Doctoral Thesis

Optimizing the absorption of fortification iron

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OPTIMIZING THE ABSORPTION OF FORTIFICATION IRON

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presented by

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Summary

Background: Iron deficiency is one of the major nutritional problems in the world among women of childbearing age, children, and infants, especially in developing countries. Food fortification programs are usually considered the most cost-effective and sustainable approach to combat iron deficiency. However, food fortification with iron is difficult since the best absorbable forms of iron are chemically reactive and often produce undesirable side effects in the food vehicle. Inert iron compounds on the other hand do not lead to flavor or color changes but are usually poorly absorbed.

Aim: The overall aim of this PhD project was to optimize iron bioavailability from iron fortificants currently used in iron fortification programs.

Different approaches to optimize iron bioavailability from poorly water-soluble and water-insoluble iron fortificants were evaluated, including the addition of ascorbic acid to food fortified with ferrous fumarate and ferric pyrophosphate and the addition of Na$_2$EDTA to ferrous fumarate fortified food. Furthermore, the influence of particle size on iron absorption from ferric pyrophosphate and the relative iron bioavailability (RBV; compared to ferrous sulfate) of an innovative iron compound (micronized, dispersible ferric pyrophosphate/Sunactive Fe™) were evaluated within this project. In addition, a series of studies were conducted to provide information on other relevant issues in food fortification, including the enhancing effect of erythorbic acid, a stereoisomer of ascorbic acid, on iron absorption from ferrous sulfate. Finally, the potential usefulness of NaFeEDTA as a food fortificant for fish sauce and soy sauce was investigated in studies of iron absorption and photostability.

Design: Five series of studies were made evaluating iron absorption in healthy adult women (9-10 women/iron absorption study) based on erythrocyte incorporation of stable isotopes ($^{57}$Fe and $^{58}$Fe) 14 days after intake of labeled test meals (5 mg iron/test meal). In study series 1, the effects of ascorbic acid (molar ratio ascorbic acid to added iron 4:1) and Na$_2$EDTA (molar ratio 1:1) on iron absorption from ferrous fumarate were assessed. The effect of ascorbic acid (molar ratio ascorbic acid to added iron 4:1) on iron absorption from ferric pyrophosphate was investigated in study series 2. Further, the influence of particle size on iron absorption from ferric pyrophosphate was evaluated by comparing the RBV of ferric pyrophosphate fractions.
with average particle sizes of 6.7 μm, 8.5 μm, and 12.5 μm (study series 2). In this context, iron absorption from micronized, dispersible ferric pyrophosphate was assessed in an infant cereal and a yoghurt drink (study series 3). In the fourth study series, the effect of erythorbic acid on iron absorption from ferrous sulfate at molar ratios of 2 and 4 (relative to added iron) was evaluated. In addition, the effect of erythorbic acid was compared directly to that of ascorbic acid at a molar ratio 4. Lastly, fish sauce and soy sauce were evaluated as iron fortification vehicles by comparing iron absorption from NaFeEDTA fortified fish sauce and soy sauce to a reference fortificant (ferrous sulfate) as well as comparing iron absorption from NaFeEDTA fortified fish sauce and soy sauce. Further the influence of fish- and soy sauce per se on iron absorption was evaluated (study series 5). The results from all iron absorption studies are presented as geometric means. In addition to the iron absorption studies, the photostability of NaFeEDTA in fish sauce and soy sauce stored in clear and amber glass bottles, and polyethylene terephthalate (PET) bottles under indirect sunlight/fluorescent light, natural and artificial sunlight, and in the dark was determined. NaFeEDTA degradation was assessed by measuring Fe(III)EDTA concentrations in regular intervals using HPLC techniques (study series 6).

Results:

**Study series 1:** There was no difference between iron absorption from ferrous fumarate and ferrous sulfate fortified cereal (3.0 and 3.1%, respectively, p=0.85). However, when Na₂EDTA was added, iron absorption was significantly higher from ferrous sulfate than from ferrous fumarate (5.3 vs. 3.3%, respectively, p<0.01). Further, the results showed that the addition of ascorbic acid to ferrous fumarate fortified cereal increased iron absorption significantly (6.3 vs. 10.4%, p=0.02).

**Study series 2:** The addition of ascorbic acid increased iron absorption from ferric pyrophosphate significantly (0.9 vs. 2.3%, p=0.004). However, the magnitude of the enhancing effect was significantly lower than on iron absorption from ferrous sulfate (2.6-fold vs. 3.7-fold increase). RBV of ferric pyrophosphate with an average particle size of 6.7 μm, 8.5 μm, and 12.5 μm was shown to be 52, 36, and 42%, respectively. The slight increase in RBV of ferric pyrophosphate with decreased particle size was however not significant (p>0.05).
Summary

**Study series 3:** There was no statistically significant difference between iron absorption from micronized, dispersible ferric pyrophosphate (Sunactive Fe™) and ferrous sulfate fortified infant cereal (3.4% and 4.1%, respectively, p = 0.24) and yoghurt drink (3.9% and 4.2%, respectively, p=0.72).

**Study series 4:** The addition of erythorbic acid (molar ratios of 2 and 4 relative to iron) increased iron absorption from ferrous sulfate fortified infant cereal 2.6- and 4.6-fold, respectively (from 4.1% to 10.8%, p< 0.0001 and 18.8%, p<0.0001). The addition of ascorbic acid (molar ratio 4) increased iron absorption 2.9-fold (11.7%, p=0.0004). At a molar ratio of 4, erythorbic acid was 1.6-fold (p=0.0002) more potent as an enhancer of iron absorption than ascorbic acid.

**Study series 5:** Iron absorption was similar from NaFeEDTA and ferrous sulfate fortified fish sauce (3.3 versus 3.1%) and soy sauce (6.1 versus 6.3%). Soy sauce per se was inhibitory to iron absorption from rice-based meals (8.5% without versus 6.0% with soy sauce, p<0.02) while the addition of fish sauce did not affect iron absorption significantly. No significant difference was observed when NaFeEDTA fortified fish- and soy sauce were compared directly (6.7 and 7.9%).

**Study series 6:** No significant degradation of NaFeEDTA was observed during storage of fortified soy sauce. Losses of up to 35% NaFeEDTA, however, occurred within 2-6 weeks in fortified fish sauce stored in clear bottles exposed to direct sunlight. Losses were prevented by storage in amber bottles or by storing the clear bottles in indirect sunlight or in the dark.

**Conclusions:** A number of important conclusions can be drawn from the results of these studies. Firstly, the findings show that iron absorption from ferrous fumarate and ferric pyrophosphate fortified foods can be increased by adding ascorbic acid. Na₂EDTA on the other hand was shown to have no effect on iron absorption from ferrous fumarate which emphasizes the need to evaluate the effect of enhancers and inhibitors on iron absorption from poorly water-soluble and water-insoluble iron compounds. Secondly, the results from studies 2 and 3 indicate that iron absorption from water-insoluble iron compounds can be improved by optimizing particle size.

Erythorbic acid, which is used in processed foods as an antioxidant, was shown to be a potent enhancer of iron absorption. However, its lack of antiscorbutic activity limits the usefulness for iron fortification programs. Nevertheless, it may be an important...
factor influencing iron bioavailability in countries where foods preserved with erythor-
bic acid are widely consumed. Lastly, the relatively high iron absorption from
NaFeEDTA fortified fish- and soy sauce indicates the potential usefulness of this iron
fortificant for fish- and soy sauce fortification programs. Since NaFeEDTA is not sta-
ble in fish sauce when stored in clear glass or PET bottles exposed to direct sunlight,
it may be necessary to protect fortified fish sauces from sunlight by using amber bot-
tles or large labels covering most of the bottle.
Zusammenfassung


**Ziel:** Das allgemeine Ziel dieser Dissertation war, die Bioverfügbarkeit der häufig in Lebensmitteln verwendeten Eisenverbindungen zu optimieren.


**Methoden:** Fünf Studienreihen wurden gemacht, in denen die Eisenabsorption jünger, gesunder Frauen (9-10 Frauen/Absorptionsstudie) gemessen wurde. Die hierfür verwendete Methode basierte auf der Inkorporation stabiler Isotope (⁵⁷Fe und ⁵⁸Fe) in Erythrozyten 14 Tage nach der Aufnahme einer markierten Testmahlzeit (5 mg Ei-
Zusammenfassung


Ergebnisse

**Studienreihe 1:** Zwischen Eisenfumarat- und Eisensulfat-angereichertem Getreidebrei konnte kein Unterschied hinsichtlich der Eisenabsorption festgestellt werden (3.0 bzw. 3.1%, p=0.85). Die Eisenabsorption aus Eisensulfat war allerdings signifikant höher als aus Eisenfumarat, nachdem dem Brei Na₂EDTA zugegeben wurde (5.3 bzw. 3.3%, p<0.01). Ferner zeigten die Resultate, dass die Zugabe von Ascorbinsäu-
Zusammenfassung

Zur fortifizierten Getreidebrei die Eisenabsorption signifikant erhöhte (6.3 vs. 10.4%, p=0.02).

**Studienreihe 2:** Der Zusatz von Ascorbinsäure zu Eisenpyrophosphat-angereichertem Getreidebrei hat die Eisenabsorption signifikant erhöht (0.9 vs. 2.3%, p=0.004). Allerdings war das Ausmass des Effektes signifikant geringer als bei Brei, welcher mit Eisensulfat fortifiziert worden war (2.6-fache Steigerung verglichen mit 3.7-facher Steigerung). Die RBV von Eisenpyrophosphat mit einer durchschnittlichen Partikelgrösse von 6.7μm, 8.5 μm und 12.5 μm waren 52, 36 beziehungsweise 42%. Der geringe Anstieg der RBV in Abhängigkeit von der verringerten Partikelgrösse war nicht signifikant (p>0.05).

**Studienreihe 3:** Es gab keinen signifikanten Unterschied zwischen der Eisenabsorption aus mit mikronisiertem und dispergierbarem Eisenpyrophosphat (Sunactive Fe™) oder Eisensulfat (3.4% bzw. 4.1%, p=0.24) fortifiziertem Getreidebrei. Ferner konnte auch kein Unterschied in der Eisenabsorption aus einem Yoghurtdrink, fortifiziert mit den gleichen Eisenkomponenten, festgestellt werden (3.9% bzw. 4.2%, p=0.72).

**Studienreihe 4:** Der Zusatz von Isoascorbinsäure (molares Verhältnis Isoascorbinsäure zu Eisen 2:1 und 4:1) bewirkte eine 2.6-fache beziehungsweise 4.6-fache Steigerung der Eisenabsorption aus einem Babybrei, der mit Eisensulfat fortifiziert worden war (von 4.1% auf 10.8%, p<0.0001 und 18.8%, p<0.0001). Der Zusatz von Ascorbinsäure (im molaren Verhältnis 4:1) steigerte die Eisenabsorption nur um das 2.6-fache. Isoascorbinsäure förderte dementsprechend die Eisenabsorption um das 1.6-fache (p=0.0002) gegenüber Ascorbinsäure.

**Studienreihe 5:** Die Eisenabsorptionen aus Fischsauce und Sojasauce, die mit NaFeEDTA beziehungsweise Eisensulfat fortifiziert worden waren, haben sich als vergleichbar erwiesen (3.3 und 3.1% bzw. 6.1 und 6.3%). Des Weiteren hat sich gezeigt, dass die Sojasauce per se eine hemmende Wirkung auf die Eisenabsorption hatte (8.5% ohne vergl. mit 6.0% mit Sojasauce, p<0.02). Fischsauce hingegen hatte keinen Einfluss auf die Eisenabsorption. In Bezug auf die Eisenbioverfügbarkeit konnte im direkten Vergleich der mit NaFeEDTA angereicherten Fisch- und Sojasauce kein Unterschied festgestellt werden (6.7 und 7.9%).
Zusammenfassung

**Studienreihe 6:** Es wurde kein Abbau von NaFeEDTA während der Lagerung der fortifizierten Sojasauce festgestellt. NaFeEDTA Verluste von bis zu 35% wurden allerdings in fortifizierten Fischsaucen beobachtet, die in klaren Flaschen direktem Sonnenlicht ausgesetzt worden waren. Diese Verluste konnten durch Vermeidung direkten Sonnenlichts bzw. durch Lagerung in braunen Flaschen verhindert.

**Schlussfolgerungen:**

Introduction

In the 17th century, iron deficiency anemia was known as “the green sickness” or chlorosis and was believed to be associated with being in love because mainly young women suffered from the condition. A common treatment for those who were lethargic and pale was to lay a sword out overnight where it would get wet with dew, and in the morning to scrape off the rust and disperse it in wine. This was found to be a stimulant that restored skin color and vitality. The connection between iron and the successful treatment of “chlorosis” was however not understood until the 19th century. A French Professor, Pierre Blaud, reported in 1832 on the successfulness of treating young women whom “coloring matter was lacking in the blood” with iron tablets. Iron fortification of foods as a preventive measure for iron deficiency anemia was demonstrated in 1928 by Mackay who showed that anemia during infancy could be decreased by fortifying milk powder with iron (as reviewed by Carpenter, 1990; and Yip, 2001).

Although strategies for treatment and prevention of iron deficiency have been known for such a long period of time, iron deficiency is today assumed to be the most common micronutrient deficiency world-wide. Food fortification programs are presently considered the most cost-effective and sustainable approach to combat iron deficiency. However, iron is the most difficult mineral to add to foods as the most bioavailable forms of iron are chemically reactive and often produce undesirable organoleptic changes to the food vehicle. Inert iron compounds on the other hand do not lead to flavor or color changes but are usually poorly absorbed as they do not dissolve completely in the gastrointestinal tract during digestion. A further problem is the presence of inhibitors in the food vehicles and overall diet. It is therefore challenging to develop iron fortified foods and design efficacious iron fortification programs.

The present thesis focuses on improving the bioavailability of iron fortificants and is based on 6 manuscripts and a literature review. Within the literature review, the current knowledge regarding iron deficiency, iron absorption, and food fortification with iron is reviewed. The first three manuscripts describe the evaluation of various strategies to increase iron absorption from poorly water-soluble and water-insoluble
Introduction

iron compounds. In the fourth manuscript a study made to investigate the effect of erythorbic acid on iron absorption is presented. The last two manuscript focus on NaFeEDTA fortified fish sauce and soy sauce as fortification vehicles, more precisely on iron bioavailability from these vehicles and the photostability of NaFeEDTA in fish sauce and soy sauce.
1 Literature Review

1.1 Iron

Iron is an essential trace element for the human body and plays an important role in oxidative energy production. Body iron which transports and uses oxygen in the production of energy is referred to as functional iron and can be found in hemoglobin, myoglobin, iron-dependent enzymes, and respiratory chain proteins (Bothwell et al. 1979). Both inadequate iron supply and excessive iron accumulation in the body lead to increased morbidity. Iron overload has been associated with several pathological conditions, including liver and heart diseases (Yang et al. 1998; Milman et al. 2001; Rasmussen et al. 2001), cancer (Beckman et al. 1999; Parkkila et al. 2001), neurodegenerative disorders (Sayre et al. 2000; Berg et al. 2001), diabetes (Perez de Nandoles et al. 2000; Ellervik et al. 2001; Parkkila et al. 2001), hormonal abnormalities (Wilkinson, 1996), and immune system abnormalities (Li et al. 2000; Walker & Walker, 2000). Far more people are however affected by iron deficiency. Iron deficiency has been estimated to affect over 1 billion people in developing as well as industrialized countries and is described in the first part of this review along with the causes, consequences, and prevalence of iron deficiency. Thereafter, the review will focus iron fortification, which is generally considered the most cost-effective and sustainable approach to prevent iron deficiency (Cook & Reusser, 1983).

1.2 Iron Deficiency

Iron deficiency (ID) develops in three overlapping stages (Cook & Finch, 1979). Storage iron depletion is the first stage of ID, which is characterized by the absence of iron stores without the loss of functional iron. Therefore, this stage is not associated with any adverse physiological consequences. The amount of storage iron can be determined by measuring the concentration of serum ferritin, which provides a quantitative estimation of the size of iron stores in liver, bone marrow, and spleen (Cook & Finch, 1979; Lynch & Green, 2001). Each 1 μg/L serum ferritin indicates about 8 mg storage iron in adults (Bothwell et al. 1979) and 0.14 mg in children (Finch & Huebers, 1982). When serum ferritin levels in adults fall below 12 μg/L iron stores are
Literature review

considered to be depleted (Institute of Medicine, 2002). The second stage of ID is known as early functional ID or ID erythropoiesis. During this stage the amount of iron supplied to bone marrow and other tissues is only marginally inadequate (Lynch & Green, 2001). As hemoglobin concentrations do not fall below levels considered indicative of anemia, this stage is also referred to as ID without anemia. It can be determined by measuring the saturation of serum transferrin, the main iron-binding protein responsible for the transport of iron in the plasma (Brody, 1999), which decreases from 30-35% to below 16%. Further, free erythrocyte protoporphyrin concentrations greater than 70 μg/dL of erythrocytes indicate that the formation of heme from protoporphyrin IX is impaired due to insufficient iron supply. Lastly, the lack of iron leads to an increase in transferrin receptors on the surface of all tissues resulting in soluble serum transferrin receptor concentrations above 8.5 mg/L (Institute of Medicine, 2002). The final stage of ID is iron deficiency anemia (IDA), which is diagnosed on the basis of hemoglobin concentrations below 120 g/L in menstruating women above 15 years of age and 130 g/L in men above 15 years of age. The critical levels for the diagnosis of anemia in pregnant women are slightly lower (110 g/L) as are the values for infants and children (6 months to 5 years: 110 g/L, 5-11 years: 115 g/L, and 12 to 14 years: 120 g/L) (WHO/UNICEF/UNU, 2001). IDA however occurs only in association with abnormal values for the indicators for ID, since decreased hemoglobin levels are not a specific indicator for IDA. Hemoglobin levels can be decreased for a number of reasons, for example further nutritional deficiencies such as vitamin A, folic acid, riboflavin, or vitamin B₁₂ deficiency (Allen & Casterline-Sabel, 2001), infections such as malaria (Fleming, 1981), and chronic inflammatory disorders (Yip & Dallman, 1988).

1.2.1 Determinants of Iron Balance

In order to describe the causes of ID it is necessary to have knowledge of the factors upon which iron balance depends. There are three major determinants of iron balance. Firstly, iron requirements which consist of basal iron losses from interior and exterior surfaces of the body, menstrual blood and therefore iron losses, and addi-
tional iron needs for growth and during pregnancy and further vary according to age and gender. Secondly, iron balance is dependent on the amount of iron consumed with the diet and on the amount of iron which can be absorbed from the diet (Baynes, 1994). Since iron absorption is discussed later in detail (section 1.3) it will only briefly be mentioned here.

1.2.1.1 Iron Requirements

Basal Iron Losses
The calculation of basal iron losses in adults and children above the age of 9 years are based on studies by Green et al. (1968) and are estimated to be 14 µg per kg body weight and day. Thus, an average US-American women (64 kg) loses about 0.9 mg iron/day, an average US-American man (77 kg) about 1.1 mg (Institute of Medicine, 2002). For infants between the ages of 7 to 12 months basal loss estimates are 0.03 mg/kg/day (Garby et al. 1969). Basal iron losses for children aged 1 through 8 years are derived from the total body iron losses measured in adult men and are estimated as being 0.538 mg/m²/day (Green et al. 1968).

Menstrual Blood Losses
Menstrual blood losses are very consistent from month to month for an individual, however, they vary strongly between individuals (Hallberg et al. 1966). Nevertheless, the mean blood loss per menstrual cycle has been estimated to be 30.9 mL (Institute of Medicine, 2002). Based on an average hemoglobin concentration in non-anemic women of 135 g/l (Beaton et al. 1989) and an average iron concentration of 3.39 mg/g hemoglobin (Smith & Rios, 1974), mean menstrual iron losses have been calculated as 0.51 mg/day. It is however important to emphasize that menstrual iron losses can be considerably higher; for example in 10% of all menstruating women menstrual iron losses have been estimated to be over 1.38 mg/d (Institute of Medicine, 2002).

Additional Iron Needs
The additional iron needs for growth depend on age and are highest for infants from birth to 1 year. The infant’s additional iron needs are strongly dependent on total body iron at birth, which in turn is dependent on birth weight. For example, total body iron of infants weighing 3.5 kg at birth has been estimated to be 268 mg. An infant weighing only 2.5 kg has a total iron content of approximately 183 mg (Widdowson,
1982). At the age of 1 year total body iron content of a 10-10.5 kg heavy infant is presumably between 362 and 377 mg. Thus, the additional iron needs for infants with birth weights of 2.5 kg are approximately 0.5 mg/day while infants weighing 3.5 kg at birth only require approximately 0.3 mg/day (Fomon, 1993). In the following seven years, the growing child needs approximately 0.21 mg iron/d to cover the additional iron needs due to increased hemoglobin mass, functional tissue iron, and storage iron (Institute of Medicine, 2002). Between the age of 8 and 16 years the additional amount of iron needed for growth varies little for girls (0.36-0.38 mg/d). Boys however require nearly twice as much as girls during adolescence because of their rapid growth (0.66 mg/d) (Hallberg et al. 1993b).

During pregnancy additional iron is needed for fetal and placental iron deposition (estimated to be 300 and 50 mg, respectively) and for the increase in hemoglobin mass (approximately 450 mg). These additional needs are however not equally distributed throughout pregnancy. During the first trimester only little extra iron is needed for growth (0.27 mg/d) and iron requirements are actually less than those for menstruating women. Iron requirements in the second (3.7 mg/d) and especially third trimester (4.7 mg/d) are however very high (Hallberg et al. 1993b; Institute of Medicine, 2002).

1.2.1.2 Dietary Iron Intake
Two types of iron are present in the diet: heme iron and non-heme iron. Heme iron is derived from the hemoglobin and myoglobin in meat, poultry, and fish. Although heme iron only accounts for a small proportion of iron in the diet of industrial countries (approximately 10% (Raper et al. 1984), it plays a quantitatively important role since heme iron is well absorbed (approximately 25%) and is almost not affected by inhibitors of iron absorption (Hallberg, 1981). Non-heme iron is all iron in plants and non-cellular animals foods (for example eggs and dairy products) as well as approximately half the iron in meat, fish, and poultry (Monsen, 1988). Further non-plant sources of non-heme iron are fortification iron, which is described later in detail (Section 1.3.2) and contamination iron, for example from soil or cooking pots. Soil iron has been found in substantial amounts in foods in developing countries but is only poorly absorbable (Hallberg et al. 1983). Iron absorption of non-heme iron depends (in addition to the iron status of the individual) on the chemical form of the iron as well
as on the presence of inhibitors (phytic acid, polyphenols, calcium, and dietary protein) and enhancers (ascorbic acid and animal tissue) in the diet. Therefore, when evaluating iron intake data it is important to consider these factors.

Recommended Daily Intake Values
The variations in the amount of iron absorbed from the diet are important for the calculations of recommended daily intake values and are also the reason for the large differences between the daily intake values recommended by different organizations and different countries. As examples, the dietary reference intakes (DRI) for iron for the US and Canada and the iron intakes recommended by FAO/WHO (Food and Agricultural Organization of The United Nations/World Health Organization) are given in Tables 1 and 2. In both cases, the recommended iron intake values are calculated based on the amount of iron needed to cover iron requirements of 97.5% of the target population, as well as the amount of iron that can be absorbed from the diet (WHO/UNICEF/UNU, 2001; Institute of Medicine, 2002).

