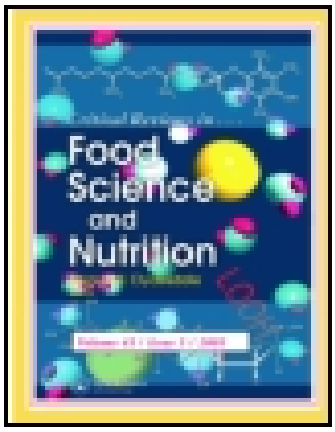


Critical Reviews in Food Science and Nutrition
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/bfsn20

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Accepted author version posted online: 01 Apr 2015.

# Iron Bioavailability in Iron Fortified Cereal Foods: The Contribution of *in vitro* Studies

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## Abstract

Iron deficiency anemia is the most common nutritional deficiency in humans. Not all dietary ingested iron, heme or non-heme, will be available to absorption and negative imbalance between iron requirements and absorption leads to iron deficiency and/or anemia. The recommended iron values usually are based on the genetic and on diet iron-bioavailability, which can be considered as the principal factor that change among the cultures and influences the distinct levels of recommendation among countries. Dietary changes present practical limitations due to be difficult to change food habits. The iron food fortification is considered more cost effective and economically more attractive than iron supplementation. There are many iron compounds available to be used in iron fortification. Cereals represent a target food group to iron fortification programs due high consumption and the *in vitro* studies can be useful to estimate the

relative iron bioavailability in large number of products in short time and with a low cost. Wheat flour baked into bread or not was the main product tested in *in vitro* bioavailability studies and ferrous sulfate was the principal iron compound used in the fortification studies. However, iron bioavailability from ferrous sulfate is lower than from other compounds, such FeNaEDTA or ferric pyrophosphate. The variables level of fortification, storage, level of extraction, baking and also the association or not with other chemical compound seems to influence the results obtained.

**Keywords:** Bioacessibility, Caco-2 cells, wheat flour, iron nutritional recommendations, food enrichment, bioavailability, iron fortificants, ferrous sulfate.

## 1. Introduction

Iron is the fourth most common element on earth. It is an essential trace mineral nutrient that participates with oxygen carriers, such as hemoglobin and myoglobin, and the iron contained within heme is essential for the redox reactions that occur in numerous cytochromes. In biological systems iron is present in one of two oxidation states, and redox inter-conversions of the ferrous ($Fe^{2+}$) and ferric ($Fe^{3+}$) forms are central to the biological properties of this element, with the ferric iron being less soluble in water than ferrous iron (Toyokuni, 1996; Shander et al., 2012).

While ferric ions are the relatively biologically inactive form of iron, ferrous ions can increase oxidative stress due to reactive oxygen species that can convert superoxide anions into highly reactive hydroxyl radicals, leading to lipid peroxidation, nucleic acid or protein damage. Studies have demonstrated a direct interrelationship between the intracellular free iron pool and oxidative stress (Toyokuni, 1996). The iron high affinity for different iron-binding proteins maintains low levels of free iron in the blood stream and/or cells, minimizing the chances of oxidative stress and permitting iron delivery to the organic target tissues. There is no evidence of physiological benefits in having iron stores higher than the minimum to guarantee adequate iron procurement to the functional compartments. The adult human body usually contains at least 3000 mg (45 mg/kg) elemental iron, with females in reproductive age generally having lower levels than males due to iron loss during menses, pregnancy, and lactation (Alleyne et al., 2008).

Homeostatic mechanisms can alter intestinal iron absorption by supplying iron preferentially to functional compartments in response to deficiency or excess. Healthy individuals present daily iron absorption of 1 to 2 mg of iron that is balanced by similar amount

of iron loss from skin and gut, and from menstruation and pregnancy (Shander et al., 2012). The imbalance between iron requirements and absorption leads to iron deficiency or overload. The former, depending on severity, may lead to iron anemia. Iron overload is associated with cancer risk in humans, such as colorectal cancer and liver cancer by molecular mechanisms (Huang, 2003; Hurrell et al., 2010; Xue and Shah, 2013).

Iron deficiency anemia is the most common nutritional deficiency in humans, affecting 1.62 billion people globally. Individuals more vulnerable to iron deficiency are infants over 6 months, children, women of fertile age, pregnant women and older people (de Benoist et al., 2008). Iron deficiency anemia was defined by the World Health Organization (WHO) as a hemoglobin level below 13 g/dL in men, below 12 g/dL in non-pregnant women over the age of 15 years, and below 11 g/dL in pregnant women (de Benoist et al., 2008).

The main effects of iron anemia are impaired physical performance due to low levels of hemoglobin and myoglobin, resulting in reduced activity of iron-dependent cytochromes and of ATP production. Iron deficiency impairs psychomotor development in children and cognitive performance among children and adults. Body thermoregulation and cellular immune function could also be impaired in cases of severe iron deficiency (WHO, 2006; Shenkin, 2008; Beard and Han, 2009; Rattehalli et al., 2013).



## 2. Dietary iron

Dietary iron is comprised by two iron types: the non-heme (inorganic iron-salts) which are present in plants, food and animal tissues, and the heme iron which comes from hemoglobin and myoglobin present in animal food sources. The latter contributes around 10-15% of total iron

consumption in omnivorous subjects, and is absorbed by a separate pathway and more efficiently than non-heme iron. Heme iron has higher bioavailability (estimate at 15-35%) and also of the total absorbed iron. With the exception of a few iron fortificant compounds, all non-heme iron present in food forms a common iron pool in the digestive tract and is absorbed to the same extent, with the absorption efficiency linked to the balance between the absorption inhibitors, enhancer compounds and the iron status of the individual (Heimbach et al., 2000).

Research has shown that the form of iron and the role of enhancers and inhibitors of iron absorption may be more important than total iron intake in determining iron status (Nair and Iyengar, 2009). People consuming foods high in iron absorption inhibitors and lower in iron absorption promoter compounds, and also followers of vegetarian diets, had reduced assumed efficiency of absorption from 18 to 10%. As the absorption of non-heme iron found in plants foods is lower than from meat and meat products, vegetarians need to consume twice as much iron to meet the daily requirement (IOM, 2001). Considering the intake data and isotope studies, iron bioavailability has been estimated to be around 14-18% for mixed diets and 5-12% for vegetarian diets in subjects with no iron stores. These values have been used to generate dietary reference values for all population groups (Hurrell and Egli, 2010).

Not all ingested heme or non-heme iron will be available to be absorbed. The fraction absorbed will be influenced by individual factors and also by the complexation chemistry rules in the intestinal lumen (Schümann et al., 2007). The absorption of heme-iron is much less affected by dietary compounds with the exception of calcium, where calcium phosphate is the strongest inhibitor of heme and non-heme iron, compared to calcium carbonate and calcium citrate compounds (Cook et al., 1991). Among the enhancers factors are found the ascorbic acid a

nutrient influences positively the non-heme iron bioavailability. Nojeim and Clydesdale (1981) showed that ascorbic acid acts as a reducing agent, contributing to increased ionization and favors the ferrous valence state at pH 2.7. The ionic state of iron is linked to it solubility and by consequence to its bioavailability.

Computing all influences over the iron bioavailabitility the estimated average fractional iron absorption for a typical western diet is about 18% and 15% for IOM and EU-SCF, respectively (Scientific Committee on Food, 1992; IOM, 2001). The FAO/WHO (2004), using more recent data, set iron bioavailability as 5% for a strict vegetarian diet, at 10% when some meat and ascorbic acid was added, and at 15% for diets rich in meat and fruits.

Since dietary habits change between cultures it is reasonable to assume that the nutritional iron recommendation will be different among distinct countries as a function of the level of iron, inhibitors and enhancers in the diet (**Table 1**). The body also acts to adjust intestinal mucosa cells involved in the iron uptake, regulating the number of the binge and transport iron proteins. This process is considered as a regulatory mechanism to prevent iron overload and to achieve iron homoeostasis in accordance with functional requirements (Benito and Miller, 1998; Schümann et al., 2007).



