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In Vitro Bioavailability of Iron from the Heme Analogue Sodium Iron Chlorophyllin

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The use of heme analogues from vegetable origin could provide an alternative iron source of potentially high bioavailability. Sodium iron chlorophyllin is a water-soluble semisynthetic chlorophyll derivative where the magnesium in the porphyrin ring has been substituted by iron. We have used an in vitro model that combines gastric and intestinal digestion followed by intestinal iron uptake in Caco-2 cells to determine the bioavailability of iron from sodium iron chlorophyllin. Our results demonstrate that sodium iron chlorophyllin is stable under simulated gastrointestinal conditions and is able to deliver bioavailable iron to Caco-2 cells. Similar to the heme, the bioavailability of iron from sodium iron chlorophyllin is dependent on the food matrix, and it was inhibited by calcium. Potentially, sodium iron chlorophyllin could be used as an iron fortificant from vegetable origin with high bioavailability.

KEYWORDS: Caco-2 cells; calcium; digestion model; ferritin; hemoglobin; iron fortification; iron sulfate; pH; porphyrin

INTRODUCTION

Iron deficiency is the most common nutrient deficiency in the world. The World Health Organization estimates that the number of anemic people worldwide to be a staggering two billion and that approximately 50% of all anemia can be attributed to iron deficiency (*1*). Functional consequences associated with iron deficiency anemia are cognitive impairment, decreased physical performance, and reduced immunity (*2*−*4*). Diversification of the diet and fortification of foods are considered the more plausible approaches to combat iron deficiency (*5*).

Iron in the diet is present as nonheme and heme iron, both of which are absorbed in the duodenum (*6*). Nonheme iron accounts for more than 85% of the total iron in the diet. Nevertheless, it shows low bioavailability (2−7%) as it is strongly affected by several dietary factors. Phytates, phenolic compounds, and calcium have been shown to inhibit nonheme iron transport, while ascorbic acid can enhance nonheme iron uptake. However, heme iron is considered to be highly bioavailable (10−20%), and it is less affected by meal composition. Heme iron is iron (Fe^{2+}) that is bound to the iron protoporphyrin IX prosthetic groups of proteins, mainly hemoglobin and myoglobin, which are present in animal tissue. Heme is released from hemoglobin during digestion so that it can be taken up by the duodenal enterocytes. The intact iron porphyrin is transported across the brush border membrane by the Heme Carrier Protein 1 (HCP1) (*7*). It is in fact this porphyrin ring that is recognized by the transporter and not the complexed iron. Once inside the cell, the iron is released, and it is then likely to enter the low molecular weight pool of iron.

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Iron fortification of foods generally has used nonheme iron sources, as these are cheap and easily available. Nevertheless, these iron sources have poor bioavailability and often affect the organoleptic characteristics of the product. The use of heme-iron as a fortificant has been limited (*8*−*11*). This probably responds to a myriad factors including the elevated costs of hemoglobin or hemoglobin extracts, the intense color of hemoglobin, the large amounts of hemoglobin required, and its animal origin, which means that it might not be consumed in certain regions of the world. Plant hemoglobins such as leghemoglobin from the soy root nodule have also been considered as potential fortificants (*12*), but the relatively low concentration of iron in this protein limits its use.

The use of heme analogues from vegetable origin could provide an alternative iron source of potentially high bioavailability. Nelson and Ferruzzi have explored the use of chlorophyll derivatives as potential iron sources (*13*). They demonstrated that these compounds were present in mixed micelles after in vitro digestion. Sodium iron chlorophyllin is a water-soluble semisynthetic chlorophyll derivative where the magnesium in the porphyrin ring has been substituted by iron (**Figure 1**). This compound is commercially available, and it is used in the food and pharmaceutical industry as a colorant. However, its potential as an iron source has not been studied.

