

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Table 1. Composition of the Fbs Analyzed[a]

component (g/100 g)	sample			
	FbFe	FbFeZn	FbMFe	FbMFeZn
osmosis water	58.716	58.716	57.676	57.676
apricot puree	24.5	24.5	24.5	24.5
grape concentrate	7.15	7.15	7.15	7.15
orange concentrate	4.15	4.15	4.15	4.15
sugar	5.076	5.076	5.076	5.076
skimmed milk powder			1.04	1.04
pectin	0.354	0.354	0.354	0.354
L-ascorbic acid	0.054	0.054	0.054	0.054
Fe-sulfate	0.003	0.003	0.003	0.003
Zn-sulfate		0.0016		0.0016

[a] Fb = fruit beverage (grape + orange + apricot). Fe, supplemented with Fe sulfate (3 mg/100 mL of Fb). Zn, supplemented with Zn sulfate (1.6 mg/100 mL of Fb). M, skimmed milk 11% (v/v).

To our knowledge, this is the first work that determines iron bioavailability in fortified Fbs with iron and/or zinc and/or skimmed milk through Caco-2 ferritin formation via ELISA measurement. Thus, the aim of the present study was to assess iron bioavailability as influenced by the presence of known dietary promoter and inhibitory factors in fortified Fbs.

MATERIALS AND METHODS

Samples. Four Fbs were used (all of them containing grape, orange, and apricot) with iron (Fe sulfate 3 mg/100 mL of Fb), with/without zinc (Zn sulfate 1.6 mg/100 mL of Fb), and with/without skimmed milk (M) (11% v/v): FbFe, FbFeZn, FbMFe, and FbMFeZn. The composition of these Fbs is reported in **Table 1**.

In Vitro Digestion: Solubility Method. The in vitro digestion protocol was applied to Fbs as described previously by Glahn et al. (*10*) with minor modifications as indicated by Perales et al. (*14*) and comprising two sequential steps (gastric and intestinal). Briefly, initial weights of 80 g for each sample were adjusted to pH 2.0 with 6 M HCl (GLP 21 pH meter, Crison, Barcelona, Spain). The pH was checked after 15 min and if necessary readjusted to 2.0. Then, an amount of freshly prepared demineralized pepsin solution sufficient to yield 0.02 pepsin/g sample was added. The samples were made up to 100 g with cell culture-grade water (Aqua B Braun, Braun Medical, Barcelona, Spain) and incubated in a shaking water bath at 37 °C/120 strokes per minute for 2 h (SS40-2, Gran Instruments, Cambridge, United Kingdom). The gastric digests were maintained in ice for 10 min to stop pepsin digestion.

For the intestinal digestion stage, the pH of the gastric digests was raised to pH 6.5 by dropwise addition of 1 M $NaHCO_3$. Then, an amount of freshly prepared and previously demineralized pancreatin−bile salt solution sufficient to provide 0.005 g pancreatin and 0.03 g bile salt/g 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 then adjusted to 7.2 by dropwise addition of 0.5 M NaOH.

Aliquots of 20 g of sample were transferred to polypropylene centrifuge tubes (50 mL, Costar, New York) and centrifuged at 3500*g* for 1 h at 4 °C (GT422 centrifuge, Jouan, Saint Nazaire, France), and supernatants were used to determine the iron bioaccessible content (soluble mineral content). The iron contents of Fbs and bioaccessible fractions were determined by atomic absorption spectrophotometry (AAS) (model 2380; Perkin-Elmer, Norwalk, CT). Prior to iron determination, the organic matter of samples was destroyed by ashing at 450 °C (model K1253A; Heraeus Instruments, Hanau, Germany). The bioaccessible fraction (soluble fraction) was used in the Caco-2 cell ferritin assays.

Cell Culture. Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC 86010202, Salisbury, United Kingdom) and were used between passages 30 and 36. The cells were cultured and maintained according to Viadel et al. (*13*) under low iron conditions.