## 3. Iron nutritional recommendations

Recommendations for dietary iron intake for both genders at different ages are given by FAO/WHO (2002), by the EU Scientific Committee (1992), and also by countries and or groups of countries. Some of these recommended values are exhibited in **Table 2**. The proposed values

are usually grounded in body iron-losses, diet iron-bioavailability and iron-requirements for metabolism and growth (Schümann et al., 2007). The iron recommendations are linked to the organic requirements that can be estimate in different ways. The recommended values usually are based on the genetic factor of the population and also the diet iron-bioavailability, which can be considered the principal factor that changes between cultures, and influences the different levels of recommendation.

Cuervo et al. (2009) reviewed the definitions, data and methodology of nutritional reference values for nutrients, including iron, from some selected countries, groups of countries and agencies. They concluded that the bulk of them define major concepts in the same way, but with differing nomenclature. However, they did note that there are remarkable differences in the nutritional recommendations amongst the countries and groups of countries: these differences included age groups, nutrients covered, methodology, review periodicity and values proposed. Such differences can also be found when iron is analyzed alone among distinct countries, groups of countries and agencies (**Table 2**).

In all consulted data, the highest recommended values for iron were for women of childbearing age (except for Brazil) and pregnant women, with the WHO recommending iron supplements in Philippines, Italy, if the iron bioavailability in the diet is low. The recommended values by age are very distinct among countries, groups of countries and agencies, with preschool-age children having high iron recommended ingestion values. The highest iron ingestions values recommended (**Table 2**) are applied to the groups of individuals with greater prevalence of anemia (de Benoist et al., 2008). Some individuals have increased iron requirements, such as endurance athletes, blood donors, individuals with pathological blood loss

and post-menopausal women that are using hormone replacement therapy (IOM, 2001; Whiting and Barabash, 2006).

Assessment of iron-related risks use reports of adverse health effects which were used in an attempt to derive an upper safe level for dietary iron intake. Not all countries or country groups quote values for iron upper safe limits. Of the countries that do quote values, that of the United States is noteworthy, with a value of 40 mg for individuals until 13 years old, and of 45 mg for all others (IOM, 2001). A particularly sensitive subpopulation for iron overload are homozygotes for hereditary hemochromatosis, an autosomal recessive disease with estimated prevalence in the population of 2 in 1000 in Caucasians, with lower incidence in other races. Approximately 3-7% of the US Caucasian population are heterozygotes and 3-5% are homozygotes. These individuals are susceptible to iron overload even at normal dietary iron intakes due to an accelerated rate of intestinal iron absorption and progressive iron deposition in various tissues. It has been estimated that the fraction of homozygotes with clinical symptoms was less than 1%. The majority of homozygotes are not diagnosed and can be at risk of iron overload (Moyer et al., 2011).



**4. Evaluation of iron bioavailability**

There is no universally accepted definition of bioavailability, and different researchers have defined it in different ways. A definition that has gained wide acceptance defines bioavailability as the amount of a nutrient that is available for normal metabolic and physiologic processes. Bioavailability also influences a nutrient's beneficial effects at physiologic values of ingestion as well as affecting the nature and severity of toxicity due to excessive consumption.

Absorption and/or retention of mineral nutrients are often used as indicators of bioavailability but they are not synonymous with bioavailability, which integrates the various processes whereby an ingested nutrient becomes available: digestion, absorption, transport, utilization and, elimination (Van Campen and Glahn, 1999; Wienk, et al., 1999; Hambidge, 2010).

Bioavailability can be considered as an important factor in the nutrition field due to its variations with different foods, food components and gastrointestinal conditions. Some factors that may affect bioavailability include the concentration of a nutrient, dietary factors, chemical form, supplements taken separately from meals, nutrition and health of the individual, excretory losses, and nutrient–nutrient interactions. The Dietary Reference Intake (DRI) publications point out three principal factors that must affect the bioavailability of iron: chemical form, dietary factors (see **Table 2**) and concentration (IOM, 2001; Hambidge, 2010).

While there is less information available on mineral nutrient bioavailability, including iron, than its chemical concentrations, collectively can be found significant amount of data. Nevertheless, these data were studied and collected by many different techniques and procedures and under a diversity of variable conditions. In many cases, these data cannot be compared as they were obtained by different methods and/or variables (Van Campen and Glahn, 1999). Iron is the only micronutrient for which there is a direct measure of bioavailability with 80-90% of the absorbed iron used to the hemoglobin synthesis and low daily metabolic excretion which allow the uses of measure of iron absorption as predictable bioavailability (Fairweather-Tait, 2001).

The bioavailability of iron from fortificants is dependent on the solubility of the fortificant and the composition of the diet, and in particular on the proportion of inhibitors of iron absorption in the diet as phytates and polyphenols (WHO, 2006). Experiment shows that are

interactions with added iron in phytate-rich, fiber-rich fraction of wheat bran under gastrointestinal pH conditions, where most of the iron was bound to the insoluble fiber fraction (Platt and Clydesdale, 1987). On the other hand, the addition of ascorbic acid or NaFeEDTA [sodium iron ($Fe^{3+}$) ethylenediaminetetraacetic acid] and the removal of phytates, all of which reduce the effect of the inhibitors, can be effective ways of increasing the total amount of iron absorbed from iron-fortified foods (WHO, 2006).

The current information about the bioavailability of commonly used iron fortificants is found in researches conducted by using single-meal studies. These bioavailability estimates could be less accurate and have less meaning in practical way due to the lack of the whole diet studies, particularly over longer time periods. Long-term studies could be very useful to assess true bioavailability and bioefficacy of food fortificants (Casgrain et al., 2010), and also help to analyze the influence of gut microbiota and understand the influence of other dietary factors (enhancers/inhibitors/micronutrient interactions), as well as the dietary patterns of the individuals (e.g., vegetarians), which can have an effect on iron status.



**5. Iron fortification and the role of cereals**

Food fortification or enrichment means the "addition of one or more essential nutrients to a food whether or not it is normally contained in the food for the purpose of preventing or correcting a demonstrated deficiency of one or more nutrients in the population or specific population groups" (Codex Alimentarius Commission, 1987) and it is among the four principals strategies for minimizing nutritional iron deficiency. The other three include dietary modifications and/or diversification to improve iron bioavailability, selective plant breeding or

genetic engineering to increase the iron content or to reduce absorption inhibitors in dietary staples, and iron supplementation with pharmacological doses (provision of micronutrients, usually in higher doses), usually without food (Hurrell et al., 2010). Dietary changes are the preferred method, but it presents practical limitations due to difficulties in changing food habits (Lynch, 2005). Iron fortification is considered economically more attractive than iron supplementation and appears to be more cost effective than iron supplementation, regardless of the geographic coverage of fortification (Baltussen, et al., 2004).

In comparison, food fortification at the present time is safer, and a more economical, flexible, social acceptable and effective approach to improving nutrition iron status among vulnerable individuals in a number of developed countries where these people consume significant quantities of industrially manufactured foods. Milk, margarine, cheese, yogurt, condiments and seasoning powder, salt, sugar, and cereals, with emphasis on wheat and maize flour and rice are among the common staple foods used as vehicle for iron fortification (Martínez-Navarrete et al., 2002; Hurrell et al., 2010, Akhtar et al., 2011). Fortified cereal-based foods with iron are the principal target in food fortification because this food is present regularly in the human diet (Topping, 2007). Products derived from cereal flours (e.g. bread, cereal snacks and breakfast cereals) are also useful food vehicles, but the amount of iron provided via this route will depend on the quantity of food eaten and on the level of fortification (WHO, 2006).