In the present study, the in vitro bioavailability of iron from sodium iron chlorophyllin has been determined using a method that models gastric and intestinal conditions and measures the fraction of iron (ionized or in the porphyrin group) released by foodstuffs. Intestinal cellular uptake of the released iron was modeled in vitro using Caco-2 cells, which are human cells derived from the colon (*14*). When cultured in adequate conditions, Caco-2 cells can spontaneously differentiate into cells that have the

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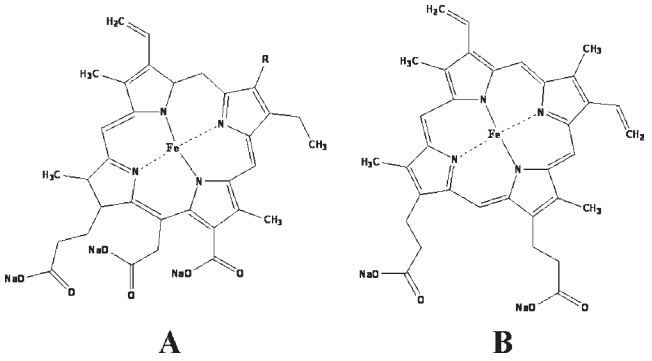

Figure 1. Structure of sodium iron chlorophyllin (**A**) and heme (**B**). In sodium iron chlorophyllin, R corresponds to CH_3 for chlorophyllin a and CHO for chlorophyllin b.

morphology and functionality resembling those of intestinal enterocytes (*15*−*17*). Iron uptake in Caco-2 cells can be determined by the measurement of ferritin formation, as it has been shown to be a good marker of the iron absorbed by the cell (*18*).

The aim of this study was (i) to determine the in vitro bioavailability of sodium iron chlorophyllin from different food matrixes, (ii) to compare the in vitro bioavailability of iron from sodium iron chlorophyllin to that of iron sulfate and hemoglobin, and (iii) to identify the possible dietary factors that can affect its bioavailability.

MATERIALS AND METHODS

Chemicals. Unless stated otherwise, all chemicals were purchased at Sigma-Aldrich (Zwijndrech, The Netherlands). Sodium iron chlorophyllin was obtained at Chemfar (Hangzhou, China) as a mulberry leaf extract. Dulbecco's modified Eagle's medium (DMEM) with 25 mmol/L glucose and 4 mmol/L L-glutamine, and the penicillin/streptomycin solution were obtained at Lonza (Breda, The Netherlands). Heat-inactivated fetal bovine serum, nonessential amino acids, and powdered minimal essential medium (MEM) were purchased at Gibco, Invitrogen (Breda, The Netherlands). The immunoglobulin standard used for protein determination was from BioRad (Veenendaal, The Netherlands).

Foodstuff Preparation. The food matrixes considered in this study included water, dough, powder drink, and chocolate. All food products contained iron as iron sulfate ($FeSO_4$), hemoglobin, or sodium iron chlorophyllin. The amount of $FeSO_4$, hemoglobin, or sodium iron chlorophyllin added to each food product was normalized to the total amount of iron present in the compound. In hemoglobin, the amount of iron present, as indicated by the supplier, was 3.2 mg/g. The total amount of iron present in sodium iron chlorophyllin was 29.1 mg/g as measured by ICP-AES (see below for analysis details).

Water solutions containing 6 mg of iron for 100 mL of water were prepared on the day of the experiment. Dough was prepared by mixing 50 g of wheat flour (Meneba # Pelikaan, The Netherlands) with milli-Q water solutions of $FeSO_4$, hemoglobin, or sodium iron chlorophyllin. The final iron concentration was 2 mg of iron for a 10 g portion of dough. The dough was subsequently divided into portions and stored at −20 °C until used.

To prepare the drinks, 2 mg of iron (as $FeSO_4$, hemoglobin, or sodium iron chlorophyllin) and 10 mg of powder were diluted in 80 mL of milli-Q water at 70 °C and then stirred briefly for complete dissolution. The powder was prepared at Unilever R&D Vlaardingen (The Netherlands) and contained 26.8% semiskimmed milk powder, 30.6% maltodextrin, 23.8% icing sugar, 10.2% refined soybean oil, 5.1% wheat starch, 2.7% potassium hydrogenophosphate, and 0.8% vanilla flavor. The powder contained 350 mg of calcium per 100 g; the molar ratio of iron to calcium was 1:25. The drinks were prepared on the day of the experiment.