Table 2. Total (μg/g Fb) and Bioaccessible Iron Content (μg/g Fb)[a]

samples	total content μg/g Fb	bioaccessible μg/g Fb	bioaccessibility[b] (%)
FbFe	33.49 ± 1.87	32.61 ± 1.13	97.38 ± 3.38 a
FbFeZn	36.25 ± 2.41	19.26 ± 1.19	53.13 ± 3.28 b
FbMFe	28.72 ± 0.42	25.36 ± 3.54	88.31 ± 12.31 a
FbMFeZn	28.44 ± 0.03	27.72 ± 1.34	97.46 ± 4.70 a

[a] Results are expressed as means ± standard deviations ($n = 4$). Values with no letters in common are significantly different ($p < 0.05$). [b] Bioaccessibility (%) = 100 × bioaccessible iron content/total iron content.

Ferritin Assay. The ferritin measurement method was applied as previously described by Glahn et al. (*10*) but adapted to an ELISA determination instead of an immunoradiometric assay as indicated by Viadel et al. (*13*). Immediately before addition of the bioaccessible fraction, the MEM (minimum essential medium; Gibco BRL Life Technologies, Scotland) was removed from each well, and the cell monolayer was washed three times with PBS at 37 °C. Then, 1 mL of the bioaccessible fraction plus 1 mL of MEM or 2 mL of MEM (blank) was allowed to remain on the cell monolayer for 2 h at 37 °C in 5% CO_2 with 95% relative humidity. Next, solution added to the cells was removed and replaced by MEM, and cells were returned to the incubator for an additional 22 h.

Cell monolayers were washed three times with buffer solution and detached with trypsin-EDTA solution. Subsequently, cells were collected with 2 mL of deionized water at 4 °C and homogenized at 17000 rpm for 3 min at 4 °C (Polytron PT 2000, Kinematica AG). Ten microliter aliquots of the sonicated Caco-2 monolayer were used in ferritin determination (Spectro Ferritin kit, Catalog number S-22, Ramco Laboratories Inc., Stafford, TX). Control cells (blank) were used throughout the experiments. The cell protein content was determined according to Lowry et al. (*15*) for expressing the ferritin contents as ng ferritin/mg protein and to evaluate reproducibility of the cell cultures.

Cell Viability Test. The mitochondrial activity of Caco-2 cells after exposure to bioaccessible fractions of Fbs was evaluated by using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide) assay (*16*). This colorimetric method is based on the reduction of the tetrazolium ring of MTT by mitochondrial dehydrogenases, yielding a blue formazan product that can be measured spectrophotometrically; the amount of formazan produced is proportional to the number of viable cells. The conversion to insoluble formazan was measured at 570 nm with background subtraction at 690 nm. Control cells were used through each assay.

Statistical Analysis. The results obtained were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test to determine significant differences among means. Values of $p < 0.05$ were considered significant. The Statgraphics Plus version 5.1 statistical package (Rockville, MD) was used throughout.

RESULTS AND DISCUSSION

The contents of total and bioaccessible (soluble) iron of fortified Fbs are shown in **Table 2**. The total iron content of the four Fbs analyzed is in good agreement with the data provided by the manufacturer (~3 mg/100 mL Fb) (see **Table 1**).

The Fbs of the present study contain some dietary factors that interact to facilitate [organic acids—mainly ascorbic acid—and casein-derived phosphopeptides (CPPs)] or inhibit iron absorption (calcium, zinc, fiber, and polyphenols). In addition, iron absorption is correlated to its intestinal solubility; thus, mineral solubility can be used to establish trends in the bioavailability or relative bioavailability of iron, because the chance for iron uptake increases with higher solubility (*17*).

All samples offered high iron bioaccessibility (>88%), except FbFeZn, which presented a lower bioaccessible percentage (53%). This fact could be explained by iron and zinc interactions, since it is thought that some metallic ions with similar physicochemical properties could compete for the same cellular

Table 3. Caco-2 Cell Ferritin Levels from Fbs [Mean Values ± Standard Deviation ($n = 6$)] and Iron (μg) Provided by the Bioaccessible Fractions[a]

samples	Fe added to the cell monolayer (μg)	ferritin ng/mg cell protein	viability (%)[b]
blank		2.13 ± 0.45 a	100.00 ± 0.90
FbFe	22.39 ± 0.78 a	17.26 ± 2.32 b	101.00 ± 1.20
FbFeZn	13.53 ± 2.75 b	17.94 ± 4.66 b	99.70 ± 1.50
FbMFe	17.55 ± 2.45 c	53.40 ± 8.95 c	100.10 ± 1.10
FbMFeZn	19.37 ± 0.93 c	49.77 ± 8.56 c	99.30 ± 1.30

[a] Values with no letters in common are significantly different ($p < 0.05$). [b] Cell viability measured by means of the MTT test [mean values ± standard deviation ($n = 9$)].

transport systems, and as a result of such negative interaction, the mineral availability of both micronutrients could be affected as previously reviewed (*3*), and as reported by our own group using the percentage of dialysis to estimate mineral bioavailability in milk and soy-based infant formulas (*18*)—since iron absorption is especially impaired in the presence of zinc in aqueous solutions (*3*).