The DRI for the US and Canada are based on an average iron absorption of 18%, with exception of those for infants aged 7 through 12 months whose average iron absorption was estimated to be 10% (Institute of Medicine, 2002). The average iron absorption of 18% was based on the results of the following studies: Cook et al. (1991a) showed that non-heme iron absorption from self-selected diets was 7.4% for non-iron deficient healthy adults (average serum ferritin concentration: 34 μg/L). From this value non-heme iron absorption was calculated as being 16.8% for individuals with low iron stores (serum ferritin concentration: 15 μg/L). Further, heme iron absorption was assumed to be 25% (Hallberg & Rossander-Hulten, 1991), and based on data from Raper et al. (1984) it was estimated that 10% of the total iron intake would be heme iron (Overall iron absorption= Fraction of non-heme iron [0.9] x non-heme iron absorption [0.168] + fraction of heme iron [0.1] x heme iron absorption [0.25] = 17.6%).
**Table 1:** DRI as intakes for individuals for iron (mg/day) as recommended by the Food and Nutrition Board, Institute of Medicine-National Academy of Science

<table>
<thead>
<tr>
<th>Groups</th>
<th>Age</th>
<th>DRI</th>
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<tbody>
<tr>
<td>Infants</td>
<td>0-0.5 months</td>
<td>0.27</td>
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<tr>
<td></td>
<td>7-12 months</td>
<td>11</td>
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<tr>
<td>Children</td>
<td>1-3 years</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>4-8 years</td>
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<tr>
<td>Males</td>
<td>9-13</td>
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<td>14-18</td>
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<td>Females</td>
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The WHO/FAO values, which have so far not officially been published, are calculated for different types of diets with average absorption values between 5 and 15%, depending on the amount of animal products and ascorbic acid present in the diet. Very low iron absorption (5%) was assumed for diets based on cereals, roots, and tubers and containing almost no fish, poultry, meat, or ascorbic acid. When small amounts of animal tissue and ascorbic acid are present in the diet, iron absorption is estimated to be about 10% and from diets which contain generous amounts of animal tissue and ascorbic acid iron absorption is judged to be 15% (WHO/UNICEF/UNU, 2001; Institute of Medicine, 2002).
Table 2: WHO/FAO recommendations for iron intakes (mg/day) by age and gender group according to iron bioavailability in different types of diet*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Age (years)</th>
<th>Recommended iron intakes for diets of different bioavailability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5%</td>
</tr>
<tr>
<td>Children</td>
<td>0.5-1</td>
<td>18.6</td>
</tr>
<tr>
<td></td>
<td>1-3</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>4-6</td>
<td>12.6</td>
</tr>
<tr>
<td></td>
<td>7-10</td>
<td>17.8</td>
</tr>
<tr>
<td>Males</td>
<td>11-14</td>
<td>29.2</td>
</tr>
<tr>
<td></td>
<td>15-17</td>
<td>37.6</td>
</tr>
<tr>
<td></td>
<td>18+</td>
<td>27.4</td>
</tr>
<tr>
<td>Females</td>
<td>11-14 *</td>
<td>28.0</td>
</tr>
<tr>
<td></td>
<td>11-14</td>
<td>65.4</td>
</tr>
<tr>
<td></td>
<td>15-17</td>
<td>62.0</td>
</tr>
<tr>
<td></td>
<td>18+</td>
<td>58.8</td>
</tr>
<tr>
<td>Lactating</td>
<td></td>
<td>30.0</td>
</tr>
</tbody>
</table>

*psee text for detail on types of diet
*Adapted from: (WHO/UNICEF/UNU, 2001)
*a non-menstruating

1.2.2 Causes of Iron Deficiency

ID develops when the amount of iron absorbed from the diet is lower than the amount of iron needed to cover the physiological iron requirements. Consequently, the population groups most at risk to develop ID are those with the highest iron requirements. These are infants, children, adolescents, and pregnant women who have additional iron needs due to growth as well as women of childbearing age who have higher iron losses due to menstrual blood loss.

Dietary Causes of Iron Deficiency

Inadequate iron absorption can be caused by multiple factors. Low dietary iron intake is one of the reasons and can for example result from energy restriction or a diet low in iron, for example a diet based on white rice. While in developing countries energy restriction is mostly involuntary, voluntary energy restriction is relatively common in industrialized countries, in particular in young females (Baynes, 1994). The low-energy life-style in industrialized countries has further been associated with an in-
creased risk of insufficient iron intake (Hallberg, 2001). Low iron absorption can further be a result of poor dietary quality rather than low iron intake (Bothwell et al. 1989). Dietary quality, based on the content of enhancers and inhibitors of iron absorption in the diet, is frequently dependent on socio-economic factors. For example, in Venezuela the diets consumed by the different socio-economical classes were compared for iron content as well as their content of enhancing and inhibiting factors. The results showed that while iron content was higher in the diet of the lower social classes, the intake of meat and ascorbic acid was substantially lower and that of phytic acid substantially higher than in higher social classes (Taylor et al. 1995). This can be explained by the high cost of meat and fresh fruits which limits their consumption in underprivileged population groups, in developing as well as industrialized countries (Marx, 1997; Hambraeus, 1999). It is thus not surprising that ID is most common among groups of low socio-economic status (WHO/UNICEF/UNU, 2001). Further, it has been shown that in industrialized countries members of minority groups frequently suffer from IDA (Wandel, 1993), which has been associated with the lack of dietary adjustment to the new environment (Marx, 1997).

**Pathological Causes of Iron Deficiency**

ID can further be caused by increased blood loss or decreased iron absorption due to pathological causes. For example, hookworm and schistosomiasis infections cause blood loss from the intestinal mucosa. Infections with schistosomiasis can further cause hematuria (WHO, 1991; Stephenson, 1993; WHO, 1993). The abovementioned causes however mainly affect individuals in developing countries. Pathological causes of gastrointestinal blood loss that also affect individuals in industrialized countries include oesophagitis, gastritis, peptic ulcers, neoplasm, inflammatory bowel disease, and hemorrhoids (Beveridge et al. 1965; Skikne, 1988). Increased blood and therefore iron losses have further been associated with the prolonged intake of aspirin (Pierson et al. 1961). Pathological reasons for impaired iron absorption include achlorhydria and gastric surgery (Hallberg et al. 1993b). Further, gastric *Helicobacter pylori* infections have been shown to cause IDA, as reviewed by Barabino (2002), the pathogenetic mechanism has so far however not been fully understood.

**Further Causes of Iron Deficiency**

Further population groups that have an increased risk for ID due to high losses are blood donors and athletes. Blood donations result in a substantial loss of iron (200-
250 mg per donation) and have been associated with a decline in serum ferritin levels (Finch et al. 1977; Milman & Sondergaard, 1984). The increased iron need of athletes, especially of runners, is predominately due to additional gastrointestinal losses resulting from intravascular hemolysis. However, increased iron losses with sweat and increased demand for myoglobin and iron containing respiratory enzymes are further possible reasons for the so-called exercise-induced anemia (Weaver & Rajaram, 1992).

1.2.3 Consequences of Iron Deficiency Anemia

The physical manifestations of IDA include the nonspecific symptoms of anemia, such as tiredness, lethargy, and a general feeling of lack of energy. Further, the clinical symptoms of IDA include glossitis, angular stomatitis and esophageal webbing (abnormalities to the mucosa of mouth and esophagus), blue sclera (blue tinge to whites of eyes), and koilonychia (spoon nails). Also associated with IDA is the perversions of taste which can lead to the consumption of non-food items (pica) or a craving for ice (pagophagla) (Beard, 2001; Lynch & Green, 2001).

The most severe consequences of IDA are the impaired mental development and physical coordination in infants and toddlers which are presumably due to cerebral changes (Grantham-McGregor & Ani, 2001). Longitudinal studies have shown that these effects are not fully reversible by treatment with iron, since children with IDA during infancy continued to have poorer cognition and school achievements, and had more behavior problems into middle school, although IDA had successfully been treated during infancy (Lozoff et al. 2000). IDA is also associated with reversible abnormalities of immune function; however, there is no conclusive evidence that IDA per se increases the rate of infections. On the contrary, there is some evidence indicating that IDA may protect from infections with malaria (Oppenheimer, 2001). Further, reduced work performance and productivity have also been associated with IDA and are probably related to reduced oxygen transport (Haas & Brownlie, 2001). Adverse pregnancy outcomes, which include increased maternal mortality as well as low birth weight and preterm delivery, are also frequently discussed with regards to IDA. However, causal relationships between IDA and weight at birth, length of gestation and perinatal mortality have so far not been identified. Rasmussen (2001) reviewed in part these relationships and concluded that maternal hemoglobin values
during pregnancy are associated with birth weight and preterm birth in an U-shaped relationship, with high rates of babies who are small, premature or both, at low and high concentrations of maternal hemoglobin. It was however not possible to determine an association with IDA in particular. In respect to increased maternal mortality Allen (2000) concluded from a review on this issue that presently data is inadequate to determine to which extent maternal anemia might contribute to maternal mortality.

### 1.2.4 Prevalence of Iron Deficiency

IDA is one of the most common and widespread nutritional disorders in the world. It is however difficult to estimate how many people are affected and mostly the prevalence of anemia is used as an indicator for IDA and an indirect indicator of ID.

In 1985 DeMaeyer and Adiels-Tegman estimated, based on the evaluation of 523 studies, that 30% of the world’s population was anemic. Estimates of the prevalence of anemia from 1990-1995 were similar with approximately 2 billion people being anemic (over 30% of the world population) (WHO/UNICEF/UNU, 2001). However, as anemia can be due to multiple causes, such as other nutritional deficiencies as well as infections, inflammation, and malaria, it is obvious that the prevalence of IDA is not equal to the prevalence of anemia. It has been predicted that approximately 50% of all anemia is caused by ID (DeMaeyer & Adiels-Tegman, 1985). Thus, world-wide IDA affects approximately 1 billion individuals. This number can further be used to calculate the number of people affected by ID. It has been estimated that the prevalence of ID is approximately 2.5-times that of IDA, based on data from US women and children showing that 30-40% of those with ID were also anemic (Yip, 1994). This factor may however only be valid in industrialized countries, in developing countries, for example the Ivory Coast, prevalence of ID was shown to be twice that of IDA (Asobayire et al. 2001). Thus, world-wide ID may affect 2 to 2.5 billion people.

The prevalence of anemia, IDA, and ID are however not evenly distributed within a population or throughout the world. High prevalence of anemia can be found in women and children in developing countries (estimated anemia prevalence (1990-95): 39% in children aged under 5 years, 48.1% in children 5-15 years, 42% women 15-59 years). It has further been estimated that every second pregnant women in developing countries suffers from anemia. Anemia during pregnancy also affects more than 20% of all pregnant women in industrialized countries. A similarly high preva-
ence of anemia in industrialized countries has been estimated for young children (aged 0-4 years) and approximately 10% of the women aged 15-59 years suffer from anemia. In contrast, only few men of the same age are affected from anemia in industrialized countries (4%). In developing countries anemia prevalence among this group is approximately 30% (WHO/UNICEF/UNU, 2001).

While presently it is difficult to obtain valid data on the prevalence of anemia as well as IDA and ID, the global database on anemia, which is currently under revision and will be available on the WHO web-site mid-2003 (www.who.int/nut/db_mdis.htm), may simplify the search for prevalence data. This global database on anemia will compile information on anemia prevalence based on the hemoglobin concentration of different population groups (infants, children, adults, and pregnant women). Further, information available on iron status indicators, such as serum ferritin, serum transferrin receptors, and erythrocyte protoporphyrin, will subsequently be included in the database to distinguish between iron deficiency anemia, and anemia due to other causes.

1.2.5 Prevention of Iron Deficiency

Strategies to prevent ID and IDA include supplementation, dietary modification/education and food fortification. Supplementation with iron tablets is the most widely used approach to control ID (Allen, 2002a). Iron supplements can be used to treat individuals with ID/IDA, or routinely to prevent ID/IDA in population groups at high risk of developing ID/IDA, such as pregnant women, infants, and young children. Irrespective of the existing controversies regarding the effectiveness of iron supplementation during pregnancy, routine supplementation is often not successful as compliance can be low due to gastrointestinal side effects, unappealing appearance and odor of the supplements and lack of understanding regarding the importance of iron supplements. Further difficulties involved with supplementation programs can be the inadequate supply of supplements due for example to lack of funds. The delivery system can also limit the success of supplementation programs. A classic approach has been to provide iron supplements to pregnant women through antenatal care services. The success of this delivery system is however dependent on registration during early pregnancy. A more successful delivery system, which has been used in Bangladesh, is the distribution of supplements at the “door step” by a community
Literature review

workers, this is however very labor intensive (Ekström, 2001). Therefore, while iron supplementation has the advantage of enabling the targeting of high-risk population groups, it has the major disadvantage of requiring an effective system of health care delivery.

Dietary modifications are further strategies which have been suggested to prevent ID and IDA. Examples of food-based interventions include the promotion of food processing techniques which decrease the content of iron absorption inhibitors in the diets, for example phytic acid content. This can be achieved by soaking, germination or fermentation of whole grain cereals and pulses, which leads to enzymatic hydrolysis of phytic acid. A non-enzymatic method to decrease phytic acid content is milling of cereals. Further, increasing the intake of foods and beverages rich in enhancers of iron absorption with meals containing non-heme iron is assumed to improve iron status by increasing iron absorption. Similarly, decreasing the intake of foods and beverages containing inhibitors is also expected to improve iron status. While the abovementioned strategies are based on increasing iron absorption from the diet, cooking in iron pots is promoted in order to increase the iron content of the diet (Ruel & Levin, 2001). Presently, only a limited number of studies have demonstrated that dietary modifications have a positive impact on iron status. For example, the twice daily consumption of lime juice as a beverage together with meals over a period of 8 months has been shown to improved iron status in women (Garcia et al. 1998). Furthermore, the use of iron pots was shown to increase the amount of iron absorbed from the diet (Borigato & Martinez, 1998; Adish et al. 1999). The exclusion of coffee however from the toddlers diet in Guatemala did not show a significant effect on iron status (Dewey et al. 1997).

Food fortification with iron compounds is generally considered the best-long term approach to prevent iron deficiency (Cook & Reusser, 1983). The advantages of food fortification include that it reaches most segments of the population and does not require the cooperation of the individual. However, there are many difficulties which have to be overcome when fortifying the diet with iron. Most importantly there is no single fortification strategy which is suitable for world-wide use. Therefore, iron fortification programs have to be developed specifically for each country or region taking the dietary habits of the target groups/populations into account. This includes the determination of dietary iron intakes in order to estimate whether iron intakes are limited.
and/or whether iron absorption is low. The evaluation of the dietary habits is further important for the selection of an appropriate fortification vehicle. After a vehicle has been identified the most suitable iron compound for this vehicle has to be determined, taking food storage and food preparation practices in the target region into account. It is further important to evaluate the acceptability of the fortified vehicle within the target group. In addition, iron absorption from the fortified vehicle should be measured and the effect of the fortified vehicle on iron status should be determined in a long-term field study. Once a food fortification program is implemented there is the necessity for quality control of the fortified vehicle and further it is desirable to monitor iron status in the population in order to evaluate the impact of the fortification program (Cook & Reusser, 1983; Walter et al. 2001).

In the following, a number of the abovementioned aspects of food fortification will be discussed in more detail. After a short overview on iron absorption, the majority of iron compounds used for food fortification will be described, focusing on iron absorption from the various compounds. In addition, the effects of several dietary components on iron absorption from native iron as well as fortification iron will be discussed. This will be followed by a description of frequently studied iron fortification vehicles and lastly results from long-term field studies will be presented.

1.3 Iron Absorption

Iron is absorbed in the small intestine, predominately in the duodenum, by mucosal cells, which are formed in the crypts of Lieberkühn and then move towards the villous tip (Conrad & Umbreit, 2000). The available iron is absorbed in a three-step process, which includes the uptake of iron into the mucosal cell, the transport intracellularly and the transfer across the basolateral membrane into the plasma.

At least two pathways are involved in the uptake of iron into the mucosal cell: one pathway for heme iron and at least one for non-heme iron (Conrad & Umbreit, 2000). Heme iron is freed from hemoglobin and myoglobin in the duodenum by pancreatic enzymes and absorbed intact as metalloporphyrin. The iron is then released from the porphyrin ring by heme oxygenase (Conrad et al. 1966; Conrad et al. 1967). Non-heme iron is transported into the cell by the divalent metal transporter (DMT1, previously named Nramp2 and DCT1), however, in order to bind to DMT1, the iron must
be soluble and in the ferrous state (Andrews, 1999; Roy & Enns, 2000). Based on studies made by Layrisse et al. (1973) it is assumed that all non-heme iron, regardless of its origin, which is soluble in the gastric juice enters a common non-heme iron pool. The solubility of non-heme iron depends on the chemical form as well as the pH in the stomach. Ferrous iron is soluble under acidic conditions and does not precipitate until the solutions are made alkaline. However, ferric iron, which accounts for the majority of all native food iron, is insoluble in solutions with a pH above 3 and thus has to be solubilized in the gastric juice. Further, the soluble iron has to be chelated in order to prevent precipitation in the alkaline environment of the duodenum. Chelating compounds include intestinal mucins, as well as a number of dietary components such as certain amino acids, ascorbic acid, sugars, amines, and amides (Conrad & Schade, 1968; Conrad et al. 1991). Since only ferrous iron is absorbed via DMT1, ferric iron has to be reduced to ferrous iron in order to be absorbed. This is mediated by food compounds with reducing properties, for example ascorbic acid, or by the putative deheme plasma membrane protein, which is present on the surface of duodenal mucosal cells (McKie et al. 2001). However, the identification of a mucin-integrin-mobilferrin pathway has indicated that ferric iron may be absorbed without being reduced to the ferrous state (Conrad & Umbreit, 1993). Once absorbed into the mucosal cell, iron is either stored as ferritin or exported to the plasma at the basolateral surface.

Since the human body has no regulatory mechanisms to excrete excess iron once it has been absorbed, iron homeostasis is maintained by regulating iron absorption as well as iron transfer from the enterocyte into the plasma. The exact mechanisms by which iron status regulates iron absorption have however not been identified. It is assumed that while crypt cells mature into absorptive enterocytes, they are programmed via two independent regulators, the "stores regulator" and the "erythropoietic regulator", to subsequently modulate iron absorption at the villus. It has further been demonstrated that the programming depends on the iron content of the crypt cells (Roy & Enns, 2000). It is however unclear which factors are responsible for the programming of the crypt cell. The hereditary hemochromatosis protein (HFE) has been suggested to regulate iron absorption, and therefore iron stores, of the crypt cell from plasma by forming complexes with transferrin receptors. The iron stores in the enterocytes are assumed to regulate iron absorption from the duodenum via DMT1. It
is further assumed that enterocyte iron stores also regulate iron export via the baso-
lateral iron transporter IREG1, also referred to as ferroportin1 or MTP1 (Roy & Enns,
2000; Wessling-Resnick, 2000). Iron export has also been linked to a copper-
containing iron oxidase known as hephaestin. It is however not known whether it acts
alone or in conjunction with an iron transporter such as the IREG1 (Anderson et al.
2002). Recently, an additional factor has been associated with the regulation of iron
absorption, the antimicrobial peptide hepcidin. Studies in mice have shown a close
relationship between the expression of hepcidin, duodenal iron transporters, and iron
absorption (Frazer et al. 2002). Further studies are however necessary to evaluate
the importance of this factor.

1.3.1 Methods Used to Estimate Iron Absorption
A number of different methods are used to estimate iron absorption, however, iron
absorption per se, i.e. the balance between iron intake and fecal iron excretion, is
rarely measured. Instead most methods use incorporation of iron into erythrocyte as
a proxy for iron absorption. The most commonly used methods to estimate iron ab-
sorption in rats and humans are described in the following:

The commonly used method to estimate iron absorption in rats is the hemoglobin
repletion bioassay. As an example the hemoglobin repletion bioassay according to
the Association of Official Analytic Chemists is described here: Young, male rats are
depleted of iron by feeding an iron-deficient diet, so that anemia develops. After at
least 4 weeks, the rats are divided into comparable groups and receive diets con-
taining the iron compound under study in three different concentrations. The
hemoglobin repletion is measured relative to the reference source of iron, i.e. ferrous
sulfate, which is also fed in different concentrations. The hemoglobin increase will be
the result of both dietary iron concentration and the dietary iron source. Hemoglobin
levels are measured after 2 weeks of repletion and these values are plotted against
the dietary iron concentration for each iron source. The ratio of the slopes gives a
quantitative measure for the bioavailability of the iron compounds under study. The
relative bioavailability value (RBV) of the iron source is expressed relative to the
effect of ferrous sulfate (= 100%) (AOAC, 1990).

In humans, iron absorption can be assessed using a tissue retention method since
newly absorbed iron is primarily used for hemoglobin synthesis. Thus, iron absorption
from a specific food or test meal can be estimated by measuring the incorporation of stable or radioactive iron isotopes into erythrocytes 14 days after ingestion of the labeled test meal (Brise & Hallberg, 1962b; Eakins & Brown, 1966; Cook et al. 1972; Hallberg & Björn-Rasmussen, 1972; Fomon et al. 1988; Kastenmayer et al. 1994). Iron absorption is calculated based on blood volume (Brown et al. 1962) and an assumed erythrocytes incorporation of absorbed iron isotopes of 80% (Hosain et al. 1967). To overcome the influence of iron status on iron absorption, comparisons between different meals are usually made within the same subject by administering two different stable isotopes (\(^{57}\text{Fe} \text{ and } ^{56}\text{Fe}) \text{ or radioisotopes (} ^{55}\text{Fe} \text{ and } ^{59}\text{Fe}) \text{ with different test meals on consecutive days (as reviewed by van Dokkum et al. 1996). The advantage of using stable isotopes in preference to radioisotopes is the prevention of unnecessary radiation exposure. Thus, stable isotopes are particularly useful in the assessment of iron bioavailability in vulnerable groups such as infants, children, and pregnant women. However, stable isotopes have the disadvantage of being vastly more expensive than radioisotopes. Further, the large doses of isotope which need to be given for measurable enrichment of erythrocytes limit the use of stable isotopes to studies investigating iron absorption from fortification or supplementation iron unless more complicated study designs are used.

In addition to tissue retention methods, single measurements of iron retention can be performed using \(^{59}\text{Fe} \text{ and whole body counting. Using this method a whole body count is performed shortly after (usually 1 hour) oral administration of } ^{59}\text{Fe} \text{ and again after approximately 14 days and iron retention is calculated from the results of these two counts, after correcting for radioactive decay. This method can also be used in combination with erythrocyte incorporation of } ^{59}\text{Fe} \text{ and } ^{55}\text{Fe} \text{ making estimates for blood volume and erythrocytes incorporation unnecessary. It is further possible to measure iron absorption by using a fecal monitoring method with stable- or radioisotopes. However, incomplete fecal collection, which is the most common problem with this method, may lead to overestimating iron absorption (as reviewed by van Dokkum et al. 1996).}

When working with isotopically labeled iron fortification compounds (stable- or radioisotopes) it is important to ensure that the labeled compounds are comparable to their commercially available counterparts. Labeled ferrous sulfate can be prepared without many difficulties; however, special care has to be taken when preparing la-
beled iron compounds which are poorly water-soluble and water-insoluble. These labels are mostly produced by using a scaled-down manufacture procedure, making it is difficult to obtain a labeled compound with physical and chemical properties comparable to the commercial counterpart. Besides the technical difficulties associated with using a down-scaled production method, the high cost involved in the preparation of such compounds is a limiting factor, especially when stable iron isotopes are used. This explains why the vast majority of human iron absorption studies have been made with ferrous sulfate.

Throughout the present review all human iron absorption studies referenced, if not specified otherwise, were made in healthy adults using isotopic techniques based on erythrocyte incorporation of either stable or radioactive isotopes.

1.3.2 Iron Compounds

As mentioned above, iron has to be soluble in the gastric juice in order to enter the common non-heme iron pool. The bioavailability of fortification iron depends strongly upon solubility properties and iron fortificants are therefore classified according to their solubility properties (water-soluble, poorly water-soluble, and water-insoluble iron compounds).

1.3.2.1 Water-Soluble Iron Compounds

The most readily bioavailable iron compounds are water-soluble iron compounds. Of these, ferrous sulfate is the iron compound which is most commonly added to foods and it has by definition a RBV of 100%. The actual iron absorption from this compound will however vary depending on the subject’s iron status as well as on the presence of inhibitors and enhancers in meals. Ferrous sulfate is further presumably the most cost efficient iron compound (i.e. lowest price per unit absorbable iron) and is commercially available as ferrous sulfate heptahydrate (FeSO₄* 7H₂O) as well as dried ferrous sulfate (FeSO₄* xH₂O) (Hurrell, 1999). The disadvantage of ferrous sulfate is it susceptibility to cause unacceptable organoleptic changes to the fortified vehicle (Hurrell, 1984; Hurrell et al. 1989a).

Further examples of water-soluble iron compounds which can be used for food fortification are ferrous gluconate, ferrous lactate, ferric ammonium citrate and ferrous ammonium sulfate. These compounds will presumably cause similar organoleptic
problems as ferrous sulfate and are in addition more expensive than ferrous sulfate (Hurrell, 2002). Ferrous gluconate (FeCH$_2$OH-(CHOH)$_4$-COO)$_2$•2H$_2$O) is sometimes used to replace ferrous sulfate in oral iron supplements; it is however rarely used in food fortification (Hurrell, 1999). Hemoglobin repletion studies made with iron deficient rats have shown ferrous gluconate to have a RBV to ferrous sulfate between 79 and 138% (Theuer et al. 1971; Theuer et al. 1973). The RBV of ferrous lactate (Fe(CH$_3$-CHOH-COO)$_2$ • 3H$_2$O) was evaluated in the same series of studies and shown to be between 100 and 118% for rats. In humans, the RBV of ferrous lactate is reported to be comparable to ferrous sulfate (Brise & Hallberg, 1962a). Ferric ammonium citrate, which has an indefinite stoichiometry, has been shown to have a similar RBV to ferrous sulfate in rats (Hurrell, 1999). However, in humans it has been demonstrated that it is less well absorbed than native food iron and ferrous sulfate (RBV as low as 20%) (Grebe et al. 1975; Layrisse et al. 1976; Gonzalez et al. 2001). Ferrous ammonium sulfate (Fe(NH$_4$)$_2$(SO$_4$)$_2$ • 6H$_2$O) has so far not been studied in humans. It is however assumed it will be as available as ferrous sulfate since in rat studies it was shown to have a RBV of approximately 100% (Hurrell, 1999).