All chocolates were provided by Hindustan Lever Research Centre (Bangalore, India) and contained 66.7% dark chocolate (Unicolloids Impex Pvt. Ltd., Mumbai, India), 16.7% roasted nuts, 16% sugar, and 0.33% sweet orange flavor DC-27662 (Quest International, Mumbai, India) and the corresponding source of iron (as $FeSO_4$, hemoglobin, or sodium iron chlorophyllin). Each chocolate weighed 12 g and provided 10 mg of iron. The chocolates were crushed with a mortar, and one-third of each chocolate (4 g) was used in the dissolution experiments, corresponding to 3.3 mg of iron.

Water solutions containing sodium iron chlorophyllin in the absence or presence of calcium were prepared on the day of the experiment. The sodium iron chlorophyllin solution was prepared by mixing 69.3 mg of iron chlorophyllin (2 mg of iron) in 33 mL of milli-Q. The sodium iron chlorophyllin solution in combination with low calcium concentration was prepared by mixing the same amount of sodium iron chlorophyllin (2 mg iron) and 46.2 mg of calcium chloride (16.7 mg of calcium) in 33 mL of milli-Q water. The molar ratio of iron to calcium was 1:12. The sodium iron chlorophyllin solution in combination with high calcium concentration was prepared by mixing 69.3 mg of sodium iron chlorophyllin (2 mg of iron) and 555.6 mg of calcium chloride (200 mg of calcium) in 33 mL of milli-Q water. The molar ratio of iron to calcium was 1:140.

Determination of Total and Ionic Iron. Total iron present in the samples was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Briefly, samples were digested in 5 mL of 65% nitric acid and 0.5 mL of 30% hydrogen peroxide in closed vessels in a microwave oven at high temperature (200 °C) and high pressure (110 bar). After digestion, the volume was adjusted to 50 mL using demineralized water and sprayed into the inductively coupled plasma of the plasma emission spectrometer (Perkin-Elmer 3300 DV inductively coupled plasma-optical emission spectrometer). The emission of the individual elements was measured at specific wavelengths, and concentrations were quantified from standard solutions.

Ionic iron (sum of Fe^{2+} and Fe^{3+}) was determined using a Hitachi 912 analyzer and reagents for the analysis of iron in human serum based on FerroZine (Roche Diagnostics Nederland BV, The Netherlands). The analyses were performed according to the instructions of the supplier of the reagents, using the dialysate or digesta of the in vitro iron digestion assay instead of serum.

Because of the extensive acid digestion, total iron is indicative of the amount of heme and nonheme iron in the sample. However, as the FerroZine method does not react with iron bound to the porphyrin group, the iron measured with this method reflects only nonbound ionic iron. Therefore, the difference between total and ionic iron is indicative of the amount of heme-iron in the sample.

Effect of pH on the Percentage of Ionic Iron. The aqueous solutions containing 2 mg of iron as iron chlorophyllin alone or in the presence of calcium (with a molar ratio of iron to calcium of 1:12) were prepared as indicated, and the pH was adjusted to 2, 3, 4, 5, 6, 7, or 8 with HCl or NaOH. The solutions were incubated for 2 h at room temperature with agitation and then centrifuged at 13000 rpm for 5 min. From the supernatant obtained, the amount of ionic iron was measured using the FerroZine method. Results are expressed as the percentage of ionic iron in relation to the total iron present in the sample.

In Vitro Simulated Digestion. At the day of the experiment, one portion of dough was thawed and homogenized for 20 s in 80 mL of mili-Q with a Philips blender (type HR 2094/00) set at intermediate speed. Water and powder drink solutions containing 2 mg of iron per serving were prepared on the same day as indicated. The chocolates were crushed with a mortar, and 4 g was used in the dissolution experiments, corresponding to 3.3 mg of Fe.

The digestion protocol was based on the studies carried out by Miller and collaborators (*19*). All glassware were incubated overnight in 10% (v/v) HNO_3. On the day of the experiment, all glassware were washed 5 times with milli-Q to remove HNO_3. Samples were prepared as indicated and added to the vessels (100 mL), which were then placed in the dissolution apparatus type II, USP 26 (VanKel VK700, Varian, Palo Alto, CA). Subsequently, pepsin (0.5 mg/mL), water, and HCl were added to each vessel, yielding a 90 mL solution of the samples in simulated gastric fluid at pH 2.0. After 120 min of incubation at 37 °C with mixing at 100 rpm, 10 mL samples of the digests were taken for total iron determination, and the remainder was used for the simulation of the intestinal phase in an Erlenmeyer flask.