Is important to note that breakfast cereals usually are eaten with milk. Clydesdale and Nadeau (1984) studied breakfast cereals formulated with wheat, corn and three-grain (corn, wheat, and oats) that received during manufacture and before drying hydrogen-reduced elemental iron at levels of 35, 43 and 28 mg/100g, respectively, which were prepared with whole

milk and milk fractions. The whole milk, lactose-free milk, and nonfat dry milk, generated more total soluble iron than the control (water). An unexpected disappearance of the solubilization effect was noted when the deproteinized milk was used with the wheat and three-grain cereal, but not with the corn cereal, in part justified by the fact that the corn-based cereal content contains a larger amount of ionic iron than the other cereals (at the pH levels).

Iron fortification is already widely practiced in many countries. Currently about 68 countries worldwide have mandatory fortification of at least a portion of their flour, which supplies at a minimum iron and/or folic acid to their populations. Researchers found that more than 20 countries in Latin America have implemented programs of mass iron fortification, most of which involve the fortification of wheat or maize flours (Dary, et al., 2002).

The efficiency of the physiologic mechanisms for preventing the absorption of unnecessary iron has been questioned, and mandatory wheat flour fortification programs were discontinued in Denmark (1987) and Sweden (1994), in part because of concern about possible adverse effects of iron fortification (Hallberg and Hulthen, 2002), verified by significant increases in body iron stores and the prevalence of iron overload among Danish men (Milman et al., 2002). Some epidemiological evidence suggests that wheat flour fortification with iron might decrease the prevalence of iron deficiency (Hertrampf, 2002; Andang'o et al., 2007; Sadighi et al., 2009), but the effectiveness is related to the iron compound used in the fortification process (Andang'o et al., 2007).



**6. Iron compounds used in cereal fortification**

Fortification of cereal is an important subject addressed thoroughly in materials edited by Allen et al, (WHO, 2006) and also by Clydesale and Wiemer (1985). The last one addressed unique material about iron food fortification with special topics about selection of iron fortificant compounds and selection of food vehicles for fortification, among these are the cereal-based products (wheat and blended cereal, breakfast cereals and dry milled corn). The WHO publication gives guidelines on food fortification with micronutrients, including iron.

Iron compounds recommended for cereal fortification by the WHO include ferrous sulfate, ferrous fumarate, ferric pyrophosphate, and electrolytic iron powder (WHO, 2006), but many cereal foods are fortified with low-cost elemental iron powders, which are not recommended by WHO and have even lower bioavailability (Hurrell et al., 2010). Heme iron is not a regular compound used as food supplement or even in food fortification programs (Fairweather-Tait, 2001).

The freely water soluble compounds, like ferrous gluconate and ferrous lactate, exhibit relative bioavailability in humans of 89 and 106%, respectively, compared to ferrous sulfate (Akhtar et al., 2011). Ferrous sulfate is used in fresh bread and bakery products, typically products with a short shelf life, while ferrous lactate and ferrous gluconate have been used to fortify milk and soybean products, but both compounds are very expensive (Schümann et al., 1998; Akhtar et al., 2011). However, researchers reported that baked wheat flour fortified with soluble iron compounds (ferrous sulfate, ferric orthophosphate, hydrogen reduced iron, electrolytic reduced iron and carbonyl iron) produces insoluble forms of iron. That can be attributed to the fact that iron sources added to the wheat flour usually do not remain in the original chemical form after baking (Lee and Clydesdale, 1980). The use of the citric or ascorbic

acids in baked cereal base fortified products (corn-soy-milk) promotes the iron availability, and a similar improvement was obtained by boiling the corn-soy-milk fortified with orthophosphate plus cysteine, ascorbic, citric, or malic acid (Rizk and Clydesdale, 1985).

The slowly soluble iron compounds like ferric citrate and ferric sulfate have relative bioavailability of 31 and 34%, respectively, while dried ferrous is about 100%. Ferrous fumarate, ferrous succinate, ferrous tartrate and ferrous citrate, which are poorly soluble iron sources, have relative bioavailability in humans ranging of 101, 123, 62 and 74% respectively. The almost insoluble ferric orthophosphate and reduced iron exhibit bioavailabilities ranging from 5 to 60% and from 13 to 90%, respectively (Schümann et al., 1998; Akhtar et al., 2011). Nevertheless, the ferric orthophosphate has being used to fortify flour and cereal products due to its low interaction with the food matrix (Schümann et al., 1998). Nadeau and Clydesdale (1986) showed that cereal iron fortificant bioavailability could be improved by incubation of organic acid (citric and malic acids) with the iron source, particularly with elemental iron.

In model systems using different iron sources: ferrous sulfate, ferric sulfate, ferric chloride and hydrogen-reduced elemental iron, with and without acid ascorbic. Ferrous sulfate and hydrogen-reduced elemental iron systems presented an irreversible hydrolysis, resulting in polymers with ordered structures. On the other hand, added ascorbic acid increased the levels of soluble iron in all systems (Eyerman et al. 1987).

The choice of the appropriate iron source for use in the fortification and the process in question is considered as a critical point that must need in some cases an adjustment of the pH and/or additions of appropriate ligands to ensure iron solubilization, and by consequence its bioavailability. It has been shown that more reactive and potentially more bioavailable iron

sources are converted to insoluble hydroxides when stored at the pH of cereals, and become refractory and not soluble even when the pH is lowered to 2.0 (Clydesdale, 1983).

Among the iron food fortificants compounds the form with the greatest bioavailability is NaFeEDTA, which is used to fortify food as cereals, milk, sauce and sugar (Schümann et al., 1998; Heimbach et al., 2000). NaFeEDTA does not enter in the common pool of non-heme iron in the absorption process, but rather it dissociates in the gastrointestinal tract to form iron, which is bioavailable, and a NaFeEDTA salt. The absorption of the metal ion and NaFeEDTA occurs in independent processes (Heimbach et al., 2000). Sodium iron EDTA exhibits absorption levels two to three times better than those of ferrous sulfate if the phytate content of the food vehicle is high (WHO, 2006). Other compounds, such as ferrous bisglycinate and various encapsulated and micronized iron compounds, have been proposed in recent years as alternative for iron food fortification because they provide better protection against the inhibitors of iron absorption (WHO, 2006). The recommend iron compounds for distinct cereal and cereal based products with their respective characteristics and cost are displayed in the **Table 3**.

Despite the solubility and cost of iron fortificant compounds differences among iron compounds used in food fortification, is important to address the fact that foods fortified with iron exhibit increased rancidity and sometimes develop unwanted color changes. The first is due to oxidation of unsaturated lipids, while the latter usually include a green to bluish coloration in cereals, a greying of chocolate and cocoa, and darkening of salt to yellow or red/brown. These sensory changes are highly variable and difficult to predict even in the same product in different situation (WHO, 2006).

Bovell-Benjamin and Guinard (2003) critical review of sensory evaluation practices in iron fortification programs point that poor consumer acceptance, unacceptable taste, and discoloration of the iron-fortified foods being the more frequent causes of unsuccessful iron fortification programs. The authors suggest the incorporation of a thorough, organized, and unified approach to sensory evaluation practices into iron fortification programs for product optimization for improving consumer acceptance of iron-fortified foods. This latter factor is crucial for a successful iron fortification program.

Tripathi et al. (2011) evaluated the sensory quality attributes of two millet flours fortified with iron. Fortification did not cause changes in the hardness, texture and aroma of the dumplings prepared from the fortified flours in a period of 60 days following preparation. However, a discoloration was perceived in the dumplings prepared from the same flours. Nevertheless, the general quality of the products prepared was acceptable to the sensory panelists and the fortified flours appeared to be suitable as vehicles for fortification with iron. Biscuits fortified with either ferrous sulfate or NaFeEDTA equivalent to 8.8 mg of iron per 100 g of flour in combination with either citric and tartaric acids at 60, 80, or 100 mg/100 g levels were evaluated for sensory attributes by 30 panelists with the help of a scorecard specially developed for biscuits. Sensory tests indicated that NaFeEDTA-fortified biscuits were more acceptable than ferrous sulfate-fortified biscuits and that biscuits fortified with NaFeEDTA along with tartaric acid were similar to control biscuits in all sensory attributes (Govindaraj, et al., 2007).