1.3.2.2 Poorly Water-Soluble Iron Compounds

Poorly water-soluble iron compounds which are soluble in dilute acid, such as gastric acid, are interesting fortification compounds as they cause fewer organoleptic problems than water-soluble iron compounds but have a RBV similar to that of ferrous sulfate.

One of the more intensely studied iron compounds from this group is ferrous fumarate. This compound is used to fortify infant cereal manufactured by European food companies (Hurrell, 2002) and wheat and maize flours in Venezuela (Garcia-Casal & Layrisse, 2002). Further, encapsulated ferrous fumarate has been evaluated as fortification compound in “sprinkles” in Ghana (Zlotkin et al. 2001). In adults, iron absorption from various infant cereals and chocolate drink powder fortified either with ferrous fumarate or with ferrous sulfate has been shown to be comparable in the presence (Hurrell et al. 1989a; Hurrell et al. 1991) and in the absence of ascorbic acid (Hurrell et al. 2000). Further, in infants, iron absorption from ferrous fumarate has been shown to be 3-fold higher than from ferric pyrophosphate (Davidsson et al. 2000). However, when compared directly to ferrous sulfate in young Bangladeshi
children iron absorption from ferrous fumarate was shown to be only one-third of that from ferrous sulfate (Davidsson et al. 2001b). This result has been seen as an indication that young children may not absorb iron from ferrous fumarate as well as from ferrous sulfate. Further research is however needed.

Another interesting finding regarding ferrous fumarate was made by Hurrell et al. (1991). They showed that when ferrous fumarate was added to chocolate drink powder before processing, which included vacuum drying at 100 °C, iron absorption was double that from ferrous sulfate fortified chocolate drink powder, which had undergone the same treatment. When added after processing iron absorption from these two iron compounds was similar. The reason for the increased bioavailability of ferrous fumarate after heat treatment is unknown.

Further compounds with solubility properties comparable to ferrous fumarate include ferrous succinate and ferric saccharate. RBV of these compounds in humans was shown to be similar or slightly lower than that of ferrous sulfate (Hurrell et al. 1989a). These compounds are however somewhat more expensive than ferrous sulfate or ferrous fumarate, which is only 1.3-times the price of ferrous sulfate (Hurrell, 1999). Ferrous citrate is another compound with comparable solubility properties, from which iron is absorbed approximately three-fourths as well as from ferrous sulfate in rats and humans (Brise & Hallberg, 1962a; Fritz et al. 1975).

1.3.2.3 Water-Insoluble Iron Compounds

Iron is least well absorbed from water-insoluble iron fortification compounds that are only poorly soluble in dilute acid. These compounds are nonetheless frequently used for food fortification, as they do not cause changes to the color or flavor of the fortified vehicle (Hurrell, 2002). Commonly two different types of water-insoluble iron compounds are used: iron phosphate compounds and elemental iron powders.

Iron Phosphate Compounds

Ferric pyrophosphate (Fe₄(P₂O₇)₃ * xH₂O) and ferric orthophosphate (FePO₄ * xH₂O) are the main iron phosphate compounds used in food fortification. Ferric pyrophosphate is used by European food companies to fortify chocolate drink powders and infant cereals; although in infant cereals it has to some extent been replaced with ferrous fumarate (Hurrell, 1999). In adults, iron absorption from ferric pyrophosphate
Literature review

has been compared to ferrous sulfate from infant cereals as well as from chocolate drink powder. From wheat-based infant cereal its RBV varied between 39% (Hurrell et al. 1989a) and 15% (Hurrell et al. 2000). From chocolate drink powder iron absorption was 75% of that from ferrous sulfate (Hurrell et al. 1991). There is no obvious reason for the variation in RBV, although the different vehicles and their content of enhancers and inhibitors may have influenced the results. Further, the variations seen could be due to differences in the solubility properties of the labeled compounds. However, in all studies the solubilities of the labeled compounds were evaluated and reported to be similar to that of commercial ferric pyrophosphate. Since all labels were added directly before the meals were administered an influence of processing on solubility properties and thus iron absorption can be excluded.

An effect of processing on iron absorption of ferric pyrophosphate was however observed in a study made by Hurrell et al. (1991). Their results showed that RBV of ferric pyrophosphate from chocolate drink powder decreased from 75 to 21% when the fortificants were added before processing (including vacuum drying at 100 °C). On the contrary, the sterilization of soy and milk-based infant formulas has been shown to increase iron absorption in rats from ferric pyrophosphate as well as from ferric orthophosphate 2.3-fold and 1.7-fold, respectively (Theuer et al. 1971; Theuer et al. 1973). The effect of food processing on the RBV of fortification compounds has however not been evaluated systematically and thus these findings remain unexplained.

In infants, ferric pyrophosphate has so far not been compared directly to ferrous sulfate. However, a comparison with ferrous fumarate has been made, showing ferric pyrophosphate to be only one-third as well absorbed as ferrous fumarate from an infant cereal (both fortificants were added after processing) (Davidsson et al. 2000).

Iron absorption from ferric orthophosphate in humans has been shown to be between 25 and 32% of that from ferrous sulfate (Cook et al. 1973; Rios et al. 1975; Forbes et al. 1989). In rats, larger variations of the RBV have been measured. Fritz et al. (1970) evaluated four samples in rat hemoglobin repletion studies and reported RBVs between 7 and 32%. Similarly, Harrison et al. (1976) found that the RBVs of five commercial samples of ferric orthophosphate varied from 6 to 46%. In the later study, the particle sizes of the samples tested were determined and it was shown
that the 8-fold increase in RBV of ferric orthophosphate was due to a decrease in particle size from 15 µm to below 1 µm.

A innovative iron phosphate compound, which has so far not been studied in humans, is the patented compound Sunactive Fe™ (Nanbu et al. 1998). This micronized ferric pyrophosphate is produced from ferric chloride using a new dispersion technique resulting in a very small particle size (ca. 0.3 µm), approximately 20-times smaller than that of conventional ferric pyrophosphate (average particle size approximately 8 µm). Further, in order to avoid the formation of agglomerates after production, emulsifiers (enzymatically hydrolyzed soy lecithin and polyglycerol fatty acid ester) are added during the production process. This has the additional advantage that Sunactive Fe™ is dispersible in liquid solutions and can for example be used to fortify milk and milk based drinks. Rat studies have shown Sunactive Fe™ to be as readily available as ferrous sulfate (Juneja et al. 2003).

**Elemental Iron Compounds**

There are three different types of elemental iron powders available for food fortification: electrolytic iron, carbonyl iron, and reduced iron. Reduced iron can further be classified into different subgroups depending on the production method: hydrogen (H)-reduced iron, carbon monoxide (CO)-reduced iron, and a reduced iron which is produced by Quebec Metal Products and is also referred to as “atomized iron” (Hurrell, 2002; Hurrell et al. 2002a).

Although elemental iron powders are, by far, the most widely used fortificants for wheat flours and other cereal products (Anderson, 1985; Fomon, 1987), it is still unclear to which extent iron is available to humans from these compounds. The main reason for the scarcity of iron absorption studies made with elemental iron powders is presumably related to the difficulties involved in producing labeled elemental iron compounds for use in human absorption studies, as previously described. Due to the manufacture procedure for elemental iron powders it is difficult to obtain a labeled compound with physical and chemical properties comparable to the commercial counterpart. This is also one of the reasons why the results of the studies which have been made with elemental iron powders need to be judged with caution.
RBV of H-reduced iron with a particle size between 5 and 10 μm was evaluated in adults by Cook et al. (1973), using the whole body counter method to measure iron absorption. Further, Björn-Rasmussen et al. (1977) evaluated four different H-reduced iron powders, which differed in respect to their solubility, using erythrocyte incorporation and whole body counter in adults. The results of these studies showed that RBV of H-reduced iron can differ between 13 and 95%. Thus, H-reduced iron can be readily available in adults although it can also be poorly available. These differences in RBV were evaluated by Björn-Rasmussen et al. (1977) and were shown to be dependent on the solubility properties of the iron powders. Unfortunately, the particle sizes of the compounds were not measured. The radioactively labeled H-reduced iron used by Cook et al. (1973) was also used to measure RBV of H-reduced iron in infants using a whole body counter and iron absorption from this compound was shown to be comparable to ferrous sulfate (Rios et al. 1975).

Iron absorption from carbonyl iron was measured in humans by Hallberg et al. (1986b) using erythrocyte incorporation and whole body counting. The labeled compound was produced by neutron irradiation of commercial carbonyl iron and was used to fortify bread rolls. These rolls were consumed with a variety of different meals and iron absorption from carbonyl iron was compared to that of native iron. The comparisons showed carbonyl iron to have RBVs between 5 and 33% compared to native iron, depending on meal composition. The results of this study were controversial since carbonyl iron had been expected to have a higher RBV (closer to 50%), based on the results from rat studies (Sacks & Houchin, 1978). It has been questioned whether the neutron irradiation and/or the 500 day storage before use could have influenced the RBV of carbonyl iron. However, since solubility in dilute acid of the labeled compound was not different to that of non-irradiated carbonyl iron powder from the same batch stored 500 days, or from newly produced carbonyl iron powder, there is little evidence to support this hypothesis.

The evaluation of electrolytic iron in a human iron absorption study showed it to have a RBV of 75% (Forbes et al. 1989) and although the labeled compound was not identical to commercial electrolytic iron, it was considered as similar enough for the estimation of RBV of electrolytic iron in humans.
More information regarding RBV of elemental iron powders is available from rat studies. These have shown electrolytic iron (RBV between 16 and 70 with a mean of 44) and carbonyl iron (RBV: 27 to 66, mean 47) to be the most readily available elemental iron powders. Lower RBVs have been shown for H-reduced iron (13 to 54, mean 30) and CO-reduced iron (12 to 32, mean: 19), as reviewed by Hurrell et al. (2002a). This rank order was confirmed in a recent study in which RBV of six commonly used elemental iron powders was compared in rats (Swain & Hunt, 2003). These comparisons showed carbonyl iron (RBV 64%) to be the most readily available elemental iron powder, followed by electrolytic iron (RBV 54 and 46%), H-reduced iron (RBV 42%), "atomized iron" (RBV 24%), and CO-reduced iron (RBV 21%). It was further shown that the RBV was dependent on physico-chemical properties as RBV of the elemental iron powders increased with increasing surface area.

A further physico-chemical property which has in rats been shown to influence RBV of elemental iron powders is particle size. Shah and Belonje (1973) for example reported that RBV of electrolytic iron increased from 12 to 32% when 99% of the particles were below 10 µm as compared to only 62%. However, the different samples of electrolytic iron used did not originate from the same batch so that factors besides the particle size, such as density and porosity, could have influenced the results. Motzok et al. (1975) avoided this source of error by separating one batch of CO-reduced iron into three different fractions (24-40 µm, 14-19 µm, and 7-10 µm) and their results show that RBV of CO-reduced iron increases with decreasing particle size in rats (RBV for the large, medium and small fraction: 11, 18 and 31%, respectively). However, decreasing the particle size of elemental iron powders does not always affect iron absorption. Verma et al. (1977) investigated the influence of particle size with elemental iron powders produced by different processes (electrolytic, H-reduced iron, CO-reduced and carbonyl) and their results showed that while RBV of H- and CO-reduced iron increased significantly with decreasing particle size, RBV of electrolytic and carbonyl iron was not affected by particle size.

In conclusion, it does not seem possible to clearly determine from the results of the above reviewed studies how readily iron will be available from foods fortified with elemental iron powders. However, since elemental iron powders are frequently used as fortificants it would seem important to further evaluate RBV of these compounds.
1.3.2.4 Novel Iron Compounds

Sodium Iron Ethylenediaminetetraacetic Acid (NaFeEDTA)

NaFeEDTA as an iron fortificant has been reviewed by the International Nutritional Anemia Consultative Group (INACG) and it was strongly recommended as the most suitable iron fortificant in developing countries (INACG, 1993). The use of NaFeEDTA as a fortification compound has a number of advantages. It is a water-soluble compound, which can be added to a number of food vehicles, for example curry powder (Ballot et al. 1989), fish sauce (Garby & Areekul, 1974), and sugar (Viteri et al. 1995), without causing organoleptic problems. However, it has been shown to cause color changes when added to some cereal based foods (Viteri et al. 1995). A further advantage of NaFeEDTA is that it has iron absorption enhancing properties, related to the chelating properties of EDTA. EDTA is a hexadentate chelator capable of combining stoichiometrically with virtually every metal in the periodic table (Chaberck & Martell, 1959). The effect of EDTA as a chelator for a particular metal depends on its stability constant at a particular pH. Under the acidic conditions in the stomach, ferric iron has the highest stability constant (West & Sykes, 1960). Therefore, when EDTA is present in the stomach it can be expected to form complexes with ferric iron and thus prevent ferric food iron from precipitating when pH rises above 3. Consequently, when iron is consumed as NaFeEDTA, iron should remain chelated to EDTA in the stomach and be prevented from precipitating (INACG, 1993). Under the alkaline pH conditions in the duodenum, the stability constant for ferric iron is lower than for many other metals, such as Cu²⁺, Zn²⁺, Ca²⁺, or ferrous iron and therefore ferric iron can be expected to be displaced by these metals. The released iron from NaFeEDTA can then be absorbed via the pathways previously described. Besides preventing precipitation, the formation of iron-EDTA complexes has the additional advantage of preventing iron from binding to phytic acid. Consequently, iron absorption from NaFeEDTA has been shown to be 2- to 3-times that of ferrous sulfate from phytic acid containing foods (INACG, 1993). Further, due to effects on iron in the common non-heme iron pool, NaFeEDTA also enhances native iron absorption (Layrisse & Martinez-Torres, 1977; MacPhail et al. 1981). The inhibitory effect of polyphenols, as present in tea and coffee, cannot be overcome completely by NaFeEDTA (MacPhail et al. 1981; Morck et al. 1983; Hurrell et al. 2000). However, although iron absorption from NaFeEDTA decreased significantly when tea was added to a meal of low-extraction wheat bread rolls, it remained approximately
2-fold higher than iron absorption from corresponding meals fortified ferrous sulfate (Hurrell et al. 2000).

Nonetheless, the use of NaFeEDTA as fortificant is controversial since concerns exist regarding its potential toxicity. It has for example been questioned whether the intake of NaFeEDTA could lead to iron overload due to uncontrolled absorption of FeEDTA. However, observations have shown that less than 1% $^{59}$Fe is excreted in the urine after oral intake of Na$^{59}$FeEDTA (MacPhail et al. 1981). This finding demonstrates that most iron must have been released from the EDTA complex before absorption as only $^{59}$Fe absorbed as intact $^{59}$FeEDTA complex would be expected to be excreted in urine. Thus, the risk of excessive iron absorption from NaFeEDTA is considered to be very low. An additional small fraction (probably less than 5%) of the EDTA moiety itself is absorbed, presumably bound to other metals, and is excreted in the urine (INACG, 1993). Further concerns have been raised regarding the influence of NaFeEDTA on metabolism of other minerals. These concerns are derived from studies made with Na$_2$EDTA (NaFeEDTA is considered to behave physiologically similarly) which have shown that intake of high amounts of Na$_2$EDTA (1000 mg per kg body weight and day) causes zinc deficiency leading to teratogenic effects in pregnant rats (Swenerton & Hurley, 1971). However, the amounts of NaFeEDTA that can be expected to be consumed when used as a food fortificant have been shown to have an enhancing effect on zinc absorption in adult women (Davidsson et al. 1994b). Further concerns have been raised regarding the effect of NaFeEDTA on the absorption of toxic metals which have high stability constants (log K), such as lead (16.8-17.7), cadmium (15.0-16.1), or mercury (20.4-22) (West & Sykes, 1960). However, NaFeEDTA has in adults been shown to have no influence on the absorption and urinary excretion of manganese (stability constant (log K) 13.5), which is a potentially toxic metal (Davidsson et al. 1998a). Its effect on the metabolism of other metals such as lead, cadmium, and mercury nonetheless still needs evaluation. Presently, NaFeEDTA is not approved as a food additive. The Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1999) has however decided that NaFeEDTA can be considered to be safe when used in supervised food fortification programs in response to a need for iron supplementation of the diet of a population as determined by public health officials. Such programs would provide daily iron intakes of approximately 0.2 mg/kg body weight.
A further disadvantage of NaFeEDTA is its susceptibility to losses due to photodegradation. EDTA is widely used in industrial, pharmaceutical, and agricultural applications. Due to its low biodegradability, EDTA often contaminates natural ground waters (Nowack et al. 1996; Sorensen & Frimmel, 1997) and studies on the photodegradability of EDTA have been focused on the development of techniques to eliminate EDTA from drinking water. The aqueous photolysis of EDTA, Fe(III)EDTA and other EDTA chelates have been examined in several studies (Carey & Langford, 1973; Lockhart & Blakeley, 1975a; Sorensen & Frimmel, 1995; Nowack et al. 1996; Sorensen & Frimmel, 1997; Nowack & Baumann, 1998; Sorensen et al. 1998) and it has been reported that Fe(III)EDTA has a half life of 2 hours in a sunlit river in summer (Kari & Giger, 1995). Further, it has been shown that Fe(III)EDTA is the most photolabile EDTA complex. The EDTA chelates of Mn(II) and Co(II) are also sensitive to photodegradation, however, to a lesser degree than Fe(III)EDTA. The chelates of Mg, Ca, Ni, Cu, Zn, Cd, and Hg have been shown to be photostable (Lockhart & Blakeley, 1975b).

The stability of NaFeEDTA in foods has so far not been studied. It would, however, seem likely that it will also degrade if aqueous foods fortified with NaFeEDTA are exposed to sunlight. Thus, it is important to consider the possible nutritional consequences of NaFeEDTA photodegradation in foods. Firstly, degradation products which could potentially be harmful to human health may be formed. The degradation products of Fe(III)EDTA have been determined as ethylenediaminetriacetate (ED3A), ethylenediaminediacetate (EDDA-N,N'; EDDA-N,N) and ethylenediaminemonoacetate (EDMA) (Lockhart & Blakeley, 1975b). EDMA is a photostable compound, which can however degrade further to iminodiacetate (IMDA), glycine, carbon dioxide, and formaldehyde (Nowack & Baumann, 1998). There is no evidence that ED3A, EDDA, EDMA, or IMDA are harmful to human health. Formaldehyde, although naturally present in a wide range of foodstuffs (Trezl et al. 1997), has been reported to be potentially carcinogenic (Ash & Ash, 1995). However, based on the evidence that formaldehyde is formed naturally in foods and is a normal mammalian metabolite and further that a threshold for carcinogenicity exist after oral administration it is believed that formaldehyde is not carcinogenic at low levels of exposure (acceptable daily intake (ADI)=20 mg/day) (Restani & Galli, 1991). Secondly, the impact of EDTA photodegradation on iron absorption should be considered. It can be expected that the
degradation products will have a lower iron binding capacity in comparison to EDTA due to the loss of carboxylic groups and thus iron absorption may be affected. However, as discussed later, EDTA to iron molar ratios of 0.5-0.7:1 have been reported to be as effective as 1:1 molar ratios in enhancing iron absorption (Hurrell et al. 2000). Therefore, the loss of carboxylic groups due to photodegradation will probably not affect iron absorption. Nonetheless, although photodegradation will presumably neither lead to adverse health effects nor affect iron absorption, NaFeEDTA photodegradation should be evaluated in liquid foods fortified with NaFeEDTA as for example sensory changes might occur.

*Ferrous Bisglycinate*

Ferrous bisglycinate is available commercially as Ferrochel (Albion Laboratories, Clearfield, UT) and consists of a molecule of ferrous iron attached to two molecules of glycine (Allen, 2002b). The exact composition along with the manufacturing process are however closely guarded secrets. Thus, there is a lack of independent evaluations of this compound. It has been claimed that iron absorption from ferrous bisglycinate is higher than from ferrous sulfate in the presence of certain inhibitors as the compound's structure protects iron from binding for example with phytic acid. This claim was based on the results of an iron absorption study showing a 4-fold higher iron absorption from ferrous bisglycinate than from ferrous sulfate fortified whole-maize meals (Bovell-Benjamin et al. 2000). However, in an earlier study made with infants, no advantageous effect of ferrous bisglycinate over ferrous sulfate in regards to iron absorption was observed (Fox et al. 1998). The main disadvantages of ferrous bisglycinate are the high cost and its tendency to provoke fat oxidation in stored cereal flours (Bovell-Benjamin et al. 1999).

**1.3.3 Inhibitors and Enhancers**

In addition to the solubility properties of native and fortification iron and the iron status of the individual, iron absorption is strongly dependent on the composition of the diet. A number of factors inhibit iron absorption while others enhance it. Beginning with the inhibitors, the majority of the dietary factors (natural food constituents and food additives) which affect iron absorption are described in the following section. However, in accordance to their relevance in this thesis, the inhibitors are described briefly whereas most enhancing factors are reviewed in greater detail. In ad-
Literature review

dition, erythorbic acid, a compound which was evaluated within this PhD project, is also reviewed.

1.3.3.1 Phytic Acid
Phytic acid, myo-inositol hexaphosphate, is widely present in cereal grains and legumes as the main phosphorous storage compound (Reddy & Sathe, 2002). It was first suspected to have an inhibitory effect on iron absorption by Widdowson and McCance (1942) and has since been shown to be the major inhibitor of native non-heme iron absorption and fortification iron absorption (ferrous sulfate) in cereal foods (Gillooly et al. 1984a; Hallberg, 1987; Hallberg et al. 1987; Rossander-Hulthen et al. 1990; Tuntawiroon et al. 1990; Cook et al. 1997) and legumes (Gillooly et al. 1983; Hurrell et al. 1992; Davidsson et al. 2001a). The inhibitory effect of phytic acid, which is related to its mineral binding properties, has been shown to be dose-dependent (Hallberg et al. 1989; Siegenberg et al. 1991). In addition, the observation has been made that partially degraded phytic acid (inositol tetra-, and pentaphosphates and to a lesser degree inositol triphosphates) also inhibits iron absorption (Sandberg et al. 1999). The inhibitory effect can be overcome by the addition of enhancers, as discussed later, as well as by phytic acid degradation (Hallberg, 1987; Hurrell et al. 1992; Davidsson et al. 1994a; Hurrell et al. 2002b).

1.3.3.2 Polyphenols
The term “polyphenol” was introduced some years ago to replace the term “vegetable tannin” and used the following definition: “Water soluble phenolic compounds having molecular weights between 500 and 3000 and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins.” However, subsequent use of the term has broadened to include lower molecular weight compounds so it now covers “natural products with more than one phenolic group” (www.ifr.bbsrc.ac.uk/polybind/polyphenols.doc). Polyphenols are known to inhibit iron absorption from native non-heme iron due to the formation of complexes between phenolic hydroxyls and iron (Brune et al. 1989). The inhibitory effect of polyphenols was first identified by Disler et al. (1975b; 1975c) who showed that the non-heme iron absorption inhibiting effect of black tea was due to the high polyphenol (tannin) content. Polyphenols were later also shown to be the non-heme iron absorption inhibiting factor in beverages such as coffee (Hallberg &
Rossander, 1982b; Morck et al. 1983; Hurrell et al. 1999), red wine (Bezwoda et al. 1985; Cook et al. 1995), cocoa, and herbal teas (Gillooly et al. 1984a; Hurrell et al. 1999). Furthermore, polyphenol containing plant foods have been shown to inhibit non-heme iron absorption (Gillooly et al. 1983; Gillooly et al. 1984a; Tuntawiroon et al. 1991) as have the extracts of green tea and rosemary (Samman et al. 2001). Besides inhibiting iron absorption from native non-heme iron, polyphenols also inhibit iron absorption from ferrous sulfate (Gillooly et al. 1984a; Bezwoda et al. 1985) as well as from NaFeEDTA (MacPhail et al. 1981; Morck et al. 1983; Hurrell et al. 2000). The extent to which polyphenols inhibit iron absorption is dependent on the dose (Brune et al. 1989; Tuntawiroon et al. 1991; Hurrell et al. 1999) as well as on the phenolic structure. Tannins from black tea have, for example, been shown to be more potent inhibitors than chlorogenic acid from coffee (Brune et al. 1989).

1.3.3.3 Calcium

It has repeatedly been observed that calcium inhibits iron absorption in humans. The inhibiting effect has been shown for native non-heme iron (Dawson-Hughes et al. 1986; Cook et al. 1991a) and heme iron (Hallberg et al. 1991; Hallberg et al. 1993a), as well as for fortification iron (ferrous sulfate) (Hallberg et al. 1991) and iron supplements (Seligman et al. 1983; Cook et al. 1991b). Further, it has been shown that doses of calcium chloride between 40 mg and 300 mg calcium cause a dose-related reduction in non-heme iron absorption from wheat rolls containing 10 mg of native calcium (Hallberg et al. 1991). The iron absorption inhibiting effect of calcium is possibly also dependent on the chemical form of the calcium (Cook et al. 1991b). In addition to calcium salts, dairy products also inhibit non-heme iron absorption and this effect has been related to their high calcium content (Deehr et al. 1990; Hallberg et al. 1991; Hallberg et al. 1992). However, the inhibitory effect of milk products on iron absorption is not only due to calcium as milk proteins have also been shown to have an inhibitory effect on non-heme iron absorption (Cook & Monsen, 1976a; Hurrell et al. 1989b; Jackson & Lee, 1992).