For the simulation of the intestinal phase, a dialysis bag (Spectra/Por 7 MWCO 8000) was filled with a water solution of $NaHCO_3$ (0.1 mol/L) and was placed in an Erlenmeyer flask. The amount of $NaHCO_3$ present in the dialysis bag was able to adjust the simulated digestion to pH 7.5. After

30 min of incubation in a water bath at 37 °C and continuous mixing (100 rpm), a mix of pancreatin (0.4 mg/mL) and bile acids solution (1.25 mg/mL) was added to the flask. The flask was further incubated with the dialysis bag for another 2 h in the same water bath at 37 °C with continuous mixing (100 rpm). Thereafter, the dialysis bag was removed, and the contents of the dialysis bag (dialysate) as well as the remaining digesta outside the dialysis bag were used for further analysis. The digests were centrifuged at 4000 rpm at 4 °C, and the supernatants were used for the determination of the amount of ionic and total iron and for iron uptake experiments in Caco-2 cells. The dialysates and digests were stored at −20 °C until further use. The results are given as bioaccessible iron and are expressed as percentages of ionic or total iron present after intestinal digestion relative to the total iron after the gastric phase.

Iron Uptake by Caco-2 Cells. The iron uptake experiments were carried out as described by Glahn et al. with slight variations (*18*). The human Caco-2 cell line was purchased from the American Type Culture Collection via its European partner LGC Standards (Teddington, UK). Stock cultures were maintained at 37 °C in complete medium in an incubator with a 5% CO_2 and 95% air atmosphere at constant humidity; the medium was changed every 2−3 days. Cell passage was maintained under 40. The complete culture medium contained DMEM with 25 mmol/L glucose and 4 mmol/L L-glutamine, supplemented with 20% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin and streptomycin, and 100 μmol/L nonessential amino acids.

For the iron uptake experiments, Caco-2 cells were seeded in 12-well plates (Corning, Schiphol-Rijk, The Netherlands) at a density of 2×10^5 cells per well. The cells were cultured in complete medium for 21 days so that they could form a monolayer of differentiated cells that resembles that of the intestinal mucosa. At this point, cells were used for iron uptake experiments.

On the day of the experiment, dialysates and digests were thawed and diluted in 10 times (10×) concentrated minimum essential medium+ (MEM+) so that 9 parts of the digest were mixed with 1 part of 10× MEM+. MEM+ concentrated media were prepared by mixing PIPES (100 mmol/L), 500 U/mL penicillin, and 500 μg/mL streptomycin, hydrocortisone (55 nmol/L), insulin (50 mg/L), selenium (0.6 μmol/L), triiodothyronine (0.52 μmol/L), epidermal growth factor (50 mg/L), 1 mmol/L nonessential amino acids, $NaHCO_3$ (262 mmol/L), NaOH (for pH correction to pH 7.0), powdered MEM, and milli-Q to a final volume of 100 mL. The concentrated MEM+ as well as the dialysate samples were sterile filtered (0.22 μm). After washing the cells twice with 1 mL of 1× MEM+, 1 mL of the diluted dyalisates or digests was applied.

Exactly 48 h after the start of the incubation at 37 °C and 5% CO_2, the cell monolayers were harvested for ferritin and protein measurements. For this, the medium covering the cells was removed carefully, and the cells were washed twice with a 1 mL rinse solution containing 140 mmol/L NaCl, 5 mmol/L KCl, and 10 mmol/L PIPES at pH 7.0. The rinse solution was then aspirated, and 250 μL of milli-Q was added. The plate was wrapped in parafilm and sonicated in a water bath at 4 °C for 15 min. After sonication, the cells were scraped and collected in Eppendorf tubes. The samples were stored at −20 °C until further use.