A Mexican study evaluated the effect of different iron sources on color values and sensory color perception in tortillas prepared with corn masa flour fortified with micronutrient premix (vitamins and zinc), and one of eight iron compounds (ferrous fumarate, ferrous sulfate,

ferric orthophosphate, ferrous lactate, ferrous gluconate, ferric pyrophosphate, NaFeEDTA, and A-131 electrolytic iron). The fortified tortillas were compared with control samples prepared without the addition of iron. All iron-fortified tortillas were significantly darker than control tortillas but the A-131 electrolytic iron had minimal effect on color and has significantly lower cost than other iron sources evaluated. On this basis this fortification was recommended by the study for fortification of corn tortillas (Richins et al., 2008). However, among children the consumption of whole maize flour fortified with electrolytic iron or NaFeEDTA resulted in no improvements or in a modest, dose-dependent improvements in their iron status, respectively, with NaFeEDTA being more suitable than electrolytic iron for diet supplementation in high-phytate flours (Andang'o et al., 2007).

The choice of the fortificant compound should be made considering factors such as the potential for organoleptic changes to the product, the bioavailability of the fortificant, the cost, stability and the shelf-life (Casgrain et al., 2010). To solve problems related to iron fortified foods such unacceptable taste, color, stability, and bioavailability, a fortification technology that prevents the iron-mediated undesirable taste and appearance of the final product while preserving stability and bioavailability was developed. The process involves iron stabilization using colloid chemistry (encapsulation), chelation, and electrochemical chemistry (redox modulation). Results from color and sensory evaluations showed that formulation of products using the fortification technology known as "GrowthPlus" eliminated unwanted effects on taste, appearance, and product stability. Bioavailability evaluation using animals and humans showed this technology does not interfere with the bioavailability of iron from either ferrous bis-glycinate or ferrous fumarate (Mehansho, 2006). However, the high cost of application of this technology makes it

inconvenient for use in fortification programs.

Another practical barrier to effective implementation of food fortification programs are policy aspects. These are important for effective program management, with legislation often being required to support and sustain iron fortification programs (Uauy et al., 2002). The regulations in various parts of the world have been adjusted for this purpose (see **Table 3**). The European Union updated the list of iron compounds approved to be used in the food enrichment in 2011 (Regulation, 2006; Commission Regulation, 2009; Regulation, 2011). The US Food and Drug Administration (FDA) include ferrous sulfate, ferrous fumarate, and ferric pyrophosphate, among the several compounds listed as 'Generally Recognized as Safe' (GRAS) and used for food iron fortification (Code of Federal Regulations, 2013). The Food Standards in the Australia New Zealand (FSANZ) include NaFeEDTA as a permitted form of iron that can be used in food fortification (Australia New Zealand Food Standards Code, 2013). Concerns about safety regarding upper limit levels must be considered in food iron fortification programs, and foods fortified may receive a special label indicating the level of fortification in accordance with specific legislations (Codex Alimentarius, 2013).



## 7. *In vitro* studies of iron bioavailability

There are four general approaches to estimate the dietary iron bioavailability: mathematical modeling (algorithmic approach), animal bioassays, *in vitro*, and human trials. *In vitro* methods have been proposed as an alternative to *in vivo* methods for estimating mineral bioavailability. Most of the *in vitro* methods have been developed to simulate the physiological conditions and the sequence of events that occur during digestion in the human gastrointestinal

tract. The *in vitro* methods, which cannot replace *in vivo* studies but are helpful as a predictive screening apparatus, exhibit certain advantages from the ethical and resources points.

Digestion and intestinal cell uptake assay are two *in vitro* methods that are very useful for the iron bioavailability studies, having, as conceptual basis whatever iron can be liberated into solution would be potentially available to be absorbed across the intestine. The first one measures the iron solubility or dyalizability under simulated gastric conditions (temperature, agitation, pH, and enzyme and chemical composition) and the other is represented by the uptake of iron using a Caco-2 cell which are a human colon adenocarcinoma cell line that exhibit characteristics of mature absorptive intestinal epithelium when differentiated (Cockell, 2007).

The gastric phase is performed with HCl or HCl-pepsin under fixed pH and temperature conditions, for a set period of time (pH 1-2, at 37ºC during 1-3 h), followed by an intestinal phase, subsequent neutralization using a base (usually NaOH or $NaHCO_3$), and incubation with pancreatic enzymes with or without bile salts (pH 7, at 37ºC during 1-5 h). This is followed by determination of the amount of soluble iron present in the supernatant obtained by centrifugation or filtration of the gastrointestinal digest of the food (solubility methods). The amount of solubilized iron can be used as a measure of bioaccessibility. Due the fact that bioavailability represents the fraction of ingested component or nutrient available for utilization in normal physiological function, the *in vitro* digestion shows only the fraction of the iron consumed available for intestinal absorption, which is known as bioacessibility (Guerra et al., 2012).

Naransiga Rao and Prabhavathi (1978) developed an *in vitro* method for measure iron availability by incubating the food matrix with pepsin-HCl at pH 1.35, followed by centrifugation and filtration. An aliquot was then adjusted to pH 7.5, with the addition of

pancreatin, and the ionizable iron was subsequently determined. Miller et al. (1981) proposed an *in vitro* digestion that measure iron solubility with accuracy for the estimation of non-heme iron availability in complex meals, with quick results and low cost. These authors digested a food sample with pepsin and HCl, after which a dialysis bag containing $NaHCO_3$ was added. This allows for a slow increase in pH before and during pancreatic digestion. The method closely parallels the events occurring when the food leaves the stomach and enters the duodenum. After incubation, pancreatin-bile addition to the digest, and another incubation period, the iron in the dialysate was analyzed. An important methodological difference with previous systems is the introduction of equilibrium dialysis. Instead of the iron fraction present in the supernatant after centrifugation, dialysable iron is measured. This eliminates the problems encountered when using centrifugation to separate soluble and insoluble components resulting from the digestion of complex meals. The use of a dialysis bag of a specific pore size also permits discrimination between high and low molecular weight soluble iron complexes. Enhancing and inhibiting factors of iron absorption such as ascorbic acid, coffee, wheat bread, tea and eggs were shown to respond *in vitro* as they affect iron absorption *in vivo*. But since this method uses iron dialyzability as a marker of availability it can significantly overestimate the iron availability (Glahn, 2009).

Simulated gastrointestinal digestion can be performed with static models where the products of digestion remain largely immobile and do not mimic physical processes such as shear, mixing and hydration. Static models are the most widespread digestive systems. However, none of these static models reproduce the dynamic processes occurring during human digestion. To overcome these limitations, several dynamic gastric models (computer-controlled systems)

haven been developed. These systems allow gradual modifications in pH and enzymes, and the removal of the dialysed components (Minekus et al., 1995; Ekmekcioglu, 2002). All these systems evaluate the aforementioned term "bioaccessibility", and can be used to establish trends in relative bioaccessibility. However, it is generally recognized that not all soluble or dialysable minerals are absorbable.