This situation is not observed in FbMFeZn, where iron bioaccessibility is much higher (97%) vs FbFeZn. CPPs produced during in vitro digestion seem to be involved in this event, due to the presence of a highly polar acidic sequence of three phosphoseryl groups followed by two glutamic acid residues, which are binding sites for minerals such as calcium, iron, and zinc. CPPs can bind iron with greater affinity than zinc and improve its bioavailability (*19*). An increase in iron solubility in the presence of CPP at intestinal level has been reviewed (*1*) and has also been reported with in vitro simulated gastrointestinal digestion studies using model solutions (*20*).

On the other hand, it has been reported that the inhibition of iron by zinc sulfate is overcome by the presence of the caseinophosphopeptide 1−25 of β-casein (*21*). In a later study (*22*), it was shown that β (1−25) allows strong reduction of the inhibiting effects of Zn on iron. This CPP [β-CN (1−25)] contains four out of the five phosphoserine residues present in the native protein, which allows 1 mol of 1,25-β-caseinophosphopeptide to bind up to 4 mol of iron. The stability constants for iron complexes with this caseinophosphopeptide are greater than the respective stability constants for calcium and zinc—thereby diminishing mineral interactions (*23, 24*). Therefore, iron binding to CPPs protects the former from inhibitory interactions with other divalent cations.

Results of ferritin contents in Caco-2 cells exposed to bioaccessible fractions of fortified Fbs together with cell viability are shown in **Table 3**. Viability values of cells preincubated for 2 h with bioaccessible fractions of digested samples, added in 1:1 proportion (v/v) with culture media, reveal important viability for all samples tested equal to control (blank) cells, in agreement with a previous study (*25*). Regarding ferritin synthesis, blank cells grown in culture medium under low iron conditions presented values of 2.13 ± 0.45 ng ferritin/mg protein, in accordance with the amount of ferritin content previously reported by Viadel et al. (*13*) of 3.58 ± 1.26 ng ferritin/mg protein under the same conditions.

The differences between the bioaccessibility of iron from FbFe and FbFeZn mentioned above (**Table 2**) were not found in the uptake of iron from the same samples measured by ferritin formation. There were no differences ($p > 0.05$) in ferritin formation by FbFe and FbFeZn samples (**Table 3**). The presence of a human-derived component, that is, Caco-2 cells, improves the system and allows more suitable results than solubility measures, since the latter are not a complete measure of iron bioavailability (*10*). It has been reported that in model solutions, $ZnCl_2$ inhibits the uptake of iron by Caco-2 cells measured by ferritin formation when present at Fe-to-Zn ratios of 1:0.5 (similar to our study, 1:0.53) and 1:1. However, pretreatment of the cells with 50 μM Zn for 48 h did not affect iron uptake (*26*). The use of model solutions that lack interacting factors, which can promote and/or hinder iron absorption, could explain the differences found with respect to the present study. It is well-known that ascorbic acid is a strong enhancer of iron absorption and promotes iron bioavailability by reducing Fe^{3+} to Fe^{2+} (the most readily absorbed form of iron) and by forming stable complexes with iron—preventing the latter from forming complexes with other dietary constituents that inhibit iron absorption, such as phytates and polyphenols, and increasing the solubility of iron by stabilizing it from oxidation and precipitation at neutral pH (*6*). Moreover, it has been reported that the beneficial promoting effect of ascorbic acid on iron availability may be most apparent in foods with high amounts of inhibitors of iron absorption (*9*), namely, in this case, zinc, fiber, and polyphenols. For many years, it has been recognized that fiber can negatively affect mineral absorption due to its capacity to bind cations. However, human and animal studies carried out with pectin (present in our Fbs) have not shown significant effects of soluble dietary fiber on mineral bioavailability (*1*). Polyphenols can negatively influence iron bioavailability due to Fe−phenolic complex formation in the gastrointestinal lumen, making it less available for absorption (*27*). The galloyl group of these compounds has been implicated as the structure responsible for such inhibition (*28*). Furthermore, to our knowledge, only one study in the literature has been carried out with fruit juices determining iron availability through ferritin formation. In this work, Boato et al. (*11*) studied iron bioavailability by means of ferritin synthesis in different fruit juices and concluded that dark-colored juices, such as red grape and prune juices, exert marked inhibitory effects on iron bioavailability, due to their high phenolic content that overwhelms the enhancing effect of ascorbic acid. Contrarily, light-colored juices such as orange and white grape juices (present in our Fbs) exhibited a promoting effect on iron uptake, since their content in galloyl groups related to phenolics is lower than in dark juices, and in consequence, ascorbic acid may counteract the negative effect of their phenolic compounds. In this study, 3.42 mg/100 g of iron was added to juices—this amount being similar to our Fbs (3 mg/100 mL Fb). Besides, ferritin formation values were ∼95 ng ferritin/mg protein and ∼70 ng ferritin/mg protein for orange and white grape juices, respectively. Ferritin contents of our samples were slightly lower (see **Table 3**) but were within the range indicated by Boato et al. (*11*) for different fruit juices (∼5 to ∼275 ng ferritin/mg protein).