Ferritin from the cell lysates was measured using a commercial ELISA kit (Adaltis, Bologna, Italy). Total cellular protein was measured with the Bradford assay and using immunoglobulin as standard. Results were expressed as ng of ferritin per mg of total cellular protein. In order to normalize results considering the amount of iron initially present in the sample, the results were also expressed as the efficiency of iron uptake in Caco-2, which is given in ng of ferritin per mg of protein per mg of total iron present in the product.

Statistical Analysis. The data presented are expressed as the mean ± SD of 2 or more experiments. Significant differences were analyzed by two-way ANOVA, and the Bonferroni posthoc test was used for comparisons (food matrix and iron source effect), or one-way ANOVA and the Bonferroni posthoc test were used for comparisons (effect of calcium) (Graph Pad Prism 4.01 software, San Diego, CA). Differences were considered significant when $p < 0.05$.

RESULTS

Effect of pH on Ionic Iron Release from Sodium Iron Chlorophyllin. The amount of ionic iron released from sodium iron chlorophyllin after 2 h of mixing at different pH was in all cases under 5% (**Figure 2**). The low amount of ionic iron shown for pH 2 to 4 is probably due to the precipitation of sodium iron chlorophyllin at pH values below 4. The amount of ionic iron present in a sample of sodium iron chlorophyllin incubated for 2 h at pH 2 and then at pH 7.0 was also measured. For this sample, the results indicate that the amount of ionic iron present after adjusting the pH back to 7.0 was 4.3 ± 0.0.4%, returning to values similar to those found at pH 7.0. Moreover, while sodium iron chlorophyllin precipitated at pH 2, when returning the precipitate to pH 7, the compound resolubilized.

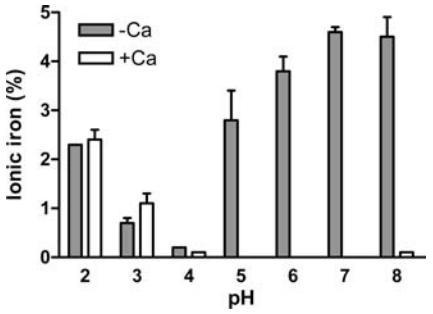

Figure 2. Percentage of ionic iron released from sodium iron chlorophyllin dissolved in water at different pH and in the absence (−Ca) or presence of calcium (+Ca) (iron to calcium molar ratio of 1:12). The absence of the dashed bar corresponds to no ionic iron released, i.e., 0%. Bars represent the mean ± SD of two experiments.

Effect of the Food Matrix on the in Vitro Iron Bioavailability. Different food matrixes were tested for the ability to modulate the in vitro iron bioavailability of $FeSO_4$, hemoglobin, or sodium iron chlorophyllin. The results from the in vitro digestion assays indicate that in all cases, only a small amount of iron from the hemoglobin and sodium iron chlorophyllin samples was present in the dialysate. Most of the iron was present in the digesta and remained bound (**Figure 3A**), as the amount of ionic iron present remained under 15% of the total iron added (**Figure 3B**). This indicates that most of the iron in hemoglobin and sodium iron chlorophyllin remained in the porphyrin group and was not released during digestion and is in line with the results observed with ionic iron release at different pH.

After in vitro digestion of the samples, these were added to the Caco-2 model to determine the cellular uptake. Cellular uptake of iron from hemoglobin was not reduced by the dough but was significantly reduced by the powdered drink and chocolate (**Figure 3C**). The iron delivery from sodium iron chlorophyllin was significantly reduced when provided as dough and powdered drink but not as chocolate (**Figure 3C**). The results showed that for all food matrixes tested both hemoglobin and sodium iron chlorophyllin were able to deliver iron to the cells and that the efficiency of iron uptake was significantly higher than that of $FeSO_4$ (**Table 1**).