Simulated digestion does not represent the complexity of the human digestion but this *in vitro* method, with and without chemical modifications, has been extensively used due to provide availability measurements that correlate well with *in vivo* models, and is also useful to predict inhibitors and enhancing dietary compounds and applied to examine the influence of food processing on iron availability (Schricker et al., 1981; Haro-Vicente, et al., 2006). Miller and Berner (1989) revised the usefulness of *in vitro* solubility methods and pointed that iron solubility following *in vitro* gastrointestinal digestion is a reliable predictor of ascorbic acid effects on bioavailability, but not of protein effects, and that the solubility of low molecular weight iron is better than simple solubility in predicting iron bioavailability. The *in vitro* iron solubility and dialyzability are indicated for large-scale screening studies (Heath and Fairweather-Tait, 2002).

Glahn et al. (1996) design an *in vitro* model to estimate iron bioavailability in foods that combine solubility or dialysability and uptake and/or transport by Caco-2 cells. In this method foods sample undergo simulated peptic digestion followed by intestinal digestion (using a dialysis method) in the presence of Caco-2 cell monolayers. $^{59}$Fe uptake by the cell line is used as an estimator of iron bioavailability. These authors adapted an *in vitro* digestion / Caco-2 cell model to assess iron availability from foods through ferritin formation by the Caco-2 cells as an

indicator of iron uptake. Ferritin formation by Caco-2 cell monolayers is highly sensitive, and accurately measures food iron availability (Garcia, et al., 1996; Glahn et al., 1998). This system provides a rapid screening tool to measure the maximum potential of iron bioavailability, since the iron can be labeled with radio- and stable isotopes (Fairweather-Tait, 2001) and the results obtained have been well correlated qualitatively to human data (Hur et al., 2011).

*In vitro* solubility seems to give results that are comparable to the rat hemoglobin incorporation bioassay, with iron isotopically labeled. For assessment of iron bioavailability in complex meals the experimental design of the rat hemoglobin incorporation bioassay is considered unsuitable and is not suitable for studying the bioavailability of iron supplements. Animal models have been used to study the mechanistic effects of additions of iron, but Caco-2 cells may be considered equally appropriate for this purpose and provide a good model of the effects observed in the intestine level. The Caco-2 cell technique can be used for faster screening to predict potential availability for iron absorption (Fairweather-Tait, 2001; Heath and Fairweather-Tait, 2002).

The collection of data using Caco-2 cells model takes three days, while the rat *in vivo* gut model requires 10 days. The cost per culture plate is around 10% of the value used to purchase six rats for *in vivo* animal model studies (Becker, 1999). Thus, the Caco-2 model offers a relatively rapid, inexpensive, and versatile system for determining the effects of various aspects of the meal itself or of the digestion conditions upon cell mineral uptake. The most serious disadvantage of the Caco-2 cell line is the transformed nature of the cells, since they are derived from colon carcinoma, and the question that arises is to what extent normal metabolic processes are maintained in these cells. On the other hand, the Caco-2 cell line lacks a mucin layer, which

may play a significant role in iron intestinal absorption, and the transepithelial resistance is much higher than in the human small intestine and resembles that of the human colon. Furthermore, these cells have low carrier expression, resulting in very low transport rates (Wienk et al., 1999).

**8. *In vitro* studies of iron bioavailability in cereals fortified with iron**

Several studies have been done to evaluate the iron bioavailability of cereals fortified with iron. Cereal is a good food target to be fortified with iron since it is the base of the human diet in many cultures, grown in over 73% of the total world harvested area and contributes over 60% of the world food production (Charalampopoulos et al., 2002). The WHO include cereals among the foods suitable for iron fortification since is consumed for the target population vulnerable to iron anemia (WHO, 2006). The use of ascorbic acid together with the iron compound fortificants in wheat products is considered suitable since this acid has the capacity to inhibit binding of ferrous iron to wheat bran (Camire and Clydesdale, 1982). The **Table 4** summarizes the results obtained in studies of iron bioavailability in cereals fortified with non-heme iron using *in vitro* methods, published since 2000.

Some variables are included in the studies, like the type of iron compound used in the fortification process, levels, coadjutants compounds added, and the iron particle size. Ferrous sulfate compound, alone or in association with other compounds, was included in the majority of the researches done with fortified cereals (13/15 studies), followed by NaFeEDTA (7/15 studies). In tests of iron-fortified cereals, ascorbic acid and NaEDTA are tested together most frequently, followed by EDTA, folic acid, citric acid and tartaric acid. Among the cereals, wheat flour, either

baked into bread or not, was the main product tested in the bioavailability studies with levels from 44 to 100mg/kg (**Table 4**).

Arredondo et al. (2006) showed that there is an inverse relationship between H-reduced particle size and iron bioavailability. In wheat flour fortification if the particle size of H-reduced iron is ≤45-$\mu$m it should be added at 3 times the level of ferrous sulfate to provide the same absolute amount of absorbed iron. More recently, researchers found that in finger millet flour fortified with ferric pyrophosphate there was a decline in the bioaccessible iron content after 30 days of storage (Tripathi and Platel, 2011).

Yeung et al (2005) evaluated two types of wheat flour, low and high-extraction, fortified with some iron powders and baked into breads, and with and without ascorbic acid. The results indicate that while ascorbic acid enhances the iron bioavailability the baking process reduces it. This result agrees with the Maekawa et al. (2006) study, that also reports that baking into bread refined wheat flour fortified with H-reduced and ferrous sulfate does not enhance the iron bioavailability. The opposite was found in finger millet flours, where the heat process improves the bioacessibility of H-reduced iron in fortified and unfortified flours (Tripathi and Platel, 2011).

Wheat flour fortified with iron plus ascorbic acid or EDTA and sodium hexametaphosphate (SHMP) and baked into chapathi Indian bread exhibits predicted iron bioavailability twice as high when ascorbic acid or EDTA was added to the bread formulation than SHMP. The same effect was observed when tested with unfortified wheat flour (Nayak and Nair, 2003). The chapathi bread made with iron fortified wheat flour presented iron

bioavailability significantly higher when the fortificant compound was NaFeEDTA rather than ferrous sulfate (Kloots et al., 2004)

About coadjutants compounds, biscuits prepared with iron-fortified wheat (Ferrous sulfate or NaFeEDTA) in combination with either citric and tartaric acids at 60, 80 or 100 mg/100g levels show that the predictable bioavailability of the iron was increased by 27 and 83.9% after the addition of the ferrous sulfate or NaFeEDTA, respectively. The addition of tartaric acid (100 mg/100 g) increases the maximum the iron availability, being 436% in ferrous sulfate formula and 386% in NaFeEDTA formula (Govindaraj et al, 2007).

In corn masa fortified with six different iron compounds and two coadjutants (ascorbic acid and $Na_2EDTA$) the increase in the dialyzability of ferrous and ferric iron compounds was improved in the formulas with EDTA (either as NaFeEDTA or as $Na_2EDTA$). This study also evaluates the effect of phytic acid, an inhibitor of iron absorption, adding phytase to the corn masa. The results show that the phytase pretreatment generally increases the iron dialysis and improves the iron availability (De-Regil et al., 2007). In infant cereals the phytate:iron ratio was not found to be the major inhibitory factor on iron bioavailability, but the lower bioavailability was attributed to the type of iron fortificant compound (Nehir El et al., 2010).

Pachón et al. (2008) evaluated the effect of food combination, including wheat fortified with iron sulfate, on the iron bioavailability. Meat, which has enhanced influence on iron absorption, increases the iron bioavailability of iron fortified foods, but iron fortified foods do not enhance total iron bioavailability when added to meat. Yeung et al. (2002) studied unbleached bread four fortified with ferrous sulfate, electrolytic iron, NaFeEDTA, ferrous bis-glycinate, and ferrous fumarate. The results suggested that ferrous fumarate and ferrous bis-

glycinate had bioavailability similar to ferrous sulfate, and that electrolytic iron and NaFeEDTA does not completely exchange with intrinsic iron in foods. The same authors in 2003 reported that NaFeEDTA is more bioavailable than elemental iron or ferrous sulfate in iron fortified cereal meal added to raisin-containing foods.