Ferritin formation by FbMFe and FbMFeZn vs FbFe and FbFeZn demonstrates that the presence of milk added in the samples positively influences iron uptake (**Table 3**). A significant ($p < 0.05$) 3-fold increase in ferritin formation in samples with milk vs nonadded samples can be noted. Intact bovine milk proteins maintain iron soluble in the digestive tract but inhibit its absorption unless they are hydrolyzed (*29*). The increase in ferritin synthesis observed could be due to the effect of CPPs formed during gastrointestinal digestion. In this sense, in vitro

studies (*20, 30*) have noted an increase in ferritin synthesis by Caco-2 cells in the presence of CPPs.

After simulated gastrointestinal digestion of bovine casein, different CPPs are released from α_{s1}, α_{s2}, and β-casein (*31*). CPPs derived from α_s caseins have a tendency to decrease the quantity of bioavailable iron, while CPPs from β-casein increase iron bioavailability. Lesser net iron absorption has been observed, assessed by the duodenal rat loop model in the presence of whole CPPs vs β-CN or β-CN (1−25)—although higher than that obtained with iron gluconate (*32*). Previously, Peres et al. (*23*) showed higher iron bioavailability in the same model when iron was bound to 1−25 β-CN vs Fe-gluconate. In addition, a significant increase in absorption (at least two times higher) and total uptake (at least three times higher) of iron complexed to β-CN (1−25) vs Fe-α_{s1}-CN has been reported, using in vivo perfused rat intestinal loop and in vitro Caco-2 cells assays (*33*).

In summary, the mechanisms by which CPPs improve iron absorption and bioavailability can be attributed to an increase in solubility, yielding soluble complexes resistant to changes in pH and ionic strength occurring in the lumen and/or to the presence of a specific absorption pathway that could reduce interactions with other divalent cations such as zinc or calcium (*1, 34*). According to this, the sum of these properties may explain the beneficial effects of milk addition on iron uptake in mineral-fortified Fbs. Thus, it can be concluded that the presence of caseinophosphopeptides derived from casein hydrolysis during digestion may confer enhancing effects on iron uptake in fortified Fbs with milk added, with respect to nonadded beverages. In addition, no negative micronutrient interaction effect can be ascribed to zinc supplementation on iron availability. Therefore, from a nutritional point of view and based on the results here obtained, the daily intake of Fbs with milk and iron (FbMFe and FbMFeZn) can be an aid to improve iron status.