Effect of Calcium on in Vitro Iron Bioavailability. The reduction of cellular iron uptake when iron was provided as powdered drink (containing a molar ratio of iron to calcium of 1:25) could indicate that calcium is a strong inhibitor of the uptake of iron from heme and heme analogues. The release of iron in the presence of calcium and at different pH showed that in the presence of calcium (molar ratio iron to calcium 1:12), iron chlorophyllin precipitated and that no iron was observed in the supernatant (**Figure 2**). Therefore, an experiment was designed to determine the in vitro bioavailability of iron from sodium iron chlorophyllin alone or in the presence of low and high levels of calcium (iron to calcium molar ratios of 1:12 and 1:140, respectively). The effect of

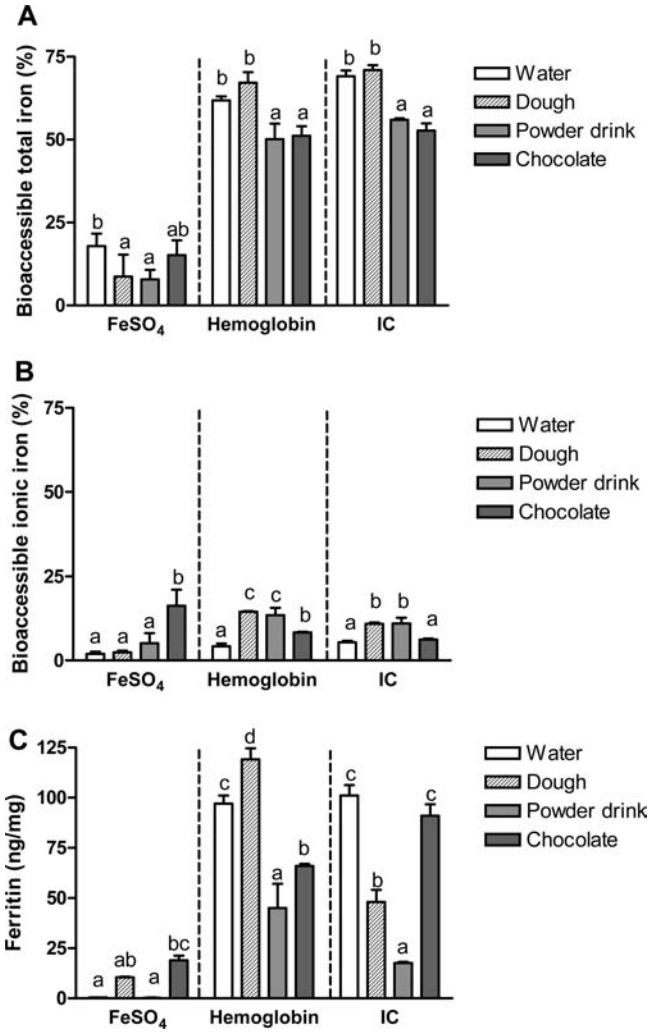

Figure 3. Bioaccessible iron determined as percentage total (**A**) or ionic (**B**) iron relative to total iron after gastric phase and cellular iron uptake determined as ferritin in Caco-2 cells (**C**). Bars represent the mean ± SD of three experiments. Bars with different letters are significantly different as measured by two-way ANOVA ($p < 0.0001$) with Bonferroni post-tests when comparing each iron source. IC, sodium iron chlorophyllin.