In commercial cereal products fortified and unfortified with iron sold in Poland was found that the first products had significantly higher potential iron bioavailability in comparison with non-fortified ones despite the discrepancy in the analytical measures with the labeled ones (Suliburska et al., 2011). Industrialized fortified iron products also can have limited information about the iron compound used. For example, in the Cagnasso et al (2010) study the authors did not have access to the kind of elemental iron used in the cereal products analyzed by the labels being H-reduced iron, electrolytic iron and carbonic iron reduced iron forms that presented distinct iron availability.



**9. Conclusion**

Different *in vitro* methods are currently available for measuring the bioaccessibility and/or bioavailability of minerals in foods, but the results are not generally comparable between methods. Differences in laboratory conditions and the lack of standardized methods are the main causes. Among the cereals, wheat flour baked into bread or not was the main product tested in *in vitro* bioavailability studies. Ferrous sulfate was the principal iron compound used in cereal fortification studies, often used in association with ascorbic acid and NaEDTA. However, iron bioavailability from ferrous sulfate is lower than from other compounds, such FeNaEDTA or ferric pyrophosphate. The variables level of fortification, storage, level of extraction, baking and

also the association or not with other chemical compound seems to influence the results obtained.

**Acknowledgements**

Quintaes, K.D. is grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - Brasil) for the researcher grant (237331/2012-8).

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**Table 1**. Dietary inhibitor and enhancer factors of iron absorption.

| | Food and food compounds | Observations |
|---|---|---|
| **Inhibitors** | -Phytate or phytic acid | Present in cereal grains, high-extraction flour, legumes, and seeds |
| | -Polyphenols | Foods that contain the most potent inhibitors (e.g. tannins) resistant to the influence of enhancers include tea, coffee, cocoa, herbal infusions (tea) in general, certain spices (e.g. oregano), and some vegetables |
| | -Calcium | Particularly from milk and milk products found as calcium phosphate, inhibit absorption of non heme and heme iron |
| | -Proteins | Animal proteins from products like milk and eggs, and albumin, casein, and soy protein (independent of the phytate content) |
| | -Inositol | Food with high inositol content |
| **Enhancers** | - Acid ascorbic | Present in fruits, juices and vegetables such as green leaves, peppers |
| | -Heme iron | Present in meat, poultry, fish and seafood |
| | -Muscle tissue, the | 30g of muscle has the enhancer propriety as 25mg of |

| | | |
|---|---|---|
| | digestion products of meat, fish or poultry | ascorbic acid, possible due to the presence of cysteine-containing peptides or a multitude of small peptides |
| | -Fermented or germinated food and condiments | Sauerkraut and soy sauce (cooking, fermentation, or germination of food reduces the amount of phytates) |
| | -Polyoxycarbonic acids | Such as citrate and malate |
| | Caseinophosphopeptides (CPPs) | The CPPs added to fruit beverage (grape and orange) appears to improve iron bioavailability. Among the CPPs compounds, αs1-CN(64–74)4P, αs2-CN(1–19)4P and β-CN(1–25)4P) increased ferritin synthesis, β-CN(1–25)4P being the most effective |

Source: Adapted from WHO, 2001; García-Nebot et al., 2010; Hurrell and Egli, 2010; Sanz-Penella et al., 2012; García-Nebot et al., 2013.

**Table 2.** Requirements of iron intake (Fe in mg/day) by ages and genders among different agencies and countries selected

| | WHO/FAO (2002) | | EU Com (1992) | | DACH (2002) | | Nordic NNR (2004) | | AU NZ (2006) | | Belgic (2006) | | Brazil (2005) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Age** | **Bioav 15%** | **Bioav 5%** | **Age** | **Fe** | **Age** | **Fe** | **Age** | **Fe** | **Age** | **Fe** | **Age** | **Fe** | **Age** | **Fe** |
| 6-12m | 6.2 | 18.6 | | | <12m | 0.5 | <6m | - | 0-6m | 0.2 | 0-3m | 1.7 | 6-11m | 0.27 |
| 1-3y | 3.9 | 11.6 | 6-11m | 6 | 1-4y | 8 | 6-11m | 8 | 7-12m | 11 | 4-5m | 4.3-10 | 1-3y | 9 |
| 4-6y | 4.2 | 12.6 | 1-3y | 4 | 4-7y | 8 | 12-23m | 8 | 1-3y | 9 | 6-11m | 10 | 4-6y | 6 |
| 7-10y | 5.6 | 17.8 | 4-6y | 4 | 7-10y | 10 | 2-5y | 8 | 4-8y | 10 | 1-10y | 10 | 7-10y | 9 |
| | | | 7-10y | 6 | | | 6-9y | 9 | | | | | | |
| **Males** | | | | | | | | | | | | | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 11-14y | 9.7 | 29.2 | 11-14y | 10 | 10-13y | 12 | 10-13y | 11 | 9-13y | 8 | 11-4y | 10 | ≥11y | 14 |
| 15-17y | 12.7 | 37.6 | 15-17y | 13 | 13-15y | 12 | 14-17y | 11 | 14-18y | 11 | 15-18y | 13 | | |
| ≥18y | 9.1 | 27.4 | >18y | 9 | 15-19y | 10 | 18-30y | 9 | ≥19y | 8 | 19-60y | 9 | | |
| | | | | | | | ≥31y | 9 | | | >60y | 10 | | |
| **Females** | | | | | | | | | | | | | | |
| 11-14*y | 9.3 | 28 | 11-14y | 22 | 10-13y | 15 | | | 9-13y | 8 | 11-14y | 10*; 22 | ≥11 | 14 |
| 11-14y | 12.5 | 37.6 | 15-17y | 21 | 13-19y | 15 | 10-13y | 11 | 14-18y | 15 | 15-18y | 9*; 21 | | |
| 15-17y | 20.7 | 62 | >18y | 20 | ≥19y | 15 | 14-17y | 15 | 19-50y | 18.0 | 19-60y | 8* 20 | | |
| ≥18y | 19.6 | 58.8 | | | | | 18-60y | 15 | | | >60y | 10 | | |
| Postmenopausal | 7.5 | 22.6 | | *** | | 10 | | 9 | | 8 | | 10 | | 14 |
| Pregnancy | | *** | | | | 30 | | | | 27 | | 10 | | 27 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lactation | 10 | 30 | 10 | 20 | 15 | 9** | 10 | 15 | |

* no menstruation; **10 in ≤18y mothers; *** iron supplement

[continue]

| **Spain (2009)** | | **France (2001)** | | **Ireland (1999)** | | **Italy (1996)** | | **Philippines (2002)** | | **United States[a] (2001)** | | **UK (1991)** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Age** | **Fe** | **Age** | **Fe** | **Age** | **Fe** | **Age** | **Fe** | **Age** | **Fe** | **Age** | **Fe** | **Age** | **Fe** |
| <6m | 7 | | | 0-3m | 1.7 | 6-12m | 7 | <6m | 0.38 | 0-6m | 0.27 | <12m | 5.4 |
| 6-12m | 7 | | | 4-6m | 4.3 | 1-3y | 7 | 6-12m | 10 | 7-12m | 11 | 1-3y | 6.9 |
| 1-3y | 7 | 1-3y | 7 | 7-12m | 7.8 | 4-6y | 9 | 1-3y | 8 | 1-3y | 7 | 4-6y | 6.1 |
| 4-5y | 9 | 4-6y | 7 | 1-3y | 8 | 7-10y | 9 | 4-6y | 9 | 4-8y | 10 | 7-10y | 8.7 |
| 6-9y | 9 | 7-9y | 8 | 4-6y | 9 | | | 7-9y | 11 | | | | |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 7-10y | 10 | | | | | | | | |
| **Males** | | | | | | | | | | | | | |
| 10-12y | 12 | 10-12y | 10 | 11-14y | 13 | 11-14y | 12 | 10-12y | 13 | 9-13y | 8 | 11-14y | 11.3 |
| 13-15y | 15 | 13-19y | 13 | 15-17y | 14 | 15-17 | 12 | 13-15y | 20 | 14-18y | 11 | 15-18y | 11.3 |
| 16-19y | 15 | 20-64 | 9 | 18-64y | 10 | 18-29y | 10 | 16-18y | 14 | ≥19y | 8 | 15-50y | 8.7 |
| ≥20y | 10 | >65 | 9 | >65y | 10 | >30y | 10 | ≥19y | 12 | | | >50y | 8.7 |
| **Females** | | | | | | | | | | | | | |
| 10-12y | 18 | 10-12y | 10 | 11-14y | 14 | 11-14y | 12*-18 | 10-12y | 19 | 9-13y | 8 | 11-14y | 14.8 |
| 13-15y | 18 | 13-15y | 16 | 15-64 | 14 | 15-49y | 18 | 13-15y | 21 | 14-18y | 15 | 15-18y | 14.8 |
| 16-49y | 18 | 16-54y | 16 | >65 | 9 | >50y | 10 | 16-49y | 27 | 19-50y | 18 | 18-50y | 14.8 |
| Postmenopausal | 10 | | 9 | | 9 | | 10 | | 10 | | 8 | | 8.7 |