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LITERATURE CITED

(1) Amaro, M. A.; Camara, F. Iron availability: An updated review. *Int. J. Food Sci. Nutr.* **2004**, *55*, 597–606.
(2) Camara, F.; Amaro, M. A. Nutritional aspect of zinc availability. *Int. J. Food Sci. Nutr.* **2003**, *54*, 143–151.
(3) Sandström, B. Micronutrient interactions: Effects on absorption and bioavailability. *Br. J. Nutr.* **2001**, *85*, S181–S185.
(4) Perales, S.; Barbera, R.; Lagarda, M. J.; Farre, R. 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. *Food Chem.* **2007**, *102*, 1296–1303.
(5) Haro-Vicente, J. F.; Martinez-Gracia, C.; Ros, G. Optimization of in vitro measurement of available iron from different fortificants in citric fruit juices. *Food Chem.* **2006**, *98*, 639–648.
(6) Salovaara, S.; Sandberg, A. S.; Andlid, T. Organic acids influence iron uptake in the human epithelial cell line Caco-2. *J. Agric. Food Chem.* **2002**, *50*, 6233–6238.
(7) Ballot, D.; Baynes, R. D.; Bothwell, T. H.; Gillooly, M.; Macfarlane, B. J.; Macphail, A. P.; Lyons, G.; Derman, D. P.; Bezwoda, W. R.; Torrance, J. D.; Bothwell, J. E. The effects of fruit juices and fruits on the absorption of iron from a rice meal. *Br. J. Nutr.* **1987**, *57*, 331–343.
(8) Fairweather-Tait, S.; Fox, T.; Wharf, G.; Eagles, J. The bioavailability of iron in different weaning foods and the enhancing effect of a fruit drink containing ascorbic acid. *Pediatr. Res.* **1995**, *37*, 389–394.
(9) Shah, M.; Griffin, I. J.; Lifschitz, C. H.; Abrams, S. A. Effect of orange and apple juices on iron absorption in children. *Arch. Pediatr. Adolesc. Med.* **2003**, *157*, 1232–1236.
(10) Glahn, R. P.; Olivia, A. L.; Yeung, A.; Goldman, M. I.; Miller, D. D. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in vitro digestion/Caco-2 cell culture model. *J. Nutr.* **1998**, *128*, 1555–1561.
(11) Boato, F.; Wortley, G. M.; Liu, R. H.; Glahn, R. P. Red grape juice inhibits iron availability: application of an in vitro digestion/Caco-2 cell model. *J. Agric. Food Chem.* **2002**, *50*, 6935–6938.
(12) Tapia, V.; Arredondo, M.; Nũnez, M. T. Regulation of Fe absorption by cultured intestinal epithelia (Caco-2) cell monolayers with varied Fe status. *Am. J. Physiol.* **1996**, *271*, G443–G447.
(13) Viadel, B.; Perales, S.; Barbera, R.; Lagarda, M. J.; Farre, R. Ferritin synthesis by Caco-2 cells as an indicator of iron bioavailability: Application to milk-based infant formulas. *Food Chem.* **2007**, *102*, 925–931.
(14) Perales, S.; Barbera, R.; Lagarda, M. J.; Farre, R. Bioavailability of calcium from milk-based formulas and fruit juices containing milk and cereals estimated by in vitro methods (solubility, dialyzability, and uptake and transport by Caco-2 cells). *J. Agric. Food Chem.* **2005**, *53*, 3721–3726.
(15) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.
(16) Laparra, J. M.; Velez, D.; Montoro, R.; Barbera, R.; Farre, R. An approach to As(III) and As(V) bioavailabilty studies with Caco-2 cells. *Toxicol. in Vitro* **2005**, *19*, 1071–1078.
(17) Zhu, L.; Glahn, R. P.; Yeung, C. K.; Miller, D. D. Iron uptake by Caco-2 cells from NaFeEDTA and $FeSO_4$: Effects of ascorbic acid, pH, and a Fe (II) chelating agent. *J. Agric. Food Chem.* **2006**, *54*, 7924–7928.
(18) Jovani, M.; leMasle, M.; Alegria, A.; Barbera, R.; Farre, R.; Lagarda, M. J.; Clemente, G. *In vitro* interactions between calcium, zinc, copper and iron in milk- and soy-based infant formulas. *Food Sci. Technol. Int.* **2000**, *6*, 25–31.
(19) Miquel, E.; Gomez, J. A.; Alegria, A.; Barbera, R.; Farre, R.; Recio, I. Identification of casein phosphopeptides released after simulated digestion in milk-based infant formulas. *J. Agric. Food Chem.* **2005**, *53*, 3426–3433.
(20) Yeung, A. C.; Glahn, R. P.; Miller, D. D. Effects of iron source on iron availability from casein and casein phosphopeptides. *J. Food Sci.* **2002**, *67*, 1271–1275.
(21) Peres, J. M.; Bouhallab, S.; Bureau, F.; Maubois, J. L.; Arhan, P.; Bougle, D. Reduction of iron/zinc interactions using metal bound to the caseinophosphopeptide 1−25 of β-casein. *Nutr. Res. (N.Y.)* **1999**, *19*, 1655–1663.
(22) Bouhallab, S.; Bougle, D. Biopeptides of milk: Caseinophosphopeptides and mineral bioavailability. *Reprod. Nutr. Dev.* **2004**, *44*, 493–498.
(23) Peres, J. M.; Bouhallab, S.; Bureau, F.; Neuville, D.; Maubois, J. L.; Devroede, G.; Arhan, P.; Bougle, D. Mechanisms of absorption of caseinophosphopetide bound iron. *J. Nutr. Biochem.* **1999**, *10*, 215–222.
(24) Miquel, E.; Alegria, A.; Barbera, R.; Farre, R. Speciation analysis of calcium, iron, and zinc in casein phosphopeptide fractions from toddler milk-based formula by anion exchange and reverse-phase high-performance liquid chromatography-mass spectrometry/flame atomic-absorption spectroscopy. *Anal. Bioanal. Chem.* **2005**, *381*, 1082–1088.
(25) Cilla, A.; Laparra, J. M.; Alegria, A.; Barbera, R.; Farre, R. Antioxidant effect derived from bioaccessible fractions of fruit beverages against H_2O_2-induced oxidative stress in Caco-2 cells. *Food Chem.* **2008**, *106*, 1180–1187.
(26) Glahn, R. P.; Wortley, G. M. Inhibition of iron uptake by phytic acid, tannic acid, and $ZnCl_2$: Studies using an in vitro digestion/Caco-2 cell model. *J. Agric. Food Chem.* **2002**, *50*, 390–395.
(27) Hurrell, R. F.; Reddy, M.; Cook, J. D. Inhibition of non-haem iron absorption in man by polyphenolic-containing beverages. *Br. J. Nutr.* **1999**, *81*, 289–295.