1.3.3.4 Dietary Protein

As mentioned, milk proteins (whole milk, casein, whey proteins derived from milk and cheese) have been shown to inhibit non-heme iron absorption. In addition, a protein related inhibition of non-heme iron absorption has been shown for eggs and egg al-

It has further been shown that protein from certain legumes and nuts have an inhibitory effect on non-heme iron absorption (Lynch et al. 1984; Macfarlane et al. 1988a; Macfarlane et al. 1988b) and the inhibitory effect of soy on non-heme iron absorption has extensively been studied (Cook et al. 1981; Hallberg & Rossander, 1982a; Morck et al. 1982; Gillooly et al. 1984b; Derman et al. 1987). This inhibitory effect of soy beans is in part due to the high phytic acid content. However, a protein related moiety of the conglycinin (7S) fraction of soy protein also inhibits non-heme iron absorption (Hurrell et al. 1992; Lynch et al. 1994). The degradation of this protein fraction is possibly the reason why MacFarlane et al. (1990) showed an inverse relationship between non-heme iron absorption and high-molecular-weight fractions of soy protein, when studying the effect of fermented and unfermented traditional oriental soy products on iron absorption. Another soy-based food in which the inhibiting factor of soy protein can be degraded during production is soy sauce and Baynes et al. (1990) even demonstrated that the addition of soy sauce to a rice-based meal had a promotive effect on non-heme iron absorption. However, Baynes et al. (1990) used a type of soy sauce known as tamari-shoyu, produced from soybeans alone without the typical addition of wheat (see section 1.4.5.3), which is only consumed locally around the Nagoya area in Japan (Fukushima, 1985). Since the more frequently consumed soy sauces differ substantially from this type of soy sauce, it is not possible to extrapolate the results obtained by Baynes et al. to other types of soy sauces.

1.3.4 Enhancers

1.3.4.1 Ascorbic Acid

Ascorbic acid is an effective enhancer of non-heme iron absorption, both in natural and in synthetic form (Hallberg et al. 1986a). By reducing ferric iron to ferrous iron and by forming soluble complexes with iron (Conrad & Schade, 1968) ascorbic acid can overcome the inhibitory effects of phytic acid (Hallberg, 1987; Hallberg et al. 1989; Siegenberg et al. 1991; Davidsson et al. 1994a; Cook et al. 1997; Davidsson et al. 2001a), calcium (Cook & Monsen, 1977), polyphenols (Disler et al. 1975b; Derman et al. 1977; Siegenberg et al. 1991), and dietary protein (Morck et al. 1982; Gillooly et al. 1984b; Stekel et al. 1986; Derman et al. 1987). This effect is dose-
dependent (Cook & Monsen, 1977; Derman et al. 1980a; Hallberg et al. 1986a; Siegenberg et al. 1991; Davidsson et al. 1998b), however, a plateau is eventually reached (Gillooly et al. 1984b; Stekel et al. 1986). The amount of ascorbic acid which is needed to have a meaningful impact on iron absorption is difficult to determine as it depends on the levels of inhibitors. It has been suggested that ascorbic acid should be added at a molar ratio of ascorbic acid to iron of approximately 2:1, as such ratios have been shown to effectively enhance iron absorption in infants, children and adults (Derman et al. 1980a; Stekel et al. 1986; Davidsson et al. 1998b). However, it may be necessary to add higher amounts of ascorbic acid to foods that contain high amounts of inhibitors, since it has also been shown that the addition of ascorbic acid at a molar ratio of 2:1 relative to iron to a soy infant formula was too low to enhance iron absorption in adult women (Gillooly et al. 1984b).

Ascorbic acid enhances iron absorption from intrinsic non-heme food iron to the same extent as from water-soluble fortification iron (ferrous sulfate) (Sayers et al. 1973; Sayers et al. 1974a). However, it has no effect on iron absorption from heme iron (Lynch et al. 1985). The enhancing effect of ascorbic acid on iron absorption is not limited to water-soluble iron compounds as the effect has also been demonstrated for ferric oxide and ferric hydroxide as well as for the water-insoluble fortificants ferric orthophosphate and electrolytic iron (Derman et al. 1977; Forbes et al. 1989).

Nevertheless, it is unclear whether ascorbic acid will enhance iron absorption from ferrous fumarate. Hurrell et al. (1991) investigated in healthy adults the effect of ascorbic acid on iron absorption from a chocolate drink and a synthetic liquid formula meal fortified with ferrous fumarate. Iron absorption from ferrous fumarate did not significantly increase after the addition of ascorbic acid at a 1.7:1 ascorbic acid to iron molar ratio to the chocolate drink. However, native iron absorption from identical test meals was also not increased by the addition of ascorbic acid at a molar ratio of 13.6:1. Therefore, these results seem to indicate that the content of inhibitors (casein and calcium from milk, phytate from malt and polyphenols from cocoa) in the test meal was not counteracted by the amount of ascorbic acid added. It does, however, not seem possible to conclude from these results that ascorbic acid has no effect on iron absorption from ferrous fumarate. Further, the studies showed that iron absorption from a synthetic liquid formula meal fortified with ferrous fumarate increased non-
significantly 1.6-fold upon the addition of ascorbic acid at an ascorbic acid to iron molar ratio of 4.4:1. The effect of ascorbic acid on iron absorption from native iron was measured simultaneously in the same test meals and a significant increase of iron absorption was seen (2.1-fold). This result led to the suggestion that perhaps ferrous fumarate does not enter the common iron pool completely and thus iron absorption from ferrous fumarate is not affected by ascorbic acid. However, since the molar ratio of ascorbic acid to iron in the test meals was about 10-times higher for native iron than for iron from ferrous fumarate (44:1 vs. 4.4:1) it is not surprising that iron absorption from native iron increased to a greater extent. Thus, the study design was not appropriate to evaluate the effect of ascorbic acid on iron absorption from ferrous fumarate. Furthermore, results showing that iron absorption from infant cereal fortified with ferrous fumarate and ferrous sulfate is not significantly different in the presence (Hurrell et al. 1989a) and in the absence of ascorbic acid (Hurrell et al. 2000) indicate that ascorbic acid enhances iron absorption from ferrous fumarate to the same extent as from ferrous sulfate.

Since ascorbic acid has repeatedly been shown to enhance native food iron absorption and fortification iron absorption from single meals, it has been assumed that ascorbic acid would also have long-term effects on iron absorption and thus iron status. However, intakes of high amounts of ascorbic acid over a long period of time (up to 2 years) failed to show positive effects on iron status in iron replete as well as iron deplete subjects (Cook et al. 1984; Hunt et al. 1994). Further, when iron absorption was measured from the complete diet over a period of 5 days, the intake of high amounts in comparison to low amounts of ascorbic acid did not influence iron absorption (Cook & Reddy, 2001). However, mean ascorbic acid intake when consuming the diet low in ascorbic acid was 51 mg/d, which may be a sufficient amount to enhance iron absorption from a Western diet.

A disadvantage of adding ascorbic acid to iron fortified foods in order to enhance iron absorption is its susceptibility to losses during food processing and storage (Hurrell, 2002). Hallberg et al. (1982) for example showed that the content of ascorbic acid in foods was decreased by prolonged warming (4h at 75°C) and that this loss of ascorbic acid resulted in decreased iron absorption.
1.3.4.2 Disodium Ethylenediaminetetraacetic Acid (Na₂EDTA)

An alternative enhancer to ascorbic acid is Na₂EDTA. This compound is an approved food additive, which is used in a wide selection of foods as a sequestering agent to prevent off flavor, discoloration, turbidity, rancidity, and texture loss which can be caused by free metals (INACG, 1993). In comparison to ascorbic acid it has the advantage of being stable during storage and food preparation. However, it has the disadvantage of not being an essential nutrient and concerns exist regarding its potential toxicity, as discussed earlier in the context of NaFeEDTA toxicity (Section 1.3.2.4). The JECFA (1974) has recommended that Na₂EDTA be permitted as a food additive at concentration providing up to 2.5 mg EDTA/kg body weight and day (ADI). This limit is based on observations made by Oser et al. (1963) that rats fed daily 100-times higher amounts of Na₂EDTA showed no adverse effects. Higher daily intakes were however not evaluated.

Similar to NaFeEDTA, Na₂EDTA has repeatedly been shown to be an effective enhancer of iron absorption from ferrous sulfate fortified foods containing phytic acid, when added at a molar ratio of Na₂EDTA to iron of 1:1 (el Guindi et al. 1988; MacPhail et al. 1994; Hurrell et al. 2000; Davidsson et al. 2001c). However, when added at Na₂EDTA to iron molar ratio greater that 1:1, Na₂EDTA has been shown to act as an inhibitor of iron absorption (Cook & Monsen, 1976b; MacPhail et al. 1994); thus the effect of Na₂EDTA on iron absorption is dose-dependent. The dose-dependency of the enhancing effect was investigated further for Na₂EDTA to iron molar ratios below 1:1. The results from these studies are however not conclusive. MacPhail et al. (1994) showed Na₂EDTA to be a more potent enhancer of iron absorption from rice-based meals fortified with ferrous sulfate when added at molar ratios of Na₂EDTA to iron of 0.25:1 and 0.5:1 than at a 1:1 molar ratio. Contrary to this, Hurrell et al. (2000) showed that iron absorption from ferrous sulfate fortified wheat-based and wheat-soybean-based infant cereals increased significantly when the molar ratios of Na₂EDTA to iron were increased from 0.33:1 to 1:1. Further, in children, Davidsson et al. (2001c) showed that increasing the molar ratio of Na₂EDTA to iron from 0.3:1 to 0.7:1 and 1:1 did not influence the enhancing effect of Na₂EDTA on iron absorption from ferrous sulfate fortified Peruvian school breakfast meals. The differences between the results of these studies indicate that, in addition to being dose-dependent, the enhancing effect of Na₂EDTA on iron absorption is dependent on fur-
ther factors. A possible influencing factor could be the presence and amount of inhibitors in the meals, as studies have shown that adding Na$_2$EDTA (molar ratio 1:1) to low inhibitory meals does not affect iron absorption (Cook & Monsen, 1976b).

A factor that has recently been shown to influence the effect of Na$_2$EDTA on iron absorption is the solubility of the iron fortificant. Studies have demonstrated that iron absorption from water-insoluble and poorly water-soluble iron compounds such as ferric pyrophosphate, elemental iron (H-reduced iron) and ferrous fumarate is not affected by the addition of Na$_2$EDTA added at a 1:1 molar ratio (Hurrell et al. 2000; Fairweather-Tait et al. 2001; Davidsson et al. 2002). The lack of effect that Na$_2$EDTA has on iron absorption from poorly water-soluble and water-insoluble iron compounds is presumably related to its chelating properties. As discussed previously, Na$_2$EDTA can bind with virtually every metal in the periodic table and, thus, in the stomach the formation of EDTA-metal complexes is not limited to iron (INACG, 1993). While iron from ferrous sulfate can be expected to enter the common non-heme iron pool rapidly, the poorly water-soluble and water-insoluble iron compounds have to dissolve in the gastric juice before iron is released and can enter the common non-heme iron pool. During this time, Na$_2$EDTA will presumably form complexes with other minerals and trace elements present in the stomach resulting in lower binding capacity of Na$_2$EDTA when iron from the poorly water-soluble and water-insoluble iron compounds becomes available.

1.3.4.3 Animal Tissue

In a number of studies, animal tissue has been shown to be an effective enhancer of non-heme iron absorption, in a dose-dependent manner (Björn-Rasmussen & Hallberg, 1979; Layrisse et al. 1984). The tissue sources, which have been investigated, include beef, chicken, calf thymus, lamb, liver, and pork (Layrisse et al. 1968; Martinez-Torres & Layrisse, 1970; Martinez-Torres & Layrisse, 1971; Martinez-Torres et al. 1974; Cook & Monsen, 1976a; Björn-Rasmussen & Hallberg, 1979; Hurrell et al. 1988). It has also been shown that animal tissue (beef) enhances non-heme iron absorption in infants (Engelmann et al. 1998). When the effect of animal tissue was studied in the presence of ascorbic acid it was observed that the promotive effects of these enhancers are not additive (Layrisse et al. 1974; Cook & Monsen, 1977).
The factors in animal tissue responsible for the enhancing effect have so far not been identified. Martinez-Torres and Layrisse (1970) showed that a mixture of amino acids similar to those present in 100 g fish could mimic the effect of the same amount of fish on iron absorption. Further, it was shown that only the sulfur-containing amino acids had an effect. This led to the conclusion that the enhancing properties of fish are strongly related to amino acids and their chemical properties. Later it was proposed that cysteine is the enhancing factor in animal tissue (Martinez-Torres et al. 1981; Layrisse et al. 1984). Taylor et al. (1986) further showed that cysteine-containing peptides, released during digestion of meat, have a enhancing effect on iron absorption. Nonetheless, the so called “meat factor” has not yet been identified.

1.3.4.4 Organic Acids

The effects of organic acids with reducing properties on iron absorption have so far not been studied extensively. Citric acid, added to test meals (1 g per meal and higher) has been shown to increase iron absorption 2- to 3-fold from a rice-based meal as well as from isolated soybean protein (Gillooly et al. 1983; Derman et al. 1987). Citric acid has, however, also been shown to decrease iron absorption by approximately 50% when added (1 g citric acid/meal) to a simple Latin American meal (Hallberg & Rossander, 1984). Lactic acid, which is formed during fermentation processes, has been shown to triple iron absorption from gruels (Derman et al. 1980b), however, again there are contradicting results showing that lactic acid has no effect on iron absorption (Baynes et al. 1990). Other acids, which can be commonly found in fruits and vegetables, are malic acid and tartaric acid and these acids have also been shown to double iron absorption when added to a rice-based meal (1 g acid/meal) (Gillooly et al. 1983).

1.3.5 Potential Enhancers

1.3.5.1 Erythorbic Acid

Erythorbic acid (syn.: isoascorbic acid, D-araboascobic acid) is a stereoisomer of ascorbic acid and differs from ascorbic acid only in the relative position of the hydrogen and hydroxyl groups on the fifth carbon atom in the molecule. Due to its strong reducing properties it has similar applications in food technology as ascorbic acid as a water-soluble antioxidant (Walker, 1991). Erythorbic acid is approved for use as a food additive in most North American and Latin American countries as well as most
Asian, Oceanian, and Central European countries (Gavard et al. 1995). Erythorbic acid has been evaluated by the JECFA and was allocated a not specified ADI (Walker, 1991) and is generally recognized as safe by the FDA (Food and Drug Administration, 2001). In the European Union (European Parliament and Council Directive, 1995) and in Switzerland (Schweiz Bundeskanzlei, 1995), however, the use of erythorbic acid is restricted to meat and fish products.

The main advantage of erythorbic acid over ascorbic acid as a food additive is its lower price and it is therefore a useful and attractive alternative to ascorbic acid as an antioxidant. It can for example be used in cured meat products to minimize the formation of nitrosamines and to reduce nitrometmyoglobin to nitrosomyoglobin, which has the characteristic red color of cured meats. Further, it can be used to prevent oxidation of unsaturated fatty acids and discoloration of fish and other seafood products by dipping these products into erythorbic acid solutions. Erythorbic acid also prevents browning of fruits and vegetables and oxidative deterioration of beer and wine. Besides the abovementioned products, erythorbic acid can also be used as an antioxidant in baked goods, fats and oils, candy, and non-alcoholic beverages (Rehwoldt, 1986; Gavard et al. 1995). A potential disadvantage of erythorbic acid in comparison to ascorbic acid is its lower stability during food processing and storage. In aqueous solutions, and in foods, erythorbic acid has been shown to oxidize more rapidly than ascorbic acid (Yourga et al. 1944; Esselen et al. 1945; Reyes & Luh, 1962). Reyes and Luh (1962) for example calculated the half-life of erythorbic acid and ascorbic acid, based their stability during thawing at 18 °C, as 26 and 38 days, respectively.

A further disadvantage of erythorbic acid is that it presumably has no antiscorbutic activity in humans, although no studies have actually investigated this. In guinea pigs, erythorbic acid is said to have one-twentieth of the antiscorbutic activity of ascorbic acid based on weight-response studies (Zilva, 1935; Yourga et al. 1944; Fabianek & Herp, 1967; Pelletier & Godin, 1969). However, using the same method, Reiff and Free (1959) failed to show any antiscorbutic effects in ascorbic acid deficient guinea pigs. It has been suspected that the reason for observed differences was due to instability of erythorbic acid in the solutions used by Reiff and Free (Pelletier & Godin, 1969). The low antiscorbutic activity, in guinea pigs, has been related to the slower absorption rate of erythorbic acid (Suzuki et al. 1991) and its lower
tissue retention in comparison to ascorbic acid (Suzuki et al. 1987; Suzuki et al. 2000; Cui et al. 2001). This is probably related to the exquisite substrate selectivity of the sodium-dependent ascorbic acid transporter which greatly favors ascorbic acid over erythorbic acid (Wang et al. 2000). Slower intestinal absorption and a lower tissue retention of erythorbic acid, and consequently a faster urinary excretion, have also been shown for humans (Wang et al. 1962; Rivers et al. 1963; Sauberlich et al. 1996). The low retention rate and higher excretion of erythorbic acid in comparison to ascorbic acid will presumably limit antioxidative effects of erythorbic acid in the body.

Results from guinea pig as well as human studies have, however, suggested that erythorbic acid might have an ascorbic acid sparing effect (Reiff & Free, 1959; Sauberlich et al. 1989). This assumption is based on the finding that after 4 weeks the body weights of guinea pigs fed erythorbic acid supplemented ascorbic acid free diets were significantly higher than those fed ascorbic acid free diets (Reiff & Free, 1959). Further, Sauberlich et al. (1989) showed in humans that the intake of erythorbic acid in addition to ascorbic acid increased plasma ascorbic acid values to a greater extent than ascorbic acid alone.

Sauberlich et al. (1996) further investigated whether erythorbic acid affects ascorbic acid metabolism in humans. No adverse effects, such as increased depletion of ascorbic acid in plasma or leukocytes, were seen with the prolonged intake of high amounts of erythorbic acid (1 g/day). Nonetheless, the presence of erythorbic acid in the diet, which has been estimated to be as high as 200 mg per day in the US (Sauberlich et al. 1996), should to be taken into account when determining plasma ascorbic acid content, as erythorbic acid can interfere with the analyses when methods are used which can not distinguish between the two acids (Sauberlich et al. 1991).

As ascorbic acid and erythorbic acid have similar physical and chemical properties (Walker, 1991) it can be expected that erythorbic acid will also enhance iron absorption. However, little information on the effect of erythorbic acid on iron absorption is available. Greger et al. (1984) reported no effect of erythorbic acid on iron absorption in healthy men based on results from 5-day balance studies. Balance studies are however not sensitive enough to detect small changes in iron absorption and, in addition, the test meals contained animal tissue. Since the promotive effects of ascorbic
acid and meat are not additive (Layrisse et al. 1974; Cook & Monsen, 1977) it would seem likely that erythorbic acid would have no effect on iron absorption in the presence of meat. Further, Lee et al. (1984) reported no effect of erythorbic acid on iron absorption in rats. However, the rat is not an appropriate animal model to predict human iron absorption since the effect of ascorbic acid on iron absorption in rats and humans is not comparable (Reddy & Cook, 1991; Reddy & Cook, 1994). Thus, it does not seem possible to conclude from these studies that erythorbic acid has no effect on iron absorption in man.

1.3.5.2 Vitamin A

Vitamin A is essential for erythropoiesis and thus a deficiency in this vitamin results in anemia in humans and animals (Hodges et al. 1978; Mejia et al. 1979; Mejia & Arroyave, 1982; Mejia & Chew, 1988; Bloem et al. 1989). Vitamin A has further been reported to have an enhancing effect on iron absorption in humans (Garcia-Casal et al. 1998). However, these findings have been discussed controversially since in comparable studies Walczyk et al. (2003) showed vitamin A to have no effect on iron absorption from ferrous sulfate fortified meals. It has thus been suggested that the enhancing effect of vitamin A reported by Garcia-Casal et al. was not due to an interaction between iron and vitamin A in the gastrointestinal tract but that differences in vitamin A status of the test subjects may explain the contradictory findings. However, vitamin A status was only determined in the Swedish students but not in the Swiss students (Walczyk et al. 2003) or the Venezuelan peasants (Garcia-Casal et al. 1998) and, therefore, there is no concrete evidence to verify this hypothesis. However, recent results reported by Davidsson et al. (2003) indicate that a relationship between vitamin A status and iron absorption exists. These results showed that in children with vitamin A deficiency iron absorption, determined by erythrocyte incorporation of iron, decreased when vitamin A (retinol) was added to a ferrous sulfate fortified meal. Yet, when, 3 weeks after receiving 200.000 IU retinol, the iron absorption study was repeated in the same children no significant influence of retinol on erythrocyte incorporation of iron was measured. It is further interesting to point out that fractional iron absorption from the test meals fortified with ferrous sulfate and vitamin A was comparable before and after vitamin A supplementation (2.6 vs. 1.9%, respectively). Iron absorption from the test meal only fortified with ferrous sulfate however decreased significantly after the supplementation (4.0 vs. 2.3%). These results could
therefore be seen as an indication that vitamin A influences the regulation of iron absorption. Further research is however necessary to fully evaluate the effect of vitamin A on iron absorption.

1.4 Iron Fortification Vehicles

When choosing a food vehicle for mass iron fortification (aimed at entire population) or targeted iron fortification (aimed a specific population group, for example infants), consideration has to be given to the pattern of its consumption as well as the technical feasibility of its fortification. In order to reach a high proportion of the vulnerable population the fortified vehicle should be consumed regularly throughout the region or country. Further, any economical bias in vehicle consumption should favor low-income groups, as ID prevalence is typically highest within these groups. In addition, it is important that the supply to the household and individuals is relatively constant and that there is little chance of individuals consuming extraordinary higher amount of the fortified vehicle. This might occur if soft drinks or snack foods are chosen as fortification vehicles. On the technical side, the selected vehicle needs to be processed to permit an entry point for the iron fortificant. This limits the use of home grown foods as fortification vehicles. Further, the task of monitoring is simplified when the vehicle is only processed in few facilities. The ideal vehicle should not be expensive and the purchase price should not increase significantly due to fortification. It is also of advantage if the vehicle has good masking qualities which will permit the use of more reactive iron compounds. Organoleptic problems are also lessened if the vehicle is not stored for prolonged periods of time, especially if the climate is hot and humid. Less suitable vehicles are foods which have inhibitory properties towards iron absorption, for example coffee (Cook & Reusser, 1983). Examples of foods which have been evaluated as fortification vehicles are cereals and cereal products, infant foods, milk products and condiments. The advantages and disadvantages of the individual vehicles are described in the following.

1.4.1 Cereal Products

Rice, corn, and wheat and their derivatives such as flour and meals are often ideal vehicles for food fortification since they are the most widely consumed staple foods in many developing countries. Rice is the dominant staple in Asia where it represents
50 to 75% of the energy intake (Walter et al. 2001). Fortification of this vehicle is however challenging since it is not frequently consumed as flour. Technologies for whole grain fortification have been developed and include the coating of rice grains with iron (Rubin et al. 1977; Peil et al. 1982) as well as use of iron fortified extruded grain analogues (Kapanidis & Lee, 1996). Grains produced by these technologies are to be added to unfortified grains in a ratio of 1:100 to 1:200. Unfortunately, besides being expensive these fortification methods have the disadvantage that the fortified grains are not fully disguised (Cook & Reusser, 1983; Kapanidis & Lee, 1996). This can be avoided by using poorly soluble iron phosphate compounds, but this will limit the amount of iron absorbed (Cook & Reusser, 1983). In addition, the coating can be removed during washing and cooking (Rubin et al. 1977; Peil et al. 1982; Cook & Reusser, 1983; Hurrell, 1999). Further development of these technologies is thus necessary before being used in fortification programs.

Cereal flours, such as wheat or maize flours, are currently the most frequently used vehicles for iron fortification. In comparison to rice, the fortification of cereal flour is technically possible with only minor changes during the milling process, using a machine known as dosifier or feeder. These machines ensure that constant amounts of fortificants are added to the flour after milling (Walter et al. 2001). The amount of iron added often depends on governmental fortification policies (mandatory or voluntary), which mostly also define which iron fortification compounds can be used. The choice of fortificants depends further on the type of flour as well as purpose of the flour.