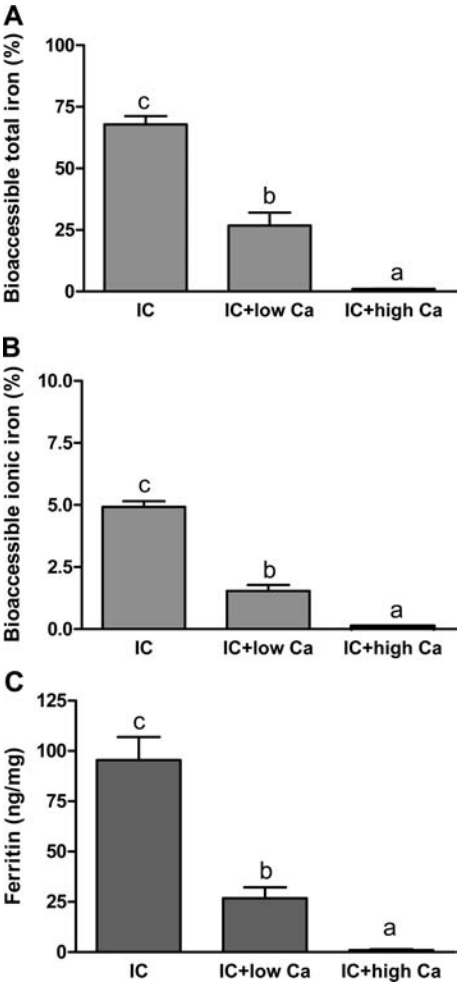

Figure 4. Percentage of available total (**A**) and ionic (**B**) iron of iron chlorophyllin alsone (IC) or iron chlorophyllin in combination with low and high calcium in aqueous solutions after gastrointestinal tract simulation (with 2 mg of iron). (**C**) Ferritin formation by Caco-2 cells from digests containing iron chlorophyllin alone (IC) or in combination with low and high calcium levels (iron to calcium molar ratios of 1:12 and 1:140, respectively). Bars represent the mean ± SD of three experiments. Bars with different letters are significantly different as measured by one-way ANOVA ($p < 0.05$).

Table 1. Efficiency of Iron Uptake (ng Ferrtin/mg Protein/mg Total Iron) in Caco-2 Cells after Product Digestion with Different Iron Sources[a]

	water	dough	powder drink	chocolate
Iron sulfate	0.2 ± 0.1 ^{a},A	9.3 ± 2.0 ^{b},A	0.1 ± 0.0 ^{a},A	4.27 ± 0.8 ^{a,b},A
Hemoglobin	49.7 ± 1.0 ^{c},B	66.8 ± 4.7 ^{d},C	26.1 ± 6.1 ^{b},C	18.2 ± 0.3 ^{a},B
Iron chlorophyllin	50.6 ± 2.4 ^{c},B	23.5 ± 1.4 ^{b},B	9.8 ± 0.2 ^{a},B	27.4 ± 2.8 ^{b},C

[a] Values are means ± SD ($n = 3$). Different lowercase letters indicate significant differences ($p < 0.05$) in the row values using two-way ANOVA. Different uppercase letters indicate significant differences ($p < 0.05$) in the column values using two-way ANOVA.

calcium was confirmed by a significant decrease in the percentage of bioaccessible total and ionic iron present after in vitro digestion (**Figure 4A** and **B**) as well as in the cellular uptake (**Figure 4C**), demonstrating a reduction of bioavailable iron.

DISCUSSION

Heme is transported across the apical membrane of the duodenal enterocytes intact via the HCP1 transporter (*7*). Therefore, the stability of iron present in the porphyrin ring to digestive conditions is a critical measure of the ability of heme analogues to function as effective iron sources. Initially, the effect of pH on sodium iron chlorophyllin was tested. The amount of ionic iron present in water solutions at different pH was indicative of the amount of iron released from the heme-like structure. Our results show that the amount of iron released from sodium iron chlorophyllin was low (under 5%) and that most of the iron remained bound to the porphyrin ring. These results were confirmed by the simulation of gastrointestinal digestion. Independent of the food matrix used, the amount of ionic iron present in the digesta remained low (under 15%). The amount of ionic iron could also not explain the increase in ferritin formation after the addition of hemoglobin or sodium iron chlorophyllin compared to that of $FeSO_4$.

The bioavailability of $FeSO_4$, hemoglobin, or sodium iron chlorophyllin was evaluated using a combination of dissolution and Caco-2 experiments. This method has been extensively used to determine the in vitro bioavailability of iron, and it is currently considered an adequate method to rank iron sources or food formulations (*20*). Nevertheless, its application in the determination of heme bioavailability has been limited (*12*). It has been

established that Caco-2 cells express the HCP1 receptor and that these cells can be used as a model for heme uptake (*21*, *22*). However, when approaching the comparison of inorganic vs heme iron sources, we observed that one method cannot be used to measure the bioavailability of the other, and slight variations were used to determine nonheme iron absorption. The absorption of inorganic iron is determined by the release of iron from the digesta and ionization during the first hours of digestion. Generally, when measuring nonheme iron absorption, a 2 h dialysis step is included to determine how much ionic iron is released from the food matrix, as only ionic iron is considered to be bioavailable (*18*). Therefore, it is physiologically relevant to use the dialysates for further experiments. However, in the case of heme-iron, the dialysate does not represent a relevant sample as heme iron, while that released from the digesta does not require release from the porphyrin and the consequent ionization. Because heme is generally linked to peptides after digestion and is transported intact by the cell, the digesta outside the dialysis membrane was used for the Caco-2 experiments as it has been done by others (*12*). Therefore, in order to obtain the most relevant results that could be translated for future in vivo studies, each physiologically relevant sample was added to the Caco-2 experiments. Our results show that when using heme or iron chlorophyllin, most of the iron was present outside the dialysis membrane, indicating that iron ions remained complexed. Moreover, as observed in **Figure 2B**, the percentage of ionic iron showed the same range for all compounds tested (both inside and outside the digesta). Therefore, the high levels of ferrtin formed in Caco-2 cells in the presence of hemoglobin and sodium iron chlorophyllin correspond to the iron bound to the porphyrin group. If the nonionic (total) iron would not be absorbed, no differences would be observed among the groups.