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| Pregnancy | 18 | 30 | 15 | 30*** | 27-38*** | 27 | 14.8 |
| Lactation | 18 | 10 | 15 | 18 | 27-30 | 10**-9 | 14.8 |

[a]UL: 0-13y: 40mg/day; >14y: 45mg/day* no menstruation; **10 in ≤18y mothers; *** iron supplements

**Source**: FAO/WHO, 2002; Deutsche Gesellschaft für Ernährung, Österreichische. Gesellschaft für Ernährung, Schweizerische Gesellschaft für Ernährungsforschung, Schweizerische V ereinigung für Ernährung; 2000; Nutrition Sub-committee of the Food Safety Authority of Ireland, 1999; Conseil Supérier d' Hygiène de Belgique, 2006; Società Italianna di Nutrizione Umana, 1998; Moreiras, et al., 2009; Scientific Committee on Food, 1992; Committee on Medical Aspects of food Policy (COMA). Departament of Health. Dietary Reference Values for Food Energy and Nutrients for the United Kingdom, 1991; Institute of Medicine, 2001. Becker et al., 2004; German Nutrition Society, 2002. Martin, 2001; BRASIL, 2005.

**Table 3.** World Health Organization suggestion of iron fortificants compounds for cereals based foods

| Iron fortificant compound | Solubility | Fe content (%) | Relative bioavailability* | Relative cost** (per mg/Fe) | Common cereal based vehicle | Legal permission of use |
|---|---|---|---|---|---|---|
| Ferrous sulfate (dry) | Water soluble | 33 | 100 | 1.0 | - Cereal-based complementary foods<br>- Low extraction (white) wheat flour or degermed corn flour<br>- Pasta<br>- Rice | EU, FDA, FSANZ |
| Ferrous sulfate plus ascorbic acid | Water soluble | 20 | 100 | 1.0 | - Pasta<br>- Rice | EU, FDA, FSANZ |
| Ferrous | Water soluble | 20 | >100 | 17.6 | - Pasta | EU |

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| bisglycinate | | | | | - Rice | |
| Ferric ammonium citrate | Water soluble | 17 | 51 | 4.4 | - Pasta<br>- Rice | EU, FDA, FSANZ |
| Sodium iron EDTA | Water soluble | 13 | >100 | 16.7 | -High extraction wheat flour, corn flour, corn masa flour | EU, FSANZ*** |
| Ferrous fumarate | Poorly water soluble, soluble in dilute acid | 33 | 100 | 2.2 | - Cereal-based complementary foods<br>- High extraction wheat flour, corn flour, corn masa flour (x2 amount)<br>- Low extraction (white) wheat flour or degermed corn flour | EU, FDA, FSANZ |
| Electrolytic iron | Water insoluble, | 97 | 75 | 0.8 | - Breakfast cereals | EU, FDA, |



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| (x2 amount) | poorly insoluble in dilute acid | | | | - Cereal-based complementary foods<br>- Low extraction (white) wheat flour or degermed corn flour | FSANZ |
| Ferric pyrophosphate (x2 amount) | Water insoluble, poorly insoluble in dilute acid | 25 | 21-74 | 4.7 | - Pasta<br>- Rice<br>- Infant cereals | EU, FDA, FSANZ |
| Encapsulated ferrous sulfate | The encapsulating agent, must be a food-grade digestible ingredient | 16 | 100 | 10.8 | - Cereal-based complementary foods<br>- High extraction wheat flour, corn flour, corn masa flour (2x amount)<br>- Low extraction (white) wheat flour or | EU, FDA, FSANZ |





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| | | | | | degermed corn flour | |
| Encapsulated ferrous fumarate (x2 amout) | The encapsulating agent, must be a food-grade digestible ingredient | 16 | 100 | 17.4 | - High extraction wheat flour, corn flour, corn masa flour | EU, FDA, FSANZ |
| Micronized ferric pyrophosphate | Poorly soluble | 25 | 21-74 | - | - Pasta<br>- Rice | EU, FDA, FSANZ |

* to hydrated ferrous sulfate, adult humans

** to dry ferrous sulfate

*** Not permitted to be added to breakfast cereals and to formulated supplementary foods for young children

EU: European Union; FDA: Food and Drug Administration; FSANZ: Food Standards in the Australia New Zealand

**Source**: Adapted from Lynch, 2005; Regulation, 2006; WHO, 2006; Commission Regulation, 2009; Commission Regulation, 2011; Akhtar et al., 2011; Australia New Zealand Food Standards Code, 2013; Code of Federal Regulations, 2013.

**Table 4**. *In vitro* studies of iron bioavailability and/or bioaccessibility in cereals fortified with non-heme iron.

| **Food Matrix (mg of iron/kg)** | **Type of the study** | **Iron compounds tested** | **Bioavailability and/or bioaccessibility** | **Reference** |
|---|---|---|---|---|
| Unbleached bread flour fortified (44 mg/kg) | Dialysis / Caco-2 cells | Ferrous sulfate<br>Electrolytic iron<br>NaFeEDTA<br>Ferrous bis-glycinate<br>Ferrous fumarate | 60 ng of FF/mg of protein<br>33 ng of FF/mg of protein<br>42 ng of FF/mg of protein<br>58 ng of FF/mg of protein<br>62 ng of FF/mg of protein | Yeung et at., 2002 |
| Indian bread (chapathi) prepared with wheat flour fortified (60 mg/kg) | Solubility | Ferrous sulfate<br>Ferrous sulfate plus acid ascorbic | 15.4%<br>22.0%<br>24.9% | Nayak and Madhavan Nair, 2003 |

| | | | | |
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| | | Ferrous sulfate plus NaEDTA<br>Ferrous sulfate plus SHMP | 22.7% | |
| Wheat bran cereal (9 mg/kg), Bread from local supermarket made with enriched flour | Dialysis / Caco-2 cells | Ferrous sulfate<br>Elemental iron (electrolytic, 100 mesh)<br>NaFeEDTA | 1.0 *<br>2.5, 4.2 *<br>18, 45 * | Yeung et al., 2003 |
| Indian bread (chapathi) prepared with the wheat flour fortified (50 mg/kg) | Dialysis / Caco-2 cells | Ferrous sulfate<br>Ferrochel amino acid chelate<br>Ferric amino acid chelate TF | 100%<br>130%<br>115%<br>73%<br>627% | Kloots et al., 2004 |

| | | | | |
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| | | Ferrous lactate | 16% | |
| | | NaFeEDTA | 64% | |
| | | Ferrous fumarate | 514% | |
| | | Ferric pyrophosphate | 44% | |
| | | SunActive Fe (Ferric | 69% | |
| | | pyrophosphate) | 72% | |
| | | Lipofer (Ferric | | |
| | | pyrophosphate) | | |
| | | Carbonyl iron | | |
| | | Electrolytic iron | | |
| Low-extraction wheat flour fortified 60 | Dialysis / | Ferrous sulfate | 100% ** | Yeung et at., |
| mg/kg) baked into bread, | Caco-2 cells | Atomet 95SP | 95, 118% ** | 2005 |
| High-extraction wheat flour fortified | | (Reduced) | 85, 120 % ** | |
| (100 mg/kg) baked into bread | | Atomet 75 (Reduced) | 105, 110 % ** | |
| | | RSI Hi-Sol (Reduced) | 104, 135% ** | |