The amount of iron added to wheat flour is often low since it is frequently added to restore the iron level in milled flour to that of the whole grain. This is also referred to as flour enrichment or restoration rather than fortification. The US Food and Drug Administration (FDA) established the first standards for enrichment of wheat flour in 1942. This was a result of President Roosevelt’s concern regarding the high rates of malnutrition, especially among members of the military, in anticipation of the US involvement in World War II (Backstrand, 2002). Until the present day, the enrichment of wheat flour with iron and a number of B-vitamins remains mandatory in the US (up to a level of 44 mg iron/kg flour) (Hurrell, 1999; Backstrand, 2002). Other countries in which wheat flour is enriched with iron include most Central and South American countries as well as the UK and Canada (Hertrampf, 2002). The iron levels in enriched flour vary in these countries between 16.5 mg iron/kg flour in the UK to 60 mg
iron/kg flour in some Central and South American countries (Hurrell, 1999; Hertrampf, 2002). Mandatory enrichment of wheat flour was also established in Denmark and Sweden but was abolished in the late 1980’s and mid 1990’s due to a number of reasons, including concerns regarding iron overload as well as doubts about the need for iron fortification (Olsson et al. 1997; Milman et al. 2002).

The choice of fortification compounds for wheat flour depends strongly on the storage time of the flour, since wheat flour is extremely sensitive to fat oxidation during storage when water-soluble iron compounds are added. Ferrous sulfate can thus only be used for wheat flour which will be stored for less than 3 months. This is usually the case for flour used for commercial bread baking. Wheat flour used for pasta can also be fortified with ferrous sulfate, due to the low moisture content (Barrett & Ranum, 1985). Wheat flour is mostly fortified with water-insoluble iron compounds such as elemental iron powder. Elemental iron powders have the disadvantage of only being poorly available (see section 1.3.2.3) and further the iron may be extracted from the flour by magnets used during milling to remove contaminants. Ferrous fumarate is being used to fortify wheat flour in Venezuela and has been recommended for the fortification of wheat flour in Latin American countries as well as the Caribbean, when ferrous sulfate cannot be used (Dary et al. 2002; Garcia-Casal & Layrisse, 2002).

Ferrous fumarate is, in combination with electrolytic iron, also being used in Venezuela for the fortification of precooked corn flour (Garcia-Casal & Layrisse, 2002). Initially this flour was to be fortified with 50 mg iron as ferrous fumarate per kg; however, due to organoleptic problems, 30 mg iron/kg flour as ferrous fumarate plus 20 mg electrolytic iron/kg flour are now being used. Another type of corn flour, which has been suggested as fortification vehicle, is lime treated corn flour also known as nixtamalized corn flour (NCF). This flour is used in Central America to prepare the main staple food tortillas. Thus, industrially produced NCF is a potentially useful fortification vehicle in these countries. NCF can be fortified with ferrous fumarate (30 mg iron/kg flour) or NaFeEDTA (15 mg iron/kg flour) without causing organoleptic changes (Dary, 2002a; Dary, 2002b). However, due to the high phytic acid content of NCF, fortification with NaFeEDTA in preference to ferrous fumarate has been recommended (Dary et al. 2002).
Other cereal products frequently fortified with iron are breakfast cereals also known as ready-to-eat cereals. Manufacturers are fortifying these products primarily with reduced iron although ferric pyrophosphate is also being used and, according to the labels, the amounts of iron added in the US vary between 29 mg iron/kg and 600 mg iron/kg cereal. However, it has been shown that the amount actually added can be considerably higher than the labeled values and concerns have been raised regarding the impact iron fortified breakfast cereals could have on iron overload (Whittaker et al. 2001). On the other hand, fortified breakfast cereal have been shown to be an important source of iron for children in Northern Ireland (McNulty et al. 1996) and further in France, Ireland, UK, and Spain fortified breakfast cereals have positively contributed to increasing iron intakes during childhood and adolescence (Serra-Majem, 2001).

1.4.2 Infant Foods

Infant formula is an excellent vehicle for iron fortification as it is consumed by the non-breastfed infant in large quantities. Further, the commercially available infant formulas, which are either milk-based or soy-based products, can be fortified with readily bioavailable ferrous sulfate without causing discoloration or fat oxidation. In addition, infant formulas always contain ascorbic acid (minimum levels approximately 50 mg ascorbic acid/L formula) which should increase the amount of available iron further (Ziegler & Fomon, 1996). Presently, formulas (for healthy term infants) in the US are commonly fortified with 12 mg iron/L, in agreement with the recommendation of the American Academy of Pediatrics' Committee on Nutrition (1976; 1989; 1999). In Europe, infant formulas should contain between 3 and 10 mg iron/L, although formulas with no added iron are permitted providing a statement is made to the effect that, when the product is given to infants over the age of 4 months, their total iron requirements must be met from other additional sources (European Economic Community, 1991; Moy, 2000). In the US "unfortified" or low-iron infant formulas contain small amounts of added iron (1.5-4.0 mg/L) (Ziegler & Fomon, 1996). It is however being discussed whether the iron fortification levels, especially those in the US, are too high (Dallman, 1989; Moy, 2000). This is firstly based on findings showing that iron absorption increases with decreasing iron content (Saarinen et al. 1977; Fomon et al. 1997). Further, long-term evaluations (0-9, 1.5-6 or 6-12 months of age) have shown that infant formulas with low iron content can be just as effective in preventing
Literature review

ID as those with high iron content (Haschke et al. 1993; Lönnerdal & Hernell, 1994; Walter et al. 1998).

In the US, as well as in Europe, cereals are typically the first solids introduced into the infant’s diet (Fomon, 1993; Ziegler & Fomon, 1996). Commercial infant cereals in industrialized countries are usually precooked, roller-dried cereal flours, which are based on cereals with low extraction rate, such as wheat, rice, and maize and are fed with milk. Complete infant cereals contain cow milk or soy protein and are fed with water. Infant cereals are commonly fortified with iron, however, since ferrous sulfate provokes off-colors as well as fat oxidation, less soluble iron compounds are usually used to avoid organoleptic problems (Hurrell et al. 1989a; Theuer, 2002). These include elemental iron powders in the US and ferric pyrophosphate and ferric orthophosphate in Europe (Hurrell, 1989). Iron phosphate compounds have however been largely replaced with ferrous fumarate due to the higher RBV of this compound. Ferrous fumarate does not provoke fat oxidation during storage, however, when acidic fruits are added to the infant cereal this compound may dissolve and cause color changes (Hurrell et al. 1989a; Hurrell, 1999). Besides being difficult to fortify with readily available iron compounds, infant cereals have the further disadvantage of containing phytic acid. The inhibitory effect of phytic acid can however be overcome by the addition of ascorbic acid (Davidsson et al. 1997).

1.4.3 Dairy Products

Iron fortification of milk as well as other dairy products has been researched extensively and thus a comprehensive review of this topic would go beyond the scope of this literature review. Therefore, only a few major finding will be reported here. Iron compounds judged as unacceptable for liquid milk fortification in at least one study include ferrous sulfate, ferrous fumarate, ferrous gluconate, ferrous lactate, ferric choline citrate, and ferrous ammonium sulfate (Demott, 1971; Edmondson et al. 1971; Kurtz et al. 1973; Wang & King, 1973). Only slight changes to the flavor of milk occur when ferric ammonium citrate or ferric pyrophosphate are used and more recently encapsulated ferrous sulfate as well as ferrous bisglycinate have also been shown to be suitable for milk fortification (Demott, 1971; Wang & King, 1973; Boccio et al. 1996; Olivares et al. 1997). Fortificants which have been shown to be suitable for non-fat milk powder are ferric ammonium citrate and ferrous sulfate (Kurtz et al. 1973).
1973; Walter et al. 2000). Further dairy products which have been evaluated as iron fortification vehicles include different cheeses, such as cheddar, mozzarella, soft white cheese, edam, and cottage cheese, as well as yoghurts. The results of these evaluations have been reviewed by Gaucheron (2000) and by Martinez-Navarrete et al. (2002).

1.4.4 Chocolate Drinks
Milk-based chocolate drink powders as well as chocolate milks have been suggested as iron fortification vehicles as these products could be targeted at children and adolescents. The disadvantage of these vehicles is the high content of polyphenolic compounds, phytic acid, and calcium which inhibit iron absorption. Further, soluble iron compounds, such as ferrous sulfate, cannot be used as fortificants since these cause discoloration and affect the taste (Douglas et al. 1981). Ferrous fumarate is also less suitable as a fortificant since it can causes off-colors (Hurrell et al. 1991). Organoleptic changes can be avoided by using ferric pyrophosphate (Hurrell et al. 1991). However, due to the high content of inhibitors, the addition of ascorbic acid would be necessary to ensure adequate iron absorption (Davidsson et al. 1998b).

1.4.5 Condiments
When the fortification of the main dietary staple is not technically possible or considered inappropriate, the fortification of condiments that are typically consumed within the target group may be an alternative. However, successful experiences with fortified condiments have so far not gone beyond experimental trails. Curry powder, fish sauce, and soy sauce as well as salt and sugar are condiments which have been evaluated as fortification vehicles and these are described in detail below. Further condiments which have been discussed as fortification vehicles are monosodium glutamate, the seasoning of instant noodles as well as bouillon cubes. However, only limited information is available regarding the potential usefulness of these vehicles (Zoller et al. 1980; Chavasit & Tontisirin, 1998; Melse-Boonstra et al. 2000).

1.4.5.1 Curry Powder
Curry powder has been evaluated as a food fortification vehicle in South Africa. The reasoning for the use of this vehicle is based on the ethnical diversity in South Africa and the resulting differences in dietary habits. While among the black South African population iron overload is a common problem due to the consumption of fermented
beverages with high iron content, the prevalence of ID is high among the Indian population. It was therefore important to select a vehicle which would almost exclusively be consumed by the iron deficient segment. Curry powder, which is also referred to as masala, was considered the most appropriate vehicle under the given circumstances. This condiment has the additional advantage of having an enhancing effect on iron absorption, as shown by Lamparelli et al. (1987). NaFeEDTA was evaluated as the fortificant and iron was shown to be approximately twice as well absorbed from NaFeEDTA as from ferrous sulfate when consumed together with a potato based meal. Further, the addition of NaFeEDTA to curry powder (1.4 mg iron/g powder) did not cause organoleptic changes considered unsatisfactory, although some darkening of prepared foods did occur (Lamparelli et al. 1987).

1.4.5.2 Fish Sauce
Fish sauce is a clear brown liquid with a salty taste (average sodium chloride content is 22-30%) and mild fishy flavor (Lopetcharat et al. 2001). It is a popular condiment in Southeast Asia, especially in Vietnam, Thailand and Cambodia where it is used in cooking as a substitute for salt and serves as a basic flavoring in numerous dishes (Ang, 1999; Luh, 1999; Lopetcharat et al. 2001). Due to the frequent consumption and the difficulties involved in fortifying rice, this condiment is a potentially useful iron fortification vehicle for most Southeast Asian countries.

In Vietnam fish sauce is called Nuoc-Mam and is consumed by approximately 80% of the population (Mannar & Gallego, 2002). It is generally manufactured from small fish, although shrimp can also be used, which are kneaded and pressed by hand and then placed in layers with salt (fish to salt ratio 3:1) in earthenware jars or fermentation tanks. Fermentation time for small fish is approximately 6 months but extends to 18 months if larger fish are used. The first supernatant collected from the tanks is referred to as high-quality fish sauce. Lower quality fish sauce is produced by adding hot brine to the residue. The same brine can be used to extract several fermentation tanks and caramel, molasses, roasted maize, or barley can be added to the fish residue before extraction to improve the color of lower quality fish sauce. Further, high quality fish sauce may be added to enhance color and flavor (Lopetcharat et al. 2001). Thai fish sauce, Nampla, is produced in a similar way. One major difference, however, is that the lower quality fish sauce is extracted with BX-water. This is a by-
product of monosodium glutamate production and a rich source of glutamic acid and thus improves nitrogen content of lower quality fish sauce (Lopetcharat et al. 2001).

During fermentation, the fish protein is hydrolyzed by endogenous enzymes and microorganisms into small peptides and amino acids. Further, ammonia and trimethylamine are formed and are responsible for the ammoniacal note of fish sauce. Low molecular weight volatile fatty acids (VFA), in particular acetic, ethanolic, propionic, n-butyric, and isovaleric acid have been identified to contribute to the cheesy note of fish sauce. The VFA are formed by autooxidation of polyunsaturated acids and by bacterial action on amino acids. The meaty aroma of fish sauce is not attributed to a particular compound but appears to be due to a large number of volatiles (Lopetcharat et al. 2001).

Fish sauce was first investigated as an iron fortification vehicle by Garby et al. (1974). Their studies showed that fish sauce could be fortified with NaFeEDTA (1 mg iron/mL sauce) without causing precipitation of peptides. Further, sensory testing showed that fortifying fish sauce with NaFeEDTA did not influence the taste of meals prepared with this fish sauce. Other soluble iron compounds, such as ferrous sulfate and ferrous lactate have been shown to rapidly cause peptide precipitation. However, recent studies showed that the addition of citric acid (150 mg/100 mL) to ferrous sulfate and ferrous lactate fortified fish sauce (50 mg iron/100 mL) can prevent peptide precipitation. It was also shown that ferric ammonium citrate does not cause precipitation in fish sauces stored for 3 months (unpublished data, Ronja Sakata, Semesterarbeit 2001/2002, Laboratory for Human Nutrition).

1.4.5.3 Soy Sauce
Soy sauce is a dark brown liquid extracted from a fermented mixture of salt, soybeans, and wheat, or salt and soybeans alone (average sodium chloride content: 16-18%). It has a salty taste and sharp flavor and has been used as an all-purpose seasoning in Asia for thousands of years (Liu, 1999; Lopetcharat et al. 2001).

There are many different types of soy sauces. Based on the preparation methods soy sauce can be divided into 3 groups: fermented soy sauce, chemical soy sauce, and semi-chemical soy sauce. Further, soy sauces differ depending on the raw materials used as well as their geographical origin (Liu, 1997).
Soy sauce is traditionally made by fermentation and the basic manufacturing methods for all fermented soy sauces only differ slightly. The manufacturing method of modern type Chinese soy sauce is described here: The raw ingredients, in this case defatted soy meal and wheat bran at a ratio of 60:40 (low-grade wheat flour can additionally be added in order to increase sugar content of the soy sauce), are mixed and soaked in water before being autoclaved. Following this procedure, a mold seed culture, 80% Aspergillus oryzae and 20% Aspergillus sojae, is added, and the resulting inoculated material is cultured for 24 hours at 30-35 °C. This procedure is also referred to as Koji making. The mature Koji is then mixed with brine and the resulting mash (salt content: 17-19%) is fermented at relatively high temperatures (40-45 °C). The amount of brine added when making modern type of soy sauce is only about half as much as used for traditionally produced Chinese soy sauce and only about three-quarters as much salt is added. Under these conditions (low-water, low-salt, high temperature) it only takes 3 weeks to complete the fermentation. In comparison, when soy sauce is produced using the traditional method fermentation takes about 6 months. After fermentation, the mash is transferred into another tank, mixed with further brine and heated to approximately 80 °C. The resulting liquid after filtration, is raw soy sauce, which is then pasteurized and frequently sodium benzonate is added as a preservative before being bottled (Xu, 1990). The modern method described here has been adopted by most commercial soy sauce producer in China. The traditional method for making soy sauce, using soybeans and a small amount of wheat flour, which can however also be excluded, is now predominately only used at a domestic level (Liu, 1999).

In Japan, there are five officially recognized types of fermented soy sauce. Koikuchi shoyu, the most popular type of Japanese soy sauce, and Usukushi shoyu are made from equal amounts of soybeans and wheat. The difference between these two sauces is that the fermentation of Usukushi shoyu is controlled in order to prevent color development. The three remaining types of soy sauces are produced and consumed only in isolated localities for special uses. Saishikomi shoyu is produced from an equal amount of soybeans and wheat. It however differs from the abovementioned since raw soy sauce instead of brine is added during the manufacturing. Higher wheat to soybean ratios are used to make Shiro shoyu and the fermentation is controlled in order to prevent color formation. The last type of Japanese soy sauce,
Literature review

Tamari shoyu, is made by using a higher soybean to wheat ratio (9:1) or soybeans alone and is characterized by a higher content of amino acids (Fukushima, 1985; Liu, 1999).

Soy sauce can further be made by acid hydrolysis (hydrochloric acid) of defatted soy products for 8-12 hours. The hydrolyzate is neutralized with sodium carbonate and the resulting product is known as chemical soy sauce. To improve the quality of chemical soy sauce it can be blended with fermented soy sauce and such products are referred to as semi-chemical soy sauces (Liu, 1999).

Soy sauce has only recently been suggested as a vehicle for food fortification and it has been shown that NaFeEDTA can be added to soy sauce without cause unacceptable changes to the vehicle. Further, a study made by Baynes et al. (1990) indicated that soy sauce (Tamari shoyu) per se might have an enhancing effect in iron absorption, as discussed earlier (Section 1.3.3.4).

1.4.5.4 Salt
Salt has successfully been used for iodine fortification since 1922, when it was first introduced in Switzerland. It is potentially also an ideal vehicle for iron fortification and in India the technical feasibility of salt fortification has been researched extensively. The advantages of salt as an iron fortification vehicle are the regular consumption of salt in relatively constant amounts. Further, in many developing countries it is one of the only processed dietary items which is bought by the majority of the population. This is for example the case in India where no other suitable vehicle for iron fortification has yet been identified (Sivakumar et al. 2001). However, the fortification of salt with iron is technically challenging for a number of reasons. Water-soluble iron compounds react with moisture and impurities in the salt and cause unacceptable changes in color (Sayers et al. 1974b; Mannar & Diosady, 1998). This can be avoided by using water-insoluble iron compounds such as ferric orthophosphate or ferric pyrophosphate (Narasinga Rao et al. 1972; Sayers et al. 1974b; Narasinga Rao & Vijayasarathy, 1975). Studies have further indicated that the addition of sodium hexametaphosphate (SHMP) stabilizes ferrous sulfate and prevents color changes (Ranganathan, 1992). Additional problems arise when the salt is fortified with iron and iodine since the stability of iodine is decreased in the presence of iron. It has however been shown that iodine losses can to some extent be prevented by
adding SHMP to salt fortified with ferrous sulfate and potassium iodate or potassium iodine (Sivakumar et al. 2001). Further, the encapsulation of iodine with dextrin has also proven to effectively prevent iodine loss in dual fortified salt (Diosady & Venkatesh Mannar, 2000).

1.4.5.5 Sugar
Sugar is a condiment which has also been evaluated for its use as iron fortification vehicle. It has been used successfully as a vitamin A fortification vehicle in Central America and studies have shown that additional fortification with NaFeEDTA is possible without affecting the stability of vitamin A (added as retinyl palmitate) (Viteri et al. 1995). NaFeEDTA (130 mg iron/kg sugar) has been shown to be a satisfactory fortificant for sugar which does not segregate and only causes discoloration when fortified sugar is stored under extreme conditions (Viteri et al. 1995). However, although NaFeEDTA does not affect the acceptability of sugar, it has been shown to affect the color of some food items prepared with fortified sugar. This includes discoloration of certain corn puddings and gruels as well as blackening of tea and coffee. Discoloration of tea has also been shown to occur when sugar fortified with ferrous sulfate is used. Only the use of ferric orthophosphate as fortificant has been shown to avoid this problem (Disler et al. 1975a).

1.5 Impact of Iron Fortification on Iron Deficiency

Presently, numerous food items are being fortified with iron, especially in industrialized countries. However, the impact of these fortification programs on iron status and public health is largely unknown. Wheat flour has for example been fortified in the US for over half a century and during this time the prevalence of IDA has decreased. However, in the same period of time, the standard of living improved, meat intake increased and the use of iron supplements became more widespread. Thus, it is not possible to relate the improvement in iron status exclusively to iron fortification (Lynch, 2002). Overall, surprisingly few studies have been made evaluating the efficacy (the extent to which a specific intervention, procedure, regime, or service produces a beneficial result under ideal conditions (Last, 1988)) or the effectiveness (the extent to which a specific intervention, procedure, regime, or service, when deployed in the field, does what it is intended to do for a defined population (Last, 1988)) of food fortification programs.
1.5.1 Cereal Flour

Some information on the effectiveness of flour fortification programs can be derived from ID prevalence data from Venezuela before and after the introduction of iron fortification and from studies made in Sweden and Denmark evaluating the impact of the abolishment of wheat flour iron fortification (Olsson et al. 1997; Milman et al. 2002).

In Venezuela, after only 1 year of wheat and maize flour fortification with iron, prevalence of ID (based on serum ferritin values) decreased from 37% to 16% and remained at comparable levels during the following 5 years. The prevalence of anemia also decreased during the first year of flour fortification. After three years, however, anemia prevalence had increased again to values comparable to those seen before the introduction of flour fortification and remained high over the following 2 years. The reasons for the increase of anemia prevalence to pre-fortification levels have not been identified. Factors which have been discussed as possible causes are the decreased consumption of maize flour, which is additionally fortified with vitamin A, as well as the further deterioration of quality of life (Garcia-Casal & Layrisse, 2002).

In Denmark and Sweden, iron fortification of flour was abolished in 1987 and 1995, respectively, due to a number of reasons, including concerns regarding iron overload as well as doubts about the need for iron fortification (Olsson et al. 1997; Milman et al. 2002). In Denmark, the impact of flour iron fortification on public health was estimated by cohort comparisons of changes of iron status parameters before (1984) and after (1994) the mandatory flour fortification (30 mg carbonyl iron/kg flour). These comparisons were made in women and men aged 40 through 70 years and showed that during this period, despite the abolition of flour iron fortification, iron stores increased in men as did the prevalence of iron overload. In women, the prevalence of iron overload also increased slightly whereas the prevalence of ID and IDA was unchanged (Milman et al. 2000; Milman et al. 2002). One conclusion drawn from these evaluations was that food iron fortification had no positive effect on iron status within the monitored population groups. In Sweden, where flour iron fortification levels were the highest world-wide (60 mg carbonyl iron/kg flour), the influence of the withdrawal on iron status is still being evaluated. Presently, indicative data is only available from a study investigating the effects of the changed policy in individuals suffering from a genetic iron overload disorder (hemochromatosis) (Olsson et al. 1997). The results of
this study showed that after flour fortification with iron was discontinued, average iron absorption in these subjects decreased by 0.65 mg/d (from 4.27 to 3.63 mg iron/d), which resulted in a decreased phlebotomy frequency (phlebotomies are an effective treatment to lessen the negative consequences of iron overload). Further, iron absorption from carbonyl iron was calculated and shown to be higher than previously believed (38%; iron absorption in subjects with hemochromatosis is thought to correspond to iron absorption of iron deficient subjects). The abolition of flour iron fortification was therefore of benefit for subjects with hemochromatosis. At the same time, since the carbonyl iron was more available than previously reported, it was concluded that the withdrawal may result in higher ID prevalence within groups with high iron requirements (menstruating females).

### 1.5.2 Infant Foods

More evidence exists regarding the effectiveness of iron fortified infant formula. A number of studies have shown that the consistent use of ferrous sulfate fortified formula in comparison to “unfortified” formula (see section 1.4.2) during the first 9 to 15 months of the infants life results in higher serum ferritin and hemoglobin levels as well as a decreased prevalence of ID and IDA (Marsh et al. 1959; Saarinen, 1978; Haschke et al. 1988; Moffatt et al. 1994). Further, the introduction of iron-fortified formulas in the US has been associated with decreased prevalence of ID and anemia among infants (Ziegler & Fomon, 1996). Nevertheless, it has been suggested that iron-fortified infant formula only has a modest impact on iron status during the first 6 months of life due to the infant’s low iron requirements (Fomon, 2001). However, Moffatt et al. (1994) showed that the prevalence of anemia at the age of 6 months was significantly higher (28% vs. 8%) when unfortified infant formula (1.1 iron mg/L formula) was fed in comparison to fortified formula (12.8 mg iron/L formula). The use of a formula with less fortification iron would possibly have been as effective since Lönnerdal and Hernell (1994) have shown that iron status at the age of 6 months was satisfactory in infants fed formulas fortified with 4 mg and 7 mg iron/L formula.

Further evaluations on the effect of iron fortified infant formula on hemoglobin concentrations have been made in Chile, where milk powder is distributed to a large percentage of infants by the National Health Service free of cost. Fortifying this milk powder with ferrous sulfate (15 mg iron/100g powder) has been shown to decrease
Literature review

the prevalence of anemia significantly when given to infants from 3 to 15 months of age (anemia prevalence at 15 months of age: 34.6% unfortified milk powder group vs. 12% fortified milk powder group). Similar results were observed when the acidified milk powder, fortified with ferrous sulfate (15 mg iron/100g powder) and ascorbic acid, was given to infants from 3 to 15 months of age. These results were further confirmed in a large regional field study with the aim to evaluate the effect of fortifying acidified milk powder with ferrous sulfate under the conditions of normal distribution. However, since the comparison between fortified and unfortified milk powder were made in two cohorts (infant born June and July 1978 vs. infants born August and September 1978, respectively) the result of this later study can not easily be evaluate (Walter et al. 2000).