The results indicate that in all food matrixes tested both hemoglobin and iron chlorophyllin delivered significantly more iron than $FeSO_4$. The uptake of iron from hemoglobin was inhibited when provided in chocolate and powdered drink and chocolate, while for iron chlorophyllin, this only occurred for dough and powdered drink. It is well established that the bioavailability of iron from heme is not affected by luminal factors that generally influence nonheme iron uptake such as phytate or ascorbic acid (*23*). However, the effects of other factors have not been extensively studied. Surprisingly, heme-iron uptake seemed to be inhibited by chocolate. Polyphenols have been shown to inhibit nonheme iron uptake, but no effect on heme uptake has been described. Interestingly, the effect of polyphenols was not observed when sodium iron chlorophyllin was used. Polyphenols from wine and tea have been shown to increase pepsin activity (*24*), and this could influence the digestion of hemoglobin and the solubility of the released heme. Peptides derived from hemoglobin digestion are known to maintain heme solubility and to allow heme uptake (*25*). Extensive digestion of the peptides could therefore decrease heme solubility and consequently, heme-iron bioavailability. However, other potential interactions between polyphenols and hemoglobin cannot be discarded.

The bioavailability of iron from both hemoglobin and iron chlorophyllin is significantly inhibited when the food matrix contains calcium. Preliminary experiments have shown that when sodium iron chlorophyllin is added to milk a precipitate is formed (data not shown). A decrease in the in vitro iron bioavailability was observed when hemoglobin and sodium iron chlorophyllin were present in a powdered drink containing calcium (iron to calcium molar ratio of 1:25). This was also confirmed in an aqueous solution containing calcium (as calcium chloride) where sodium iron chlorophyllin precipitated even at low calcium concentrations (iron to calcium molar ratio of 1:12). This precipitation due to the presence of calcium was later on confirmed by dissolution and Caco-2 experiments, where the bioavailability of iron derived from sodium iron chlorophyllin was greatly reduced in the presence of calcium (at both molar ratios of 1:12 and 1:140). A reduction in heme-iron bioavailability in the presence of calcium has also been shown in vivo by Roughead and collaborators (*26*, *27*). Their results indicate that this inhibition occurs at the apical side of the intestine and not at the basolateral side, suggesting some limiting factor during uptake. This is most likely due to the fact that the heme or heme analogue structures form aggregates in the presence of calcium and that these aggregates precipitate, limiting the bioavailability of the heme iron.

It should be noted that sodium iron chlorophyllin is intensely green, and therefore, it dramatically affects the color of the food matrixes where it is added. Coloration could be masked in the presence of cocoa both in chocolate bar formats as well as in drink products (data not shown). Addition of sodium iron chlorophyllin to fruit drinks containing strawberry was also able to mask the intense green color (not shown). Nevertheless, it is clear that for this compound color is a major factor and that strategies should be considered for masking or linking it to particular green flavors such as pistachio or kiwi.

In summary, our results demonstrate that sodium iron chlorophyllin is stable under simulated gastrointestinal conditions and is able to deliver bioavailable iron to Caco-2 cells. Similar to heme, the bioavailability of iron from sodium iron chlorophyllin was inhibited by calcium. Potentially, sodium iron chlorophyllin could be used as an iron fortificant from vegetable origin with high bioavailability similar to that of heme. Adequate product formulation and in particular the absence of calcium would be essential to ensure iron delivery. These iron bioavailability results should be corroborated in human intervention studies.

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