(28) Brune, M.; Rossander, L.; Hallberg, L. Iron absorption and phenolic compounds: importance of different phenolic structures. *Eur. J. Clin. Nutr.* **1989**, *43*, 547–558.
(29) Hurrell, R. F.; Lynch, S. R.; Trinidad, P. Y.; Dassenko, S. A.; Cook, J. D. Iron absorption in humans as influenced by bovine milk proteins. *Am. J. Clin. Nutr.* **1989**, *49*, 546–552.
(30) Argyri, K.; Miller, D. D.; Glahn, R. P.; Zhu, L.; Kapsokefalou, M. Ppetides isolated from in vitro digests of milk enhance iron uptake by Caco-2 cells. *J. Agric. Food Chem.* **2007**, *55*, 10221–10225.
(31) Miquel, E.; Gomez, J. A.; Alegria, A.; Barbera, R.; Farre, R.; Recio, I. Identification of casein phosphopeptides after simulated gastrointestinal digestion by tandem mass spectrometry. *Eur. Food Res. Technol.* **2006**, *222*, 48–53.
(32) Bouhallab, S.; Cinga, V.; Ait-Oukhatar, N.; Bureau, F.; Neuville, D.; Arhan, P.; Maubois, J. L.; Bougle, D. Influence of various phosphopeptides of caseins on iron absorption. *J. Agric. Food Chem.* **2002**, *50*, 7127–7130.
(33) Kibangou, I. B.; Bouhallab, S.; Henry, G.; Bureau, F.; Allouche, S.; Blais, A.; Guerin, P.; Arhan, P.; Bougle, D. L. Milk proteins and iron absorption: contrasting effects of different caseinophosphopeptides. *Pediatr. Res.* **2005**, *58*, 731–734.
(34) Aït-Oukhatar, N.; Bouhallab, S.; Bureau, F.; Arhan, P.; Maubois, J. L.; Drosdowsky, M. A.; Bougle, D. L. Bioavailability of caseinophosphopeptide bound iron in the young rat. *J. Nutr.Biochem.* **1997**, *8*, 190–194.

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