While the effectiveness of iron fortified formula has been demonstrated only few studies have evaluated the effectiveness of iron fortified infant cereal. Infant cereals are typically fortified with poorly available iron compounds and thus it has to be questioned whether iron absorption from these cereals will be high enough to prevent ID. Walter et al. (1993) studied the effect of feeding iron fortified infant cereal (55 mg reduced iron/100 g dry cereal) on anemia prevalence in breastfed and formula fed infants from the age of 4 months until the age of 15 months. The formula fed infants received either fortified or unfortified cereal together with unfortified formula (no added iron). Breastfed infants also received either fortified or unfortified cereal (unfortified formula was supplied when infant had been weaned) and a fifth group received unfortified cereal together with fortified formula (12 mg iron/L formula). The results of this study showed that at 15 months of age the prevalence of anemia among infants receiving unfortified cereal was significantly higher than among those fed fortified cereal, regardless if the child was breastfed or formula fed. The results also showed that the use of iron-fortified formula was as effective as the use of fortified cereal in regards to the prevention of anemia. It should however be noted that the consumption of cereal (20-30 g dry cereal/day) was very high. A further study by Walter et al. (2001) showed infant cereal fortified with heme iron (14 mg iron/100 g dry cereal) to effectively decrease anemia prevalence in 12 month old infants who received this cereal during the previous 8 months. Thus, the abovementioned studies showed that under conditions of a controlled field trail iron fortified infant cereals can decrease anemia prevalence and therefore indicate that feeding iron fortified infant cereals
may reduce ID and IDA. However, since 55 mg iron/100 g cereal is a very high fortification level and further heme iron is not commonly used for infant cereal fortification, it would seem important to further evaluate the efficacy of iron fortified infant cereal, especially in respect to the various iron fortificants and fortification levels used.

1.5.3 Condiments

The usefulness of iron fortified condiments has been demonstrated in efficacy and effectiveness studies. NaFeEDTA fortified curry powder was evaluated in a 2-year placebo controlled field study among Indians in South Africa and its consumption was associated with a reduced prevalence of ID (Ballot et al. 1989). Further, Garby et al. (1974) evaluated the usefulness of NaFeEDTA fortified fish sauce by monitoring changes of packed red cell volume (PCV) of inhabitants of two villages in the Nakorn Nayok Province of Thailand. The inhabitants of the villages received a free supply of either unfortified fish sauce (control village) or fortified fish sauce (1 g iron/L, test village) and after 1 year, the PCV in the test village had increased significantly by 1.5 units whereas in the control village no significant changes were seen. The efficacy of NaFeEDTA fortified fish sauce was also recently evaluated by Thuy et al. (2001) in Vietnam. The results from this placebo controlled study showed that the consumption (6 days/week) of a meal containing fortified fish sauce reduced the prevalence of IDA significantly among anemic women. Similarly, NaFeEDTA fortified soy sauce used to prepare school lunches for Chinese students aged 11 though 17 years during a 3 month study period significantly improved iron status (based on values for hemoglobin, serum ferritin, free erythrocyte protoporphyrin and total iron binding capacity) (Huo et al. 2002). Another NaFeEDTA fortified vehicle shown to be effective is sugar. In Guatemala iron fortified sugar was sold for 32 months in 3 communities and during this time serum ferritin values increased significantly within the studied populations (Viteri et al. 1995). The results from all aforementioned studies indicate that NaFeEDTA fortified condiments are potentially useful vehicles to combat ID.

The effect of iron fortified salt on anemia prevalence and/or iron status parameters has been evaluated in a number of studies. Non-iodized salt fortified with ferric orthophosphate (1 mg iron/g salt) was shown to significantly improve hemoglobin status and decrease the prevalence of anemia in Indian school children aged 5 to 15 years when used over a period of 1 year (Nadiger et al. 1980). The same salt was
evaluated in an 18-month large-scale field study in three rural and one urban area of India. Again improved hemoglobin values and decreased anemia prevalence were observed, even in areas with a high incidence of hookworm. (Working Group on Fortification of Salt with Iron, 1982). Iron fortified salt was however not introduced in India as salt was already being fortified with iodine and problems occurred with dual fortification. The impact of a thereafter developed dual fortified salt (DFS) (fortified with ferrous sulfate (1 mg iron/g salt), potassium iodate, and SHMP as stabilizer) on hemoglobin concentration was evaluated in two field trials in India. In the first trial, the results showed little or no effect of DFS on hemoglobin concentration in children and adolescents after 2 years. However, this may have been due to a poor study design rather than to the ineffectiveness of the DFS salt. In the second study, the effect of DFS on hemoglobin values of school children was evaluated in a placebo (iodized salt) controlled trial. After the study period (2 years) there was only a marginal benefit to hemoglobin: hemoglobin concentration actually decreased significantly in both groups, the decline in the group receiving DFS was however not as great (Brahmam et al. 1994; Brahmam et al. 2000; as referenced by Sivakumar et al. 2001). Unfortunately, since neither the average salt consumption was reported in the later two studies nor the dietary habits, it is difficult to estimate whether iron intake was low or iron absorption was inhibited by other dietary components.

DFS has also been evaluated in Morocco where salt was fortified with 1 mg iron/g added as encapsulated ferrous sulfate and 25 mg I/g. After 9 months, hemoglobin levels had increased and iron status had improved significantly in comparison to the group receiving iodized salt. This study also identified a further advantage of DFS as the results showed that thyroid volume decreased and plasma thyroxin levels increased to a significantly greater extent in the group receiving DFS in comparison to the group receiving iodized salt. This is due to the recently established fact that ID adversely effects thyroid metabolism and reduces efficacy of iodine prophylaxis in areas of endemic goiter (Zimmermann et al. 2002). Thus, iron fortification may not only have a beneficial effect on iron status, it may also increase efficacy of iodine fortification.
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Literature review


Literature review


Literature review


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Chapter 2

IRON ABSORPTION FROM FERROUS FUMARATE IN ADULT WOMEN IS INFLUENCED BY ASCORBIC ACID BUT NOT BY Na₂EDTA

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Iron absorption from ferrous fumarate

Abstract
Ascorbic acid and Na₂EDTA enhance iron absorption from the water-soluble iron compound FeSO₄ but their effect on poorly water-soluble iron compounds such as ferrous fumarate is less well established. In the present study, the effects of ascorbic acid and Na₂EDTA on iron absorption from ferrous fumarate were evaluated in adult women (10 women/study) from erythrocyte incorporation of iron stable isotopes (⁵⁷Fe or ⁵⁸Fe) 14 d after administration. Two separate studies were made with test meals of iron fortified infant cereal (5 mg iron/meal). Data were evaluated by paired t-test and results are presented as geometric means. In study 1, the comparison between iron absorption from ferrous fumarate and FeSO₄ fortified cereal showed that adults absorb iron as well from ferrous fumarate as from FeSO₄ (3.0% and 3.1%, respectively, p=0.85). However, when Na₂EDTA (molar ratio Na₂EDTA/fortification iron 1:1) was added, iron absorption was significantly higher from FeSO₄ than from ferrous fumarate (5.3 vs. 3.3%, respectively, p<0.01). In study 2, iron absorption was compared from ferrous fumarate fortified meals with and without ascorbic acid added at a 4:1 molar ratio (relative to fortification iron) and the results showed that ascorbic acid increased iron absorption from ferrous fumarate significantly (6.3 vs. 10.4%, p=0.02). The results of the present studies show that iron absorption from ferrous fumarate is enhanced by ascorbic acid but not by Na₂EDTA; thus emphasising that not all findings from iron absorption studies made with FeSO₄ can be extrapolated to iron compounds which have different solubility properties.

Key words: iron fortification, ferrous fumarate, ascorbic acid, Na₂EDTA
Iron absorption from ferrous fumarate

Introduction
Ferrous fumarate is a poorly water-soluble iron compound, which is soluble in dilute acid such as gastric acid and as well absorbed as ferrous sulphate in healthy Western adults (Hurrell et al. 1989; Hurrell et al. 1991; Hurrell et al. 2000). In infants, iron absorption from this compound is significantly higher than from ferric pyrophosphate (Davidsson et al. 2000). Besides being readily bioavailable, ferrous fumarate is a useful fortification compound since it provokes fewer organoleptic changes in fortified foods during storage than freely water-soluble iron compounds such as ferrous sulphate (Hurrell, 1999). Presently, ferrous fumarate is used to fortify industrially manufactured infant cereals in Europe, maize flour in Venezuela and has been suggested as the fortificant for chocolate drink powders (Hurrell, 1999; Garcia-Casal & Layrisse, 2002).

Bioavailability of fortification iron depends on several factors, including the solubility of the iron compound in the gastric content during digestion and the composition of the meal, i.e. on the presence of enhancers and inhibitors of iron absorption. Ascorbic acid is a potent enhancer of iron absorption and can overcome the inhibitory effect of phytic acid in cereal-based meals on native iron as well as on freely water-soluble iron compounds such as ferrous sulphate (Sayers et al. 1973; Bjorn-Rasmussen & Hallberg, 1974; Sayers et al. 1974; Gillooly et al. 1984; Cook et al. 1997). Ascorbic acid has also been reported to enhance iron absorption from the water-insoluble iron fortificants ferric orthophosphate and electrolytic iron (Forbes et al. 1989). Its influence on the absorption of ferrous fumarate is, however, less well established. Hurrell et al. (1991) reported no enhancing effect of ascorbic acid on iron absorption from a ferrous fumarate fortified chocolate drink powder and only a modest enhancing effect when added to a ferrous fumarate fortified liquid formula meal.

In a similar way, Na₂EDTA has also been shown to enhance iron absorption from ferrous sulphate in inhibitory meals containing phytic acid (MacPhail et al. 1994; Hurrell et al. 2000; Davidsson et al. 2001). Its advantage over ascorbic acid is that it is stable during heat processing and storage; however, there are several recent reports that Na₂EDTA does not enhance iron absorption from poorly water-soluble and water-insoluble iron compounds. For example, when added to a ferric pyrophosphate fortified infant cereal, Na₂EDTA did not increase iron absorption (Hurrell et al. 2000). Further, iron absorption was not improved by the addition of Na₂EDTA to cornflakes forti-
Iron absorption from ferrous fumarate

ried with hydrogen reduced iron (Fairweather-Tait et al. 2001), nor when added to a typical Guatemalan meal fortified with ferrous fumarate (Davidsson et al. 2002).

The aim of the present studies was to compare the influence of Na\textsubscript{2}EDTA on iron absorption from ferrous fumarate and ferrous sulphate and to further evaluate the influence of ascorbic acid on iron absorption from ferrous fumarate. Iron absorption was measured in healthy women from a wheat-based infant cereal by using a stable-isotope technique based on the incorporation of iron stable isotopes into erythrocytes 14 d after administration.

Subjects and Methods

Subjects

Twenty apparently healthy adult women (20-30 years; max body weight 60 kg) were recruited from the student and staff population at the ETH Zurich and the University of Zurich. The subjects were randomly allocated to two separate studies (10 subjects/study). Exclusion criteria included pregnancy or lactation and known gastrointestinal or metabolic disorders. No medication (except oral contraceptives) or vitamin/mineral supplements were allowed during the study. Women regularly taking vitamin/mineral supplements discontinued the supplementation 2 weeks before the start of the study.

The study protocol was reviewed and approved by the Ethical Committee at the ETH Zurich, Switzerland. Subjects were informed orally and in writing about the aims and procedures of the study. Written informed consent was obtained from all study subjects.

Study design

Two studies were made (Table 1). In study 1, each subject received four test meals and iron absorption from ferrous fumarate and ferrous sulphate fortified meals with and without added Na\textsubscript{2}EDTA was compared. In study 2, each subject received two test meals only and iron absorption from ferrous fumarate fortified meals with and without ascorbic acid was compared. Iron absorption was based on erythrocyte incorporation of iron stable isotope labels 14 d after intake of labelled test meals. The iron compounds were labelled with $^{57}$Fe or $^{56}$Fe and added to the different test meals as described below. All test meals were fed between 0700 and 0900 hours, after an
Iron absorption from ferrous fumarate

overnight fast, under standardised conditions. A cross-over study design was used with each woman acting as her own control. On the day before intake of the first test meal (day 0), a venous blood sample was drawn after an overnight fast for determination of iron status parameters (haemoglobin (Hb), and plasma ferritin) and body weight and height were measured. In study 1, labelled test meals (meal A and B) were fed on the following 2 d (days 1 and 2). A second venous blood sample was drawn 14 d after intake of the second test meal (day 16). The second pair of labelled test meals (meals C and D) was fed on days 17 and 18 and a final blood sample was obtained 14 d after administration of the last test meal (day 32). In study 2, the first labelled test meal (meal E) was fed on day 1 and a second venous blood sample was drawn 14 d later (day 15). On day 16, the second labelled test meal (meal F) was fed and a final blood sample was obtained on day 30. No intake of food or fluids was allowed for 3 hours after test meal intake.

Test meals
All test meals consisted of 50 g roller-dried wheat-based infant cereal (Nestlé PTC, Orbe, Switzerland) fed with reconstituted milk (8 g milk powder, Sano Lait, Coop Schweiz, Basel, Switzerland, and 75 mL deionised water). The infant cereal was made from 79.7% wheat flour, partially hydrolysed, 10% sucrose, 4% honey, 3% palm oil, 3% water and 0.3% calcium carbonate. Except for calcium, no minerals or vitamins were added. Each test meal contained 5 mg of added iron, 4 mg iron as $^{58}$FeSO$_4$ plus 1 mg iron as FeSO$_4$ of natural isotopic composition or 5 mg iron as $^{57}$Fe ferrous fumarate. Deionised water (200 g) was served as a drink. To test meals C and D in study 1, Na$_2$EDTA (Akzo Nobel, Herkenbosch, The Netherlands) was added at a molar ratio of Na$_2$EDTA to fortification iron of 1:1 (26.7 mg Na$_2$EDTA). To test meal F in study 2, ascorbic acid (Merck, Darmstadt, Germany) was added at a molar ratio of ascorbic acid to fortification iron of 4:1 (63 mg ascorbic acid).

Stable isotope labels
$[^{58}$Fe]Ferrous sulphate was prepared from isotopically enriched elemental iron (Chemgas, Boulogne, France) by dissolution in sulphuric acid and dilution to appropriate concentration. $[^{57}$Fe]Ferrous fumarate was prepared in collaboration with Dr. Paul Lohmann Ltd., Emmerthal, Germany. It has been used in previous human studies (Davidsson et al. 2000; Davidsson et al. 2002).
Iron absorption from ferrous fumarate

Ascorbic acid and Na$_2$EDTA solutions
Aqueous solutions of food grade ascorbic acid and food grade Na$_2$EDTA were prepared freshly each morning and added to the test meals at the time of serving.

Quantification of iron isotopes in labelled iron fortificants
Isotope dilution mass spectrometry was used to determine the concentration of $^{57}$Fe and $^{58}$Fe stable isotopes in the ferrous fumarate and ferrous sulphate solutions. For analysis, $[^{57}$Fe]ferrous fumarate was dissolved in concentrated nitric acid. An accurately measured amount of iron of natural isotopic composition was added to aliquots taken from the prepared solutions of labelled iron fortificants. The iron standard was prepared gravimetrically from an isotopic reference material (IRMM-014, EU Institute of Reference Materials, Geel, Belgium). Isotopic analyses were performed using negative thermal ionisation mass spectrometry (NTI-MS) (Walczyk, 1997). Iron concentrations of each labelled iron fortificant were calculated based on the shift in iron isotopic abundances, the determined isotopic abundances of the pure isotopic labels and the natural iron isotopic abundances (Walczyk et al. 1997).

Iron status measurements
Venous blood samples (7 ml) were drawn in EDTA-treated tubes at each sampling. Samples were analysed for iron status indices (Hb, plasma ferritin) and for the incorporation of $^{57}$Fe and $^{58}$Fe into erythrocytes (days 15/16 and days 30/32). Whole blood samples were aliquoted for analysis of Hb and isotopic composition and plasma was separated, aliquoted and frozen for later analysis of plasma ferritin. Hb was measured by the cyanmethaemoglobin method (Sigma kit, Sigma, St. Louis, MO) and plasma ferritin by ELISA (Ramco Laboratories, Houston, Texas). Commercial quality control materials (DiaMed, Cressier sur Morat, Switzerland and Ramco Laboratories) were analysed together with samples analysed for Hb and plasma ferritin, respectively.

Quantification of iron stable isotopes in blood
Each isotopically enriched blood sample was analysed in duplicate for its iron isotopic composition as previously described by Walczyk et al. (1997). The blood samples were mineralised by microwave digestion using a mixture of nitric acid and hydrogen peroxide. Iron was separated from the matrix by anion-exchange chromatog-
Iron absorption from ferrous fumarate

Isotope analyses were performed by NTI-MS (Walczyk, 1997).

**Calculation of iron absorption**

The amounts of $^{57}$Fe and $^{58}$Fe isotopic labels in blood 14 d after test meal administrations were calculated based on the shift in iron isotope ratios and on the amount of iron circulating in the body. The calculations were based on the principles of isotope dilution and took into account that the iron isotopic labels were not monoisotopic (Walczyk et al. 1997). Circulating iron was calculated based on blood volume and Hb concentration (Kastenmayer et al. 1994). Blood volume calculations were based on height and weight according to Brown et al. (1962). For calculations of fractional iron absorption, 80% incorporation of the absorbed iron into erythrocytes was assumed (Hosein et al. 1967).

**Food analysis**

Cereal and milk powder were analysed for iron and calcium by electrothermal/flame atomic absorption spectroscopy (SpectrAA 400, Varian, Mulgrave, Australia) after mineralisation by microwave digestion (MLS-Ethos plus, Mikrowellen-Labor-Systeme, Leutkirch, Switzerland) in a HNO$_3$/H$_2$O$_2$ mixture, using standard addition technique to minimise matrix effects. Phytic acid in the infant cereal was determined by a modification of the Makower (1970) method in which cerium replaced iron in the precipitation step.

**Statistics**

Fractional iron absorption values are presented as geometric means (±1SD). Student’s paired t-test was used to compare absorption data within each study. Absorption values were logarithmically transformed before statistical analysis (Excel 2002, Microsoft Corporation, Redmond, WA, USA).

**Results**

None of the test subjects were anaemic (haemoglobin concentration <120 g/L). However, 6 women had no iron stores, indicated by low plasma ferritin values (<12 μg/L).

The test meals contained 0.6 mg native iron (1.1 mg iron/100 g infant cereal, 0.15 mg iron/100 g milk powder), 167 mg calcium (148 mg calcium /100 g infant cereal, 1159 mg calcium/100 g milk powder) and 84 mg phytic acid (168 mg phytic acid/100 g in-
Iron absorption from ferrous fumarate

The ascorbic acid content was not measured as it was assumed to be negligible.

The results of the iron absorption studies are shown in Table 1. In study 1, no significant difference between iron absorption from ferrous sulphate and ferrous fumarate fortified infant cereal was found (geometric mean 3.1 vs. 3.0%, respectively, p=0.85). When Na$_2$EDTA was added to the test meals, iron absorption was statistically significantly higher from ferrous sulphate than from ferrous fumarate fortified test meals (geometric mean 5.3 vs. 3.3%, respectively, p<0.01). The 1.7-fold increase in iron absorption from ferrous sulphate fortified cereal on addition of Na$_2$EDTA was however not statistically significant (geometric mean 3.1 without vs. 5.3% with Na$_2$EDTA, p=0.12). In study 2, the addition of ascorbic acid (molar ratio ascorbic acid to iron 4:1) to ferrous fumarate fortified cereal increased iron absorption significantly (geometric mean 6.3 vs. 10.4%, p=0.02).

Table 1: Iron absorption by healthy adult women (10 women per study) from infant cereal fortified with ferrous sulphate or ferrous fumarate (5 mg iron) with and without Na$_2$EDTA† (Study 1) and from ferrous fumarate with and without ascorbic acid‡ (Study 2)

<table>
<thead>
<tr>
<th>Study No.</th>
<th>Plasma ferritin (µg/L)*</th>
<th>Test meal</th>
<th>Iron absorption %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td>16.9 (10.5, 27.1)</td>
<td>A ferrous sulphate</td>
<td>3.1$^{ab}$ (1.3, 7.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B ferrous fumarate</td>
<td>3.0$^{ab}$ (1.6, 5.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C ferrous sulphate plus Na$_2$EDTA</td>
<td>5.3$^a$ (3.2, 8.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D ferrous fumarate plus Na$_2$EDTA</td>
<td>3.3$^b$ (2.2, 5.0)</td>
</tr>
<tr>
<td>Study 2</td>
<td>14.9 (7.7, 29.0)</td>
<td>E ferrous fumarate</td>
<td>6.3$^a$ (3.1, 13.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F ferrous fumarate plus ascorbic acid</td>
<td>10.4$^b$ (4.0, 27.0)</td>
</tr>
</tbody>
</table>

† molar ratio iron/Na$_2$EDTA 1:1; ‡ molar ratio ascorbic acid/iron 4:1; *geometric mean (-SD, +SD)

Discussion

Studies investigating the influence of enhancers and inhibitors on iron absorption from fortified foods have most often evaluated the commonly used water-soluble iron compound ferrous sulphate. Only few studies have been made with poorly water-soluble iron compounds. This is primarily due to the technical difficulties involved in producing experimentally labelled compounds. While the preparation of labelled ferrous sulphate is relatively easy, special care has to be taken when preparing poorly
water-soluble iron compounds, such as ferrous fumarate, so as to ensure that the labelled compound is similar to the commercial iron fortificant. Besides the technical difficulties associated with using a down-scaled production method, the high cost involved in the preparation of such compounds is a limiting factor, especially when stable iron isotopes are used. In our study, the labelled ferrous fumarate was produced in collaboration with a manufacturer of commercial ferrous fumarate and had a similar solubility in dilute acid (Davidsson et al. 2000).

Ascorbic acid and Na$_2$EDTA have repeatedly been shown to enhance iron absorption from ferrous sulphate (Sayers et al. 1973; Bjorn-Rasmussen & Hallberg, 1974; Sayers et al. 1974; Gillooly et al. 1984; el Guindi et al. 1988; MacPhail et al. 1994; Cook et al. 1997; Hurrell et al. 2000; Davidsson et al. 2001). However, due to the results obtained by Hurrell et al. (1991) and Davidsson et al. (2002) it is unclear whether ascorbic acid and Na$_2$EDTA also enhance iron absorption from ferrous fumarate and this study was made for clarification.

The influence of Na$_2$EDTA on iron absorption from ferrous fumarate and ferrous sulphate was compared in study 1. Our results show that healthy Western women absorb iron as well from ferrous fumarate as from ferrous sulphate. Further, the results showed that iron absorption was significantly higher from ferrous sulphate than from ferrous fumarate in the presence of Na$_2$EDTA. This indicates that Na$_2$EDTA does not enhance iron absorption from ferrous fumarate fortified infant cereal and thus confirms previous evidence that Na$_2$EDTA has no effect on iron absorption from poorly water-soluble and water-insoluble iron compounds (Hurrell et al. 2000; Fairweather-Tait et al. 2001; Davidsson et al. 2002).

The lack of effect on iron absorption after addition of Na$_2$EDTA to ferrous fumarate fortified foods is probably related to the solubility of this iron compound. Iron from ferrous sulphate can be expected to enter the common non-heme iron pool rapidly, while ferrous fumarate has to dissolve in the gastric juice before iron is released and enters the iron pool. During this time, Na$_2$EDTA can form complexes with other minerals and trace elements, resulting in a reduced binding capacity of Na$_2$EDTA with the more slowly solubiilised iron compounds. EDTA-iron complexes, which are assumed to be responsible for the enhancing effect of Na$_2$EDTA, would thus not be formed.
The results from study 2 show that ascorbic acid added at a 4:1 molar ratio (relative to iron) enhances iron absorption from ferrous fumarate significantly. Thus, iron does not seem to be competing with other minerals and trace elements or with other food components present in the gastric juice for the reducing and chelating properties of ascorbic acid, which are thought to be responsible for its enhancing effect on iron absorption (Conrad & Schade, 1968).

Until now, the enhancing effect of ascorbic acid on iron absorption from ferrous fumarate had not been demonstrated directly. Results from earlier studies had indicated that ascorbic acid enhanced iron absorption from ferrous fumarate to the same extent as from ferrous sulphate, as there was no significant difference between iron absorption from infant cereals fortified with these compounds in the presence (Hurrell et al. 1989) and in the absence of ascorbic acid (Hurrell et al. 2000). However, another study had reported ascorbic acid to have no significant effect on iron absorption from a chocolate drink powder and a liquid formula meal fortified with ferrous fumarate (Hurrell et al. 1991). These findings can probably partly be explained by the relatively low molar ratio of ascorbic acid to iron (1.7:1) and by the high level of iron absorption inhibitors in the chocolate drink (casein and calcium from milk, phytate from malt and polyphenols from cocoa). This assumption is supported by the observation in the same study that native iron absorption from the unfortified chocolate drink was not increased even though the molar ratio of ascorbic acid to iron was approximately 14:1. Further, although iron absorption from a ferrous fumarate fortified liquid formula meal did not increase significantly on addition of ascorbic acid at a 4.4:1 ascorbic acid to iron molar ratio, a 1.6-fold increase (p=0.14) was observed.