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| | | Ac-325 (H-reduced)<br>RSI-325 (CO-reduced)<br>MH300.29 (CO-reduced)<br>Ferronyl (Carbonyl)<br>OF (Carbonyl)<br>A-131 (Electrolytic)<br>Electrolytic iron | 76, 111% **<br>63, 68% **<br>75, 160% **<br>90, 140% **<br>70, 98% **<br>70, 80% ** | |
| Wheat flour (*Sharbati* variety) fortified bread (60 mg/kg) | Dialysis / Caco-2 cells | H-reduced iron (8-$\mu$m)<br>H-reduced iron (≤45-$\mu$m) | 68.2%<br>31.1% | Arredondo et al., 2006 |
| Wheat flour (300 mg/kg) baked into bread | Dialysis / Caco-2 cells | Ferrous sulfate, 7-hydrate granular<br>H-reduced iron | 85 to 105 ng of FF/mg of protein<br>50 to 118 ng of FF/mg of protein | Maekawa et al., 2006 |

| | | | | |
|---|---|---|---|---|
| Corn masa gruel (10 mg/L) with and without phytase treatment | Dialysis | Ferrous sulfate | 1.4, 2.8% | Del Regil et al., 2007 |
| | | Ferrous sulfate + ascorbic acid | 1.4, 3.4%<br>12.3, 27.4% | |
| | | Ferrous sulfate + $Na_2EDTA$ | 1.1, 3.6%<br>2.4 - 4.4% | |
| | | Ferrous bis-glycinate | 11.7, 40.1% | |
| | | Ferrous bis-glycinate + ascorbic acid | 1.7, 2.1%<br>1.2, 5.8% | |
| | | Ferrous bis-glycinate + $Na_2EDTA$ | 19.8, 41.4%<br>0.6, 5.5% | |
| | | Ferrous fumarate | 1.5, 7.8% | |
| | | Ferrous fumarate + ascorbic acid | 18.1, 34.7%<br>0.7, 2.6% | |
| | | Ferrous fumarate + $Na_2EDTA$ | 2.9, 6.2%<br>18.9, 43.4% | |
| | | Ferric chloridric | 11.2, 42.4% | |





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| Ferric chloridric + ascorbic acid | 12.1, 39.3% - |
| Ferric chloridric + $Na_2EDTA$ | |
| Ferric ammonium citrate | |
| Ferric ammonium citrate + ascorbic acid | |
| Ferric ammonium citrate + $Na_2EDTA$ | |
| NaFeEDTA | |
| NaFeEDTA + ascorbic acid | |
| NaFeEDTA + $Na_2EDTA$ | |



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| Iron-fortified wheat biscuits (88 mg/kg) | Solubility | Ferrous sulfate | 27% | Govindaraj et al., 2007 |
| | | Ferrous sulfate + 60mg citric acid | 55% | |
| | | | 104% | |
| | | Ferrous sulfate + 80mg citric acid | 53% | |
| | | | 311% | |
| | | Ferrous sulfate + 100 mg citric acid | 397% | |
| | | | 436% | |
| | | Ferrous sulfate + 60 mg tartaric acid | 83% | |
| | | | 101% | |
| | | Ferrous sulfate + 80 mg tartaric acid | 117% | |
| | | | 63% | |
| | | Ferrous sulfate + 100 mg tartaric acid | 86% | |
| | | | 201% | |
| | | NaFeEDTA | 338% | |
| | | NaFeEDTA + 60mg citric acid | | |





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| | | NaFeEDTA + 80mg citric acid | | |
| | | NaFeEDTA + 100 mg citric acid | | |
| | | NaFeEDTA + 60 mg tartaric acid | | |
| | | NaFeEDTA + 80 mg tartaric acid | | |
| | | NaFeEDTA + 100 mg tartaric acid | | |
| Gluten free bread fortified with iron | Dialysis | Ferrous sulfate | 3.1% | Kiskini et al., 2007 |
| | | Ferric pyrophosphate | 4.1% | |
| | | Ferrous bis-glycinate | 3.8% | |
| | | Ferrous gluconato | 3.0% | |
| | | Ferrous lactate | 2.0% | |

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| | | Elemental iron | 2.3% | |
| | | NaFeEDTA | 4.0% | |
| Iron-fortified wheat flour (50 mg/kg) | Dialysis / Caco-2 cells | Ferrous sulfate | 25 ng of FF/mg of protein | Pachón et al., 2008 |
| Commercial cereal iron-fortified and also fortified in the laboratory, prepared with and without milk | Dialysis | Ferrous sulfate<br>Elemental iron<br>NaFeEDTA | 4 - 15%<br>1 - 15%<br>19 - 28% | Cagnasso et al., 2010 |
| Fortified infant cereals purchased from supermarket | Dialysis | Iron disphosphate<br>Iron sulfate | 1.19 - 6.25%<br>0.80 - 9.81% | El Nehir et al., 2010 |
| Corn flakes (36 mg/kg)<br>Honey flakes (21 mg/kg)<br>Flakes *Shelly Crups* (51 mg/kg) | Solubility | Iron reduced | 36.8%<br>15.5%<br>14.1% | Suliburska et al., 2011 |





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| Balls *Nesquik* (36 mg/kg) | | | 19.2% | |
| Muesli *Fitness Fruits* (84 mg/kg) | | | 41.4% | |
| Flakes *Cookie Crips* (36 mg/kg) | | | 37.8% | |
| Flakes *Dotty Churps* (35 mg/kg) | | | 24.0% | |
| Cookies *Miskopty* (60 mg/kg) | | | 37.7% | |
| Cookies *Go! Musli = owacami* (29 mg/kg) | | | 50.0% | |
| | | | 18.8% | |
| Cookies *Go! Kakao* (35 mg/kg) | | | 49.8% | |
| Cookies *Go! 4=boza + mleko* (29 mg/kg) | | | | |
| Finger millet flour (60 mg/kg), and dumpling and roti preparations | Dialysis | Ferrous fumarate | 0.29 mg of Fe/100g | Tripathi and Platel, 2011 |
| | | Ferrous fumarate + folic acid | 0.40 - 1.09 mg of Fe/100g | |
| | | Ferrous fumarate + EDTA | 2.25 - 2.55 mg of Fe/100g | |
| | | Ferrous fumarate + | 2.35 - 2.36 mg of | |

| | | |
|---|---|---|
| | citric acid + EDTA | Fe/100g |
| | Ferrous fumarate + | 1.13 - 1.17 mg of |
| | folic acid + citric acid | Fe/100g |
| | Ferric pyrophosphate | 0.27 mg of Fe/100g |
| | Ferric pyrophosphate + | 0.35 mg of Fe/100g |
| | folic acid | 2.4 mg of Fe/100g |
| | Ferric pyrophosphate + | |
| | EDTA | |

SHMP = Sodium hexametaphosphate

FF: Ferritin formation

* ferritin formation in relation a ferritin formation in wheat bran cereal not fortified.

** ferritin formation (5) in relation a $FeSO_4$ (100%).