Based on the results from the present study, showing that ascorbic acid enhances iron absorption significantly from ferrous fumarate, it would seem that the lack of statistical evidence in the earlier study was presumably due to the small sample size (8 subjects) and the large variations within the group.

The results of the present studies confirmed that healthy adults absorb iron as well from ferrous fumarate as from ferrous sulphate and thus indicate that, in adults, ferrous fumarate dissolves completely in the gastric juice. Further, the results showed that ascorbic acid enhances iron absorption from ferrous fumarate, while Na₂EDTA has no significant influence on iron absorption from ferrous fumarate. Thus, our results emphasise that not all findings from iron absorption studies made with ferrous
Iron absorption from ferrous fumarate

sulphate can be extrapolated to iron compounds which have different solubility properties.

References


Iron absorption from ferrous fumarate


Chapter 3

**EFFECT OF ASCORBIC ACID AND PARTICLE SIZE ON IRON ABSORPTION FROM FERRIC PYROPHOSPHATE IN ADULT WOMEN**

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Abstract
The effects of added ascorbic acid and particle size on iron absorption from ferric pyrophosphate were evaluated in adult women (9-10 women/study) based on erythrocyte incorporation of iron stable isotopes ($^{57}$Fe or $^{58}$Fe) 14d after administration. Three separate studies were made with test meals of iron fortified infant cereal (5 mg iron/meal) and the results are presented as geometric means and relative bioavailability values (RBV, FeSO$_4$=100%). The results of study 1 showed that iron absorption was significantly lower from ferric pyrophosphate (mean particle size 8.5 µm) than from FeSO$_4$ in meals without ascorbic acid (0.9 vs. 2.6%, p<0.0001, RBV 36%) and in the same meals with ascorbic acid added at a 4:1 molar ratio relative to fortification iron (2.3 vs. 9.7%, p<0.0001, RBV 23%). Ascorbic acid increased iron absorption from ferric pyrophosphate slightly less (2.6-fold) than from FeSO$_4$ (3.7-fold) (p<0.05). In studies 2 and 3, RBV of ferric pyrophosphate with an average particle size of 6.7 µm and 12.5 µm was not significantly different at 52 and 42% (p>0.05), respectively. In conclusion, the addition of ascorbic acid increased fractional iron absorption from ferric pyrophosphate significantly, but to a lesser extent than from FeSO$_4$. Decreasing the mean particle size to 6.7 µm did not significantly increase iron absorption from ferric pyrophosphate.

Keyword: iron absorption, humans, ferric pyrophosphate, ascorbic acid, particle size, food fortification, stable isotopes
Improving ferric pyrophosphate absorption

Introduction

Ferric pyrophosphate, an iron fortificant which is insoluble in water and only poorly soluble in dilute acid, is currently used by European food companies to fortify infant cereals and chocolate drink powders. Its main advantage is that it causes no adverse color and flavor changes to the food vehicle as do readily water-soluble iron fortificants such as ferrous sulfate [1]. However, due to its limited solubility, iron absorption can be expected to be low; studies in adults have reported iron absorption from ferric pyrophosphate to be in the range of 15 to 75% of that from ferrous sulfate, depending on batch and processing conditions [2-4]. In infants, iron absorption from ferric pyrophosphate fortified foods has so far not been compared directly to that from ferrous sulfate. However, iron absorption from ferric pyrophosphate fortified wheat-soy infant cereal has in 6-12 month old infants been shown to range between <0.7 and 2.7% (geometric mean 1.3%) and was further shown to be only one-third as well absorbed as ferrous fumarate [5]. These low iron absorption values were somewhat surprising since ascorbic acid had been added at a 3:1 molar ratio relative to iron. The enhancing effect of ascorbic acid on iron absorption has been demonstrated for ferrous sulfate in several studies [6-9] but very little is known about the effect on water-insoluble iron fortificants. Ascorbic acid has been shown to enhance iron absorption from ferric orthophosphate and elemental iron [10] but the effect on iron absorption from ferric pyrophosphate has so far not been evaluated.

Furthermore, a factor which influences iron absorption from water-insoluble iron fortificants is particle size [11-14] and we have recently reported that iron absorption from micronized, dispersible ferric pyrophosphate (Sunactive Fe™) is not significantly different to that from ferrous sulfate [15]. This fortificant has an average particle size of 0.3 μm, which is over 20-times smaller than regular commercial ferric pyrophosphate (average particle size approximately 8 μm). Decreasing the particle size of hydrogen and carbon monoxide reduced iron powders by about 50-60%, to sizes between 7 and 10 μm, has been shown to increase iron absorption from these fortificants by approximately 50% in rats [13, 14].

The aims of the present studies were to investigate the effect of added ascorbic acid on iron absorption from ferric pyrophosphate and to evaluate the impact of ferric pyrophosphate particle sizes within the range of approximately 7-13 μm on iron absorption. Erythrocyte incorporation of stable isotopes 14 days after administration of la-
Improving ferric pyrophosphate absorption

beled test meals was used to estimate iron absorption in healthy women. An infant cereal was used as the standardized test meal.

Subjects and Methods

Subjects

Twenty-eight apparently healthy adult women (20-30 years; max body weight 60 kg) were recruited from the student and staff population at the ETH Zurich and the University of Zurich. The subjects were randomly allocated into three separate studies (9-10 women/study). Exclusion criteria included pregnancy or lactation and known gastrointestinal or metabolic disorders. No medication (except oral contraceptives) or vitamin/mineral supplements were allowed during the study. Women regularly taking vitamin/mineral supplements discontinued the supplementation 2 weeks before the start of the study.

The study protocol was reviewed and approved by the Ethical Committee at the ETH Zurich, Switzerland. Subjects were informed orally and in writing about the aims and procedures of the study. Written informed consent was obtained from all women.

Study design

Three studies were made (Table 1). In study 1, each subject (10 women) received four labeled test meals and iron absorption from ferric pyrophosphate (average particle size 8.5 μm) and ferrous sulfate fortified meals with and without added ascorbic acid was compared. In studies 2 and 3, each subject (9 women/study) received two meals only and iron absorption from fine (average particle size 6.7 μm) and coarse (average particle size 12.5 μm) ferric pyrophosphate fractions were compared to that from ferrous sulfate, respectively. Iron absorption was based on erythrocyte incorporation of iron stable isotope labels 14 days after intake of labeled test meals. The iron fortificants were labeled with 57Fe or 58Fe and added to the different test meals as described below. All test meals were fed, after an overnight fast, under standardized conditions. A cross-over study design was used with each woman acting as her own control. On the day before intake of the first test meal (day 0), a venous blood sample was drawn after an overnight fast for determination of iron status parameters (hemoglobin (Hb), and plasma ferritin) and body weight and height were measured. Two test meals were fed on the following days (days 1 and 2) between 0700 and 0900 hours. No intake of food or fluids was allowed for 3 hours after test meal intake. A
Improving ferric pyrophosphate absorption

second venous blood sample was drawn 14 days after intake of the second test meal (day 16). In study 1, a second pair of labeled test meals (meals C and D) was fed on days 17 and 18 and a final blood sample was obtained 14 days after administration of the last test meal (day 32).

**Test meals**
The test meals consisted of 50 g roller-dried wheat-based infant cereal (Nestlé PTC, Orbe, Switzerland) fed with reconstituted milk (8 g milk powder Sano Lait, Coop Schweiz, Basel, Switzerland, and 75 mL deionized water). The infant cereal was made from 79.7% partially hydrolyzed wheat flour, 10% sucrose, 4% honey, 3% palm oil, 0.3% calcium carbonate and 3% water. Except for calcium, no minerals or vitamins were added to the infant cereal. Each test meal contained 5 mg added iron, 4 mg iron as $^{58}\text{FeSO}_4$ plus 1 mg iron as FeSO$_4$ of natural isotopic composition or 5 mg iron as $^{57}\text{Fe}$ ferric pyrophosphate. In study 1, the labeled ferric pyrophosphate used was comparable to commercial ferric pyrophosphate. Fine and coarse particle size fractions of ferric pyrophosphate, as described below, were used in study 2 and 3, respectively. To test meals C and D in study 1, food grade ascorbic acid (Merck, Darmstadt, Germany) was added at the time of serving as an aqueous solution at a molar ratio of ascorbic acid to fortification iron of 4:1 (63 mg ascorbic acid). Aqueous ascorbic acid solutions were prepared freshly each morning. Deionized water (200 g) was served as a drink.

**Stable isotope labels**
$^{58}\text{Fe}$Ferrous sulfate was prepared from isotopically enriched elemental iron (Chemgas, Boulogne, France) by dissolution in sulfuric acid and dilution to appropriate concentration. $^{57}\text{Fe}$Ferric pyrophosphate (hereafter referred to as un-separated ferric pyrophosphate) was prepared in collaboration with Dr. Paul Lohmann Ltd., Emmerthal, Germany. It has been used in previous human studies [5]. Part of the un-separated labeled ferric pyrophosphate was used to prepare the fine and coarse particle size fractions. The coarse fraction was collected by trapping large particles by means of filtration. For this, un-separated labeled ferric pyrophosphate was suspended in deionized water and filtered through a polyamide monofilament screening fabric (pore size 11 μm, Sefar Nitex 03-11/6, Sefar Inc., Rueschlikon, Switzerland). The filter cake representing the coarse particle size fraction was washed thoroughly with deionized water and dried on the filter at room temperature. The fine fraction
was subsequently separated from the filtrate by sedimentation. Deionized water was added to the filtrate to a total volume of 2L and this suspension was filled into a tall beaker (24 cm) and left for 20h at room temperature. After this time period, the particles which remained in suspension were recovered by filtering the suspension through a 0.45 μm filter (Alltech Associates, Inc., Deerfield, IL, USA). The resulting filter cake (fine particle size fraction) was dried on the filter at room temperature. Particle size distribution of the un-separated, fine and coarse ferric pyrophosphate fractions was measured by laser light diffraction (Mastersizer X, Malvern Instruments Ltd., Malvern, UK). Average particle size, calculated as surface area moment mean (Sauter mean diameter), of the un-separated, fine and coarse ferric pyrophosphate fractions was 8.5 μm, 6.7 μm and 12.5 μm, respectively (Figure 1). The Sauter mean diameter, which is based on surface area as well as volume, was considered to be the most appropriate parameter to describe average particle size [16, 17].

Figure 1: Particle size distribution shown as cumulative volume percentage frequency curve of fine (--), un-separated (-o-), and coarse (-△-) ferric pyrophosphate fractions measured by laser diffraction (Mastersizer X, Malvern Instruments Ltd., Malvern, UK).

Quantification of iron isotopes in labeled iron fortificants
Isotope dilution mass spectrometry was used to determine the concentration of $^{57}$Fe and $^{58}$Fe stable isotopes in the ferric pyrophosphate and ferrous sulfate solutions. For analysis, $[^{57}$Fe]ferric pyrophosphate was dissolved in concentrated nitric acid. An accurately measured amount of iron of natural isotopic composition was added to ali-
Improving ferric pyrophosphate absorption

Quotes taken from the prepared solutions of labeled iron fortificants. The iron standard was prepared gravimetrically from an isotopic reference material (IRMM-014, EU Institute of Reference Materials, Geel, Belgium). Isotopic analyses were performed using negative thermal ionization mass spectrometry (NTI-MS). Iron concentrations of each labeled iron fortificant were calculated based on the shift in iron isotopic abundances, the determined isotopic abundances of the isotopic labels, and the natural iron isotopic abundances [18, 19].

Iron status measurements
Venous blood samples (7 ml) were drawn in EDTA-treated tubes at each sampling. Samples were analyzed for iron status indices (Hb, plasma ferritin) and for incorporation of $^{57}$Fe and $^{58}$Fe into erythrocytes (day 16 and day 32). Whole blood samples were aliquoted for analysis of Hb and isotopic composition and plasma was separated, aliquoted and frozen for later analysis of plasma ferritin. Hb was measured by the cyanmethemoglobin method (Sigma kit, Sigma, St. Louis, MO) and plasma ferritin by ELISA (Ramco Laboratories, Houston, Texas). Commercial quality control materials (DiaMed, Cressier sur Morat, Switzerland and Ramco Laboratories) were analyzed together with samples analyzed for Hb and plasma ferritin, respectively.

Quantification of iron isotope in blood
Each isotopically enriched blood sample was analyzed in duplicate for its iron isotopic composition as previously described by Walczyk et al. [19]. The blood samples were mineralized by microwave digestion using a mixture of nitric acid and hydrogen peroxide. Iron was separated from the matrix by anion-exchange chromatography and a solvent-solvent extraction step into diethyl ether. Isotopic analyses were performed by NTI-MS [18].

Calculation of iron absorption
The amounts of $^{57}$Fe and $^{58}$Fe isotopic labels in blood 14 days after test meal administrations were calculated based on the shift in iron isotope ratios and on the amount of iron circulating in the body. The calculations were based on the principles of isotope dilution and took into account that the iron isotopic labels were not monoisotopic [19]. Circulating iron was calculated based on blood volume and Hb concentration [20]. Blood volume calculations were based on height and weight according to Brown
et al. [21]. For calculations of fractional iron absorption, 80% incorporation of the absorbed iron into erythrocytes was assumed [22].

**Food analysis**
Cereal and milk powder were analyzed for iron and calcium by electrothermal/flame atomic absorption spectroscopy (SpectrAA 400, Varian, Mulgrave, Australia) after mineralization by microwave digestion (MLS 1200) in a HNO$_3$/H$_2$O$_2$ mixture and using standard addition technique to minimize matrix effects. Phytic acid in the infant cereal was determined by a modification of the Makower method [23] in which cerium replaced iron in the precipitation step.

**Statistics**
Fractional iron absorption values are presented as geometric means (–1SD, +1SD). Student’s paired t-test was used to compare absorption data within each study, Student’s unpaired t-test was used to compare iron absorption ratios between studies. Absorption values were logarithmically transformed before statistical analysis (Excel 2002, Microsoft Corporation, Redmond, WA, USA).

**Results**
None of the women participating in the study were anemic (hemoglobin concentration <120 g/L). However, 8 women had no iron stores as indicated by low plasma ferritin values (<12 μg/L).

The test meals contained 0.6 mg native iron (1.1 mg iron/100 g infant cereal, 0.15 mg iron/100 g milk powder), 167 mg calcium (148 mg calcium/100 g infant cereal, 1159 mg calcium/100 g milk powder) and 84 mg phytic acid (168 mg phytic acid/100 g infant cereal). The ascorbic acid content was not measured as it was assumed to be negligible.

The results of the iron absorption studies are presented in Table 1 as geometric mean iron absorption and as relative bioavailability values (RBV) (RBV of ferrous sulfate = 100%). In study 1, the expected low bioavailability of ferric pyrophosphate was confirmed by the direct comparison of iron absorption from ferric pyrophosphate (un-separated) and ferrous sulfate fortified infant cereal (geometric mean 0.9 vs. 2.6%, respectively, p<0.0001, RBV 36%). When ascorbic acid was added to the test meals, iron absorption from ferric pyrophosphate (un-separated) and ferrous sulfate in-
Improving ferric pyrophosphate absorption

creased 2.6-fold and 3.7-fold, respectively (geometric mean 2.3 vs. 0.9%, p=0.01 and 9.7 vs. 2.6%, p<0.001, respectively, RBV 23%). RBV from fine (study 2) and coarse (study 3) fractions of ferric pyrophosphate was 52 and 42%, respectively (1.2% (fine) vs. 2.3% (ferrous sulfate), p=0.004 and 2.0% (coarse) vs. 4.9% (ferrous sulfate), p<0.001). Decreasing mean particle size from 8.5 to 6.7 µm did not affect iron absorption significantly (RBV 36 vs. 52%, p=0.09). Further, increasing the particle size from 8.5 to 12.5 µm had no significant effect on RBV (36 vs. 42%, p=0.47).

Table 1: Iron absorption by healthy adult women from an infant cereal fortified with ferrous sulfate or un-separated ferric pyrophosphate* (5 mg iron) with and without ascorbic acid* (study 1) and with ferric pyrophosphate of fine* (study 2) and coarse* particle size (study 3)

<table>
<thead>
<tr>
<th>Study no</th>
<th>n</th>
<th>Plasma ferritin* (μg/L)</th>
<th>Test meal</th>
<th>Iron absorption, %*</th>
<th>Relative bioavailability to ferrous sulfate, %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>26.6 (17.1, 41.4)</td>
<td>A ferrous sulfate</td>
<td>2.6a (1.3, 5.2)</td>
<td>36 (24, 55)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B ferric pyrophosphate (un-separated)</td>
<td>0.9b (0.4, 2.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C ferrous sulfate + ascorbic acid†</td>
<td>9.7c (5.1, 18.5)</td>
<td>23 (16, 35)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D ferric pyrophosphate (un-separated) + ascorbic acid†</td>
<td>2.3a (1.0, 4.9)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>17.8 (8.5, 37.8)</td>
<td>E ferrous sulfate</td>
<td>2.3a (1.2, 4.3)</td>
<td>52 (32, 85)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F fine particle size ferric pyrophosphate</td>
<td>1.2b (0.7, 2.0)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>13.4 (7.4, 24.4)</td>
<td>G ferrous sulfate</td>
<td>4.9a (2.5, 9.5)</td>
<td>42 (26, 66)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H coarse particle size ferric pyrophosphate</td>
<td>2.0b (0.8, 5.3)</td>
<td></td>
</tr>
</tbody>
</table>

average particle sizes: 8.5 µm, 6.7 µm and 12.5 µm; † molar ratio ascorbic acid/iron of 4:1; n: number of test subjects; *geometric mean (-SD, +SD); a, b, c, d: within separate studies mean values not sharing a common superscript letter were significantly different (p < 0.05)

Discussion

Ferric pyrophosphate is currently used by European food companies to fortify infant cereals and chocolate drink powders as this fortificant does not cause unacceptable changes to the color and flavor [24]. However, until now only few human iron absorption studies have been made with this fortificant. This is primarily due to the technical difficulties involved in producing labeled iron fortificants. While the preparation of labeled ferrous sulfate is relatively easy, special care has to be taken when preparing labeled water-insoluble iron fortificants, such as ferric pyrophosphate, so as to ensure that the labeled fortificant is similar to the commercial iron fortificant. Besides the technical difficulties associated with using a down-scaled production method, the high cost involved in the preparation of such fortificants is a limiting factor, especially when stable iron isotopes are used. In our study, the labeled ferric pyrophosphate
Improving ferric pyrophosphate absorption

(un-separated) was produced in collaboration with a manufacturer of commercial ferric pyrophosphate and had a similar solubility to commercial ferric pyrophosphate in dilute acid [5].

The importance of evaluating iron absorption from forticants besides ferrous sulfate has been emphasized by the results of recent studies showing that iron absorption enhancing effects demonstrated with ferrous sulfate can not necessarily be extrapolated to water-insoluble iron forticants. For example, although the enhancing effect of Na$_2$EDTA has repeatedly been demonstrated on iron absorption from ferrous sulfate in humans [4, 25, 26] the addition of Na$_2$EDTA to a ferric pyrophosphate fortified infant cereal had no effect on iron absorption [4]. Until now, the effect of ascorbic acid on iron absorption from ferric pyrophosphate had not been evaluated.

The results from the present study show that ascorbic acid added to infant cereal at a 4:1 molar ratio relative to fortification iron enhances iron absorption 2.6-fold from ferric pyrophosphate (un-separated). These results therefore support previous evidence that ascorbic acid has an enhancing effect on iron absorption from water-insoluble iron forticants such as ferric orthophosphate and electrolytic iron [10]. However, the magnitude of the enhancing effect of ascorbic acid on iron absorption was significantly higher from test meals fortified with ferrous sulfate (3.7-fold) than with ferric pyrophosphate (un-separated) (2.6-fold). This resulted in a significantly lower RBV for ferric pyrophosphate (un-separated) in the presence of ascorbic acid as compared to the absence of ascorbic acid (23 vs. 36%, respectively, p<0.05). Forbes et al. [10] similarly reported a greater enhancing effect of ascorbic acid (5:1 molar ratio relative to iron) on iron absorption from ferrous sulfate than from electrolytic iron (3-fold vs. 2-fold increase in iron absorption, P<0.007). Based on these studies, it would seem that more ascorbic acid needs to be added to foods fortified with water-insoluble iron forticants than water-soluble iron forticants to get an equivalent increase in iron absorption.

The influence of ferric pyrophosphate particle size on iron absorption was also evaluated in the present study. For studies 2 and 3, the same labeled ferric pyrophosphate as used in study 1 was separated into two fractions - fine and coarse with a 2-fold difference in particle size (6.7 µm vs. 12.5 µm). By using the same batch of labeled ferric pyrophosphate in all 3 studies, the influence of other potentially important factors
Improving ferric pyrophosphate absorption

such as particle density or porosity as well as heat treatment [3, 27, 28] was mini-
mized.

Our results show that RBV of ferric pyrophosphate is not affected by mean particle
size within the range of 6.7 to 12.5 μm. Thus, small differences in the particle size of
commercial ferric pyrophosphate will not influence iron absorption significantly from
this fortificant. In an earlier study, RBV of micronized, dispersible ferric pyrophos-
phate (average particle size of 0.3 μm) added to the same infant cereal as used in
the present study was 83%, which was not significantly different from ferrous sulfate
[15]. At the present time, no conclusion can be drawn as to what extent mean particle
size should be decreased from its current approximately 8 μm to achieve a significant
increase in iron absorption from ferric pyrophosphate. With conventional grinding
procedures, however, it is difficult to decrease mean particle size below 2-3 μm. Par-
ticle size below 1 μm can be produced with special technology which includes gener-
ating ferric pyrophosphate particles in aqueous solutions and adding emulsifier to
prevent agglomeration [29]. However, this procedure markedly increases the cost of
the iron fortificant.

By evaluating the results of the present study together with those of Cook et al. [12]
the influence of particle size on iron absorption from H-reduced elemental iron pow-
der and from ferric pyrophosphate can be compared. In this earlier human study [12],
iron absorption from H-reduced elemental iron powder, with particle sizes between 5-
10 μm, was found to be equivalent to ferrous sulfate. In the present study iron from
ferric pyrophosphate with average particle sizes of 8.5 and 6.7 μm was only 40-50%
as available as from ferrous sulfate. Therefore, it is clearly not possible to extrapolate
effects of particle size from one water-insoluble iron fortificant to another.

However, particle size is presumably not the only physico-chemical property which
influences iron absorption from ferric pyrophosphate. In earlier studies, sterilization of
ferric pyrophosphate fortified infant formula was shown to increase RBV of ferric py-
rophosphate in rats [27, 28]. On the contrary, Hurrell et al. [3] reported that RBV of
ferric pyrophosphate decreased from 75 to 21% in adult human subjects as a result
of heat treatment (vacuum drying of a chocolate drink powder at 100 °C). Further-
more, when the same labeled ferric pyrophosphate was evaluated several years later
in an infant cereal, to which it was added after processing, RBV was only 15% [4].
Improving ferric pyrophosphate absorption

Although a direct comparison of these studies is not possible due to the difference in test meal composition, it is noteworthy that RBV of the same labeled batch of ferric pyrophosphate has ranged from 15% (infant cereal) to 75% (chocolate drink powder). This indicates that heat treatment and perhaps also storage duration and/or conditions influence absorption of ferric pyrophosphate.

In conclusion, the present studies showed that iron absorption from ferric pyrophosphate can be increased significantly by adding ascorbic acid. Food manufacturers should therefore ensure that adequate amounts of ascorbic acid are added to ferric pyrophosphate fortified foods. Decreasing the particle size of ferric pyrophosphate by 25% did not increase iron absorption significantly. However, the results of the present study and previous studies indicate that iron absorption from ferric pyrophosphate is dependent on particle size. Further studies are needed to investigate to what extent the particle size must be decreased to significantly increase iron absorption from ferric pyrophosphate and whether other factors such as density or surface area influence dissolution in the gastric juice and thus absorption.

References

Improving ferric pyrophosphate absorption


Improving ferric pyrophosphate absorption


Chapter 4

A MICRONISED, DISPERSIBLE FERRIC PYROPHOSPHATE WITH HIGH RELATIVE BIOAVAILABILITY IN HUMANS

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Abstract

Ferric pyrophosphate is a water-insoluble iron compound used to fortify infant cereals and chocolate drink powders as it causes no organoleptic changes to the food vehicle. However, it is only of low absorption in man. Recently, an innovative ferric pyrophosphate has been developed (Sunactive Fe™) based on small particle size ferric pyrophosphate (average: 0.3 μm) mixed with emulsifiers, so that it remains in suspension in liquid products. The aim of the present studies was to compare iron absorption of micronised, dispersible ferric pyrophosphate (Sunactive Fe™) with that of ferrous sulphate in an infant cereal and a yoghurt drink. Two separate iron absorption studies were made in adult women (10 women/study). Iron absorption was based on the erythrocyte incorporation of stable isotopes (57Fe and 58Fe) 14 d after intake of labelled test meals of infant cereal (study 1) or yoghurt drink (study 2). Each test meal was fortified with 5 mg iron as ferrous sulphate or micronised, dispersible ferric pyrophosphate. Results are presented as geometric means. There was no statistically significant difference between iron absorption from micronised, dispersible ferric pyrophosphate and ferrous sulphate fortified infant cereal (3.4% and 4.1%, respectively, p= 0.24) and yoghurt drink (3.9% and 4.2%, respectively, p=0.72). The results of these studies show that micronised, dispersible ferric pyrophosphate is as well absorbed as ferrous sulphate in adults. The high relative iron bioavailability of micronised, dispersible ferric pyrophosphate indicates the potential usefulness of this compound for food fortification.

Keywords: Iron absorption, Iron fortification, ferric pyrophosphate, Sunactive Fe™
Introduction

Food fortification programs are usually considered the most cost-effective and sustainable approach to combat iron deficiency. However, the success of an iron fortification program depends largely on the careful choice of the iron compound (Hurrell, 1997; Hurrell, 1998). A cheap and highly bioavailable iron compound that causes no organoleptic changes would be the ideal fortification compound. Unfortunately, the water-soluble compounds, which are the most bioavailable, e.g. ferrous sulphate, often cause unacceptable colour or flavour changes in the food vehicle (Hurrell and Cook 1990). Ferric pyrophosphate is a water-insoluble iron compound often used by European food companies to fortify infant cereals and chocolate drink powders. Its main advantage is that it causes no adverse colour and flavour changes to food vehicles. However, it is only poorly soluble in dilute acid, such as the gastric juice, and is thus only of mediocre absorption in man. Human studies have reported absorption values between 15-75% relative to ferrous sulphate, depending on batch and processing (Hurrell et al. 1989; Hurrell et al. 1991; Hurrell et al. 2000). A further disadvantage of ferric pyrophosphate is that it cannot be used to fortify liquid products due to its water insolubility.

Recently, a micronised, dispersible ferric pyrophosphate has been developed for food fortification. This innovative compound (Sunactive Fe™) is produced from ferric chloride and sodium pyrophosphate using a dispersion technique resulting in very small average particle sized ferric pyrophosphate (approximately 0.3 μm). Further, the formation of agglomerates is avoided by adding emulsifiers. This has the additional advantage that the micronised ferric pyrophosphate is dispersible in aqueous solutions and can be used to fortified liquid foods or drinks such as milk. Micronised, dispersible ferric pyrophosphate has been reported to have a similar bioavailability as ferrous sulphate in rat haemoglobin repletion studies (Juneja et al. 2003).

The aim of the present study was to compare iron absorption from micronised, dispersible ferric pyrophosphate (Sunactive Fe™) with ferrous sulphate added to the same foods. Iron absorption was measured in healthy women from a wheat-based infant cereal and a yoghurt drink by using a stable-isotope technique based on the incorporation of iron stable isotopes into erythrocytes 14 days after administration.
Subjects and Methods

Subjects
Twenty apparently healthy adult women (20-30 years; max body weight 60 kg) were recruited from the student and staff population at the ETH Zurich and the University of Zurich. The subjects were randomly allocated into two separate studies (10 subjects/study). Exclusion criteria included pregnancy or lactation and known gastrointestinal or metabolic disorders. No medication (except oral contraceptives) or vitamin/mineral supplements were allowed during the study. Women regularly taking vitamin/mineral supplements discontinued the supplementation 2 weeks before the start of the study.

The study protocol was reviewed and approved by the Ethical Committee at the ETH Zurich, Switzerland. Subjects were informed orally and in writing about the aims and procedures of the study. Written informed consent was obtained from all study subjects.

Study design
Iron absorption was based on erythrocyte incorporation of iron stable isotope labels 14 d after intake of labelled test meals. The iron compounds were labelled with $^{57}$Fe or $^{58}$Fe and added to the different test meals as described below. All test meals were fed, after an overnight fast, on two consecutive days under strictly standardised conditions and close supervision. A cross-over study design was used with each woman acting as her own control. On the day before intake of the first test meal (day 0), a venous blood sample was drawn after an overnight fast for determination of iron status parameters (haemoglobin (Hb), and plasma ferritin) and body weight and height were measured. The two test meals were fed on the following days (days 1 and 2) between 0700 and 0900 hours. No intake of food or fluids was allowed for 3 hours after test meal intake. A second venous blood sample was drawn 14 d after intake of the second test meal (day 16).

Test meals
The test meals in study 1 consisted of 50 g roller-dried wheat-based infant cereal (Nestlé PTC, Orbe, Switzerland) fed with reconstituted milk (8 g milk powder Sano Lait, Coop Schweiz, Basel, Switzerland, and 75 mL deionised water). The infant cereal was made from 79.7% partially hydrolysed wheat flour, 10% sucrose, 4% honey,
3% palm oil, 0.3% calcium carbonate and 3% water. Except for calcium, no minerals or vitamins were added. The test meals in study 2 consisted of a yoghurt drink made from 170 g unskimmed yoghurt (Joghurt Nature 3.5% fat, Migros Bio, Zurich, Switzerland) and 100 g unskimmed milk (Valflora 3.8% fat, Migros, Zurich, Switzerland). Each test meal contained 5 mg of added iron, 4 mg iron as $^{56}$FeSO$_4$ plus 1 mg iron as FeSO$_4$ of natural isotopic composition or 5 mg iron as micronised, dispersible $^{57}$Fe ferric pyrophosphate. Deionised water (200 g) was served as a drink in study 1.

**Stable isotope labels**

[$^{56}$Fe]Ferrous sulphate was prepared from isotopically enriched elemental iron (Chemgas, Boulogne, France) by dissolution in sulphuric acid and dilution to appropriate concentration. Micronised, dispersible [$^{57}$Fe]ferric pyrophosphate was prepared from isotopically enriched elemental iron (Chemgas, Boulogne, France) by firstly dissolving the elemental iron in concentrated hydrochloric acid. Formed $^{57}$FeCl$_2$ was oxidised to $^{57}$FeCl$_3$ by addition of hydrogen peroxide (30%). To remove impurities (iron oxides), the resulting soluble $^{57}$FeCl$_3$ was extracted into diethyl ether, followed by re-extraction into deionised water. This FeCl$_3$ solution was evaporated under vacuum at 80°C using a rotovap (Rotavapor, Buechi, Flawil, Switzerland). Thereafter, the resulting dark red paste was crystallised to bright yellow $^{57}$FeCl$_3$.6H$_2$O. From this base compound, micronised, dispersible $^{57}$Fe ferric pyrophosphate was produced by Taiyo Kagaku (Yokkaichi, Japan) by mixing $^{57}$FeCl$_3$.6H$_2$O, emulsifiers (enzymatically hydrolysed soy lecithin and polyglycerol fatty acid ester) and sodium pyrophosphate (Nanbu et al. 1998). Particle size was measured using a submicron particle sizer (NiComp 370, Particle Sizing Systems, Santa Barbara, CA, USA) and the labelled compound was found to be equivalent to commercial Sunactive Fe™ with respect to particle size distribution (average particle size 0.24 µm, Figure 1) and visual appearance. As a comparison, particle size distribution of a commercial food grade ferric pyrophosphate (Dr. Paul Lohmann Ltd., Emmerthal, Germany) was measured by laser light diffraction (Mastersizer X, Malvern Instruments Ltd., Malvern, UK; Figure 1).

**Quantification of iron isotopes in labelled iron fortificants**

Isotope dilution mass spectrometry was used to precisely determine the concentration of $^{57}$Fe and $^{56}$Fe stable isotopes in the micronised, dispersible ferric pyrophosphate and ferrous sulphate solutions. An accurately measured amount of iron of natural isotopic composition was added to aliquots taken from the prepared solutions.
Fe absorption from Sunactive Fe™

of labelled iron fortificants. The iron standard was prepared gravimetrically from an isotopic reference material (IRMM-014, EU Institute of Reference Materials, Geel, Belgium). Isotopic analysis was performed using negative thermal ionisation mass spectrometry (NTI-MS) (Walczyk, 1997). Iron concentrations in each labelled iron fortificant solution were calculated based on the shift in iron isotopic abundances, the determined isotopic abundances of the pure isotopic labels and the natural iron isotopic abundances (Walczyk et al. 1997).

Iron status measurements
Venous blood samples (7 ml) were drawn in EDTA-treated tubes at each sampling. Samples were analysed for iron status indices (Hb, plasma ferritin) and for incorporation of $^{57}$Fe and $^{58}$Fe into erythrocytes (day 16). Whole blood samples were aliquoted for analysis of Hb and isotopic composition and plasma was separated, aliquoted and frozen for later analysis of plasma ferritin. Hb was measured by the cyanmethaemoglobin method (Sigma kit, Sigma, St. Louis, MO) and plasma ferritin by ELISA (Ramco Laboratories, Houston, Texas). Commercial quality control materials (DiaMed, Cressier sur Morat, Switzerland and Ramco Laboratories) were analysed together with samples analysed for Hb and plasma ferritin, respectively.

Quantification of iron isotope in blood
Each isotopically enriched blood sample was analysed in duplicate for its iron isotopic composition as previously described by Walczyk et al. (1997). The blood samples were mineralised by microwave digestion using a mixture of nitric acid and hydrogen peroxide. Iron was separated from the matrix by anion-exchange chromatography and a solvent-solvent extraction step into diethyl ether. Isotopic analyses were performed by NTI-MS (Walczyk, 1997).

Calculation of iron absorption
The amounts of $^{57}$Fe and $^{58}$Fe isotopic labels in blood 14 d after test meal administrations were calculated based on the shift in iron isotope ratios and on the amount of iron circulating in the body. The calculations were based on the principles of isotope dilution and took into account that the iron isotopic labels were not monoisotopic (Walczyk et al. 1997). Circulating iron was calculated based on blood volume and Hb concentration (Kastenmayer et al. 1994). Blood volume calculations were based on height and weight according to Brown et al. (1962). For calculations of fractional iron
Fe absorption from Sunactive Fe™

absorption, 80% incorporation of the absorbed iron into erythrocytes was assumed (Hosein et al. 1967).

Food analysis
All test meal components (infant cereal and milk powder, milk and yoghurt) were analysed for iron and calcium by electrothermal/flame atomic absorption spectroscopy (SpectrAA 400, Varian, Mulgrave, Australia) after mineralisation by microwave digestion (MLS-Ethos plus, Mikrowellen-Labor-Systeme, Leutkirch, Switzerland) in a HNO₃/H₂O₂ mixture, using standard addition technique to minimise matrix effects. Phytic acid in the infant cereal was determined by a modification of the Makower method (1970) in which cerium replaced iron in the precipitation step.

Statistics
Fractional iron absorption values are presented as geometric means (−1SD, +1SD). Student’s paired t-test was used to compare absorption data within each study. Absorption values were logarithmically transformed before statistical analysis (Excel 2002, Microsoft Corporation, Redmond, WA, USA).

Results
None of the subjects were found to be anaemic (haemoglobin <120 g/L). However, 9 women had no iron stores indicated by low plasma ferritin values (<12 µg/L).

The test meals in study 1 (infant cereal) contained 0.6 mg iron (1.1 mg iron/100 infant cereal, 0.15 mg iron/100 g milk powder), 167 mg calcium (148 mg calcium/100 g infant cereal, 1159 mg calcium/100 g milk powder) and 84 mg phytic acid (168 mg phytic acid/100 g infant cereal). The yoghurt drink served in study 2 contained 0.06 mg iron (23 µg iron/100 g unskimmed milk, 22 µg iron/100 g unskimmed yoghurt), and 340 mg calcium (109 mg calcium/100 g unskimmed milk, 137 mg calcium/100 g unskimmed yoghurt). The ascorbic acid content was not measured as it was assumed to be negligible in both test meals.

Iron absorption from micronised, dispersible ferric pyrophosphate and ferrous sulphate fortified infant cereal and yoghurt drink is shown in Tables 1 and 2. There was no statistically significant difference between iron absorption from micronised, dispersible ferric pyrophosphate and ferrous sulphate fortified infant cereal (geometric mean 3.4% and 4.1%, respectively, p = 0.24) or from micronised, dispersible ferric
pyrophosphate and ferrous sulphate fortified yoghurt drink (geometric mean 3.9% and 4.2%, respectively, p=0.72).

Table 1: Iron absorption by healthy adult women from infant cereal (Study 1) fortified with ferrous sulphate or micronised, dispersible ferric pyrophosphate (Sunactive Fe™) (5 mg iron/meal)

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Plasma ferritin (µg/L)</th>
<th>Haemoglobin (g/L)</th>
<th>Iron absorption (%)</th>
<th>Relative bioavailability to ferrous sulphate, %</th>
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<tr>
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<td>Sunactive Fe™</td>
<td>Ferrous sulphate</td>
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<td>1</td>
<td>6.9</td>
<td>129</td>
<td>4.5</td>
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<td>11.0</td>
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<td>Geometric Mean</td>
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<td>+SD</td>
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<td>5.6</td>
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</table>

Table 2: Iron absorption by healthy adult women from yoghurt drink (Study 2) fortified with ferrous sulphate or micronised, dispersible ferric pyrophosphate (Sunactive Fe™) (5 mg iron/meal)

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Plasma ferritin (µg/L)</th>
<th>Haemoglobin (g/L)</th>
<th>Iron absorption (%)</th>
<th>Relative bioavailability to ferrous sulphate, %</th>
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<td></td>
<td>Sunactive Fe™</td>
<td>Ferrous sulphate</td>
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<td>+SD</td>
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Discussion
When measuring iron absorption from iron fortification compounds using stable or radioisotope techniques it is extremely important that the physical and chemical properties of the labelled compounds are comparable with those of their commercial counterpart. In the case of ferrous sulphate, it is relatively easy to prepare a labelled com-
Fe absorption from Sunactive Fe™

Compound with physical and chemical properties similar to commercially available ferrous sulphate. The production of labelled micronised, dispersible ferric pyrophosphate was however more complex. This was mainly due to the necessity to synthesise ferric trichloride in the hexahydrate form from isotopically enriched metal, free of acid residues and iron oxides. The labelled micronised, dispersible ferric pyrophosphate was made using a down-scaled manufacture procedure similar to the commercial procedure and the resulting compound was found to be comparable to the commercial compound (Figure 1).

**Figure 1:** Particle size distribution shown as relative volume percentage frequency curve of $^{57}$Fe labeled micronised, dispersible ferric pyrophosphate ($^{57}$Fe Sunactive Fe™) (- o -). For comparison, particle size distribution of commercial micronised, dispersible ferric pyrophosphate (Sunactive Fe™) (- ■ -) and commercial ferric pyrophosphate (Dr. Paul Lohmann Ltd., Emmerthal, Germany) (- ▲ -) are included in the figure. Particle size distribution was measured by laser diffraction (Ni-Comp 370, Particle Sizing Systems, Santa Barbara, CA, USA and Mastersizer X, Malvern Instruments Ltd., Malvern, UK).

The results of the present studies show that micronised, dispersible ferric pyrophosphate is as well absorbed as ferrous sulphate from a wheat-based infant cereal as well as from a yoghurt drink. Although not statistically different, absorption from micronised, dispersible ferric pyrophosphate relative to ferrous sulphate was lower from the infant cereal than from the yoghurt drink (Table 1). This relatively small difference in absorption from infant cereal and yoghurt drink could be related to differences in
Fe absorption from Sunactive Fe

dissolution of micronised, dispersible ferric pyrophosphate in the gastric juice as well as gastric emptying rate which both depend on meal composition (Hallberg et al. 1986).

In previous studies with adult subjects, regular ferric pyrophosphate has been reported to have a relative bioavailability (RBV) compared to ferrous sulphate (RBV = 100%) varying from 15-75%. In infant cereals, the values reported were between 15-39% (Hurrell et al. 1989; Hurrell et al. 1991; Hurrell et al. 2000). The reason for the high relative bioavailability of iron from micronised, dispersible ferric pyrophosphate is probably related to the extremely small particle size of the iron compound which is approximately 20-times smaller than regular ferric pyrophosphate (average particle size = 7.5 μm, Figure 1). In rat studies, decreasing the particle size of water-insoluble iron compounds has previously been shown to have a positive influence on iron absorption. Shah and Belonje (1973) for example showed that RBV of electrolytic iron powder increased from 12 to 32% when 99% of the particles were below 10 μm as compared to 62%. Further, Motzok et al. (1975) demonstrated that decreasing particle size of carbon monoxide reduced iron powders from 24-40 μm to 7-10 μm increased RBV from 11 to 31%. Iron absorption from ferric orthophosphate has also been shown to be dependent on particle size as RBV increased nearly 8-fold (from 6 to 46%) when particle size was decreased from approximately 15 μm to below 1 μm (Harrison et al. 1976). In humans, Björn-Rasmussen et al. (1977) reported that iron absorption from hydrogen reduced elemental iron powders was dependent on their solubility in dilute acid, which in turn was partly dependent on particle size and active surface area. In the present study, it was not technically feasible to produce labelled ferric pyrophosphate with the same particle size distribution as Sunactive Fe™ without the addition of emulsifiers. Therefore, we were not able to evaluate if the high RBV of micronised, dispersible ferric pyrophosphate was only due to the small particle size or whether the emulsifiers influenced iron absorption significantly.

Based on the results from the present studies, micronised, dispersible ferric pyrophosphate could be a very useful iron fortificant, especially since it can be expected to cause less organoleptic problems than water soluble iron compounds. Extensive organoleptic studies however still remain to be made. Presently, Sunactive Fe™ is being used in Japan to fortify milk and milk products. Milk products have previously been shown to be difficult to fortify with readily absorbable iron due to organoleptic
Fe absorption from Sunactive Fe™

problems (Demott, 1971; Edmondson et al. 1971; Kurtz et al. 1973; Wang & King, 1973). Iron fortificants which have been shown to be suitable for milk fortification are ferric ammonium citrate and ferrous bisglycinate (Edmondson et al. 1971; Wang & King, 1973; Olivares et al. 1997). While ferrous bisglycinate would be expected to be at least as well absorbed as ferrous sulphate (Fox et al. 1998) if not better (Bovell-Benjamin et al. 2000; Layrisse et al. 2000), ferric ammonium citrate has been reported to be less well absorbed than ferrous sulphate (Grebe et al. 1975; Layrisse et al. 1976; Gonzalez et al. 2001).

In addition to milk products, micronised, dispersible ferric pyrophosphate is potentially a suitable iron fortificant for vehicles which are difficult to fortify with readily available iron such as chocolate drink powders, cereal products, iodised salt, and bouillon cubes. Further, the overall acceptability of simulated rice grains (Kapanidis & Lee, 1996) may be improved by using micronised, dispersible ferric pyrophosphate instead of ferrous sulphate as less discoloration of fortified rice grains can be expected.

In conclusion, the results of the present studies show that iron absorption from micronised, dispersible ferric pyrophosphate (Sunactive Fe™) is similar to that of ferrous sulphate from a fortified infant cereal as well as from a fortified yoghurt drink. The high relative bioavailability is presumable due to the very small particle size. Micronised, dispersible ferric pyrophosphate can be expected to provoke fewer unacceptable sensory changes than water-soluble iron compound in different food vehicles; however, comprehensive sensory studies are now needed to fully evaluate the usefulness of this compound.

References


Fe absorption from Sunactive Fe


Chapter 5

ERYTHORBIC ACID IS A POTENT ENHANCER OF NON-HEME IRON ABSORPTION

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Abstract

**Background:** Erythorbic acid, a stereoisomer of ascorbic acid with similar physico-chemical properties, is widely used as an antioxidant in processed foods.

**Objectives:** The aims of the present study were to evaluate the effect of erythorbic acid on iron absorption from FeSO₄ at molar ratios 2 and 4 (relative to iron) and to compare the effect of erythorbic acid directly with that of ascorbic acid at molar ratio 4.

**Design:** Iron absorption from iron-fortified cereal was measured in ten adult women based on erythrocyte incorporation of iron stable isotopes (⁵⁷Fe or ⁵⁸Fe) 14 days after administration. Each woman consumed four FeSO₄ fortified test meals (5 mg iron/meal), with or without erythorbic acid or ascorbic acid added. Data were evaluated by paired t-test and results are presented as geometric means.

**Results:** Iron absorption from the test meal without any added enhancer was 4.1%. The addition of erythorbic acid (molar ratios 2 and 4 relative to iron) increased iron absorption 2.6- and 4.6-fold, respectively (10.8%, p< 0.0001 and 18.8%, p<0.0001). The addition of ascorbic acid (molar ratio 4) increased iron absorption 2.9-fold (11.7%, p=0.0004). At a molar ratio of 4, erythorbic acid was 1.6-fold (p=0.0002) more potent as an enhancer of iron absorption than ascorbic acid.

**Conclusion:** Although these results demonstrate that erythorbic acid is a potent enhancer of iron absorption, its lack of antiscorbutic activity limits its usefulness for iron fortification programs. However, it may play a major role in enhancing iron bioavailability from mixed diets which include foods preserved with erythorbic acid.

Keywords: erythorbic acid, ascorbic acid, iron, absorption, humans, stable isotopes
Erythorbic acid enhances iron absorption

Introduction
Ascorbic acid is a potent enhancer of non-heme iron absorption, increasing absorption of native food iron and of iron fortificants which dissolve in the gastric juice and enter the common iron pool (1). The enhancing effect appears to be due to both the reducing power and the chelating action of ascorbic acid (2). Ascorbic acid has been shown to at least partially overcome the negative effects of all major inhibitors of iron absorption, as recently reviewed by Hurrell (1). This vitamin is therefore a very important factor to enhance non-heme iron absorption and information about the intake of this vitamin is important when evaluating iron bioavailability from diets.

Ascorbic acid is commonly added to iron fortified foods to ensure adequate iron absorption but is also frequently used as an antioxidant in industrially-produced foods. Erythorbic acid (synonyms: isoascorbic acid, D-araboascorbic acid) is a stereoisomer of ascorbic acid and differs from ascorbic acid only in the relative position of the hydrogen and hydroxyl groups on the fifth carbon atom in the molecule. Due to its strong reducing properties, erythorbic acid has similar technological applications to ascorbic acid as a water-soluble antioxidant (3) and is widely used as a food additive in processed foods (4). However, the antiscorbutic activity of erythorbic acid is very limited and has been reported to be only one-twentieth of that of ascorbic acid in guinea pigs (5). In humans, neither the antiscorbutic activity of erythorbic acid nor its physiological activity as an antioxidant have been investigated. Erythorbic acid may however have an ascorbic acid sparing effect, as Sauberlich et al. (6) reported that intake of erythorbic acid together with ascorbic acid increased plasma ascorbic acid values to a greater extent than ascorbic acid alone.

As ascorbic acid and erythorbic acid have similar physical and chemical properties (3) it has been speculated that erythorbic acid will also enhance iron absorption. However, only two studies have attempted to evaluate the effect of erythorbic acid on iron absorption. Greger et al. (7) reported no effect of erythorbic acid on iron absorption in healthy men based on results from 5-day balance studies, and Lee et al. (8) reported no effect of erythorbic acid on iron absorption in rats. Although both studies report a lack of effect, it is important to stress that the chemical balance technique is not a sensitive method to evaluate iron absorption and that the rat is not an appropriate animal model to predict human iron absorption as the effect of ascorbic acid on
Erythorbic acid enhances iron absorption

Iron absorption in rats and humans is not comparable (9, 10). Conclusive data on the effect (or lack of effect) are important since it has been estimated that the average US diet may provide substantial amounts of erythorbic acid (up to 200 mg per day) from processed foods (11).

The aims of the present study were to investigate whether erythorbic acid influences iron absorption in a dose dependent manner and to evaluate the effect of erythorbic acid as compared to that of ascorbic acid. Erythrocyte incorporation of stable isotopes 14 days after administration was used to measure iron absorption in health women from a cereal based test meal fortified with ferrous sulfate.

Subjects and Methods

Subjects

Ten apparently healthy adult women (20-26 years; body weight 50-60 kg) were recruited from the student population at the ETH and the University of Zurich. Exclusion criteria included pregnancy or lactation and known gastrointestinal or metabolic disorders. No medication (except oral contraceptives) or vitamin/mineral supplements were allowed during the study. Women regularly taking vitamin/mineral supplements discontinued the supplementation 2 weeks before the start of the study. The study protocol was reviewed and approved by the Ethical Committee at the ETH Zurich, Switzerland. Subjects were informed orally and in writing about the aims and procedures of the study. Written informed consent was obtained from all study subjects.

Study design

Iron absorption was based on erythrocyte incorporation of iron stable isotope labels 14 days after intake of labeled test meals. Ferrous sulfate, labeled with $^{57}$Fe or $^{58}$Fe, was added to the different test meals as described below. All test meals were fed after an overnight fast between 0700 and 0900 hours under standardized conditions. No intake of food or fluids was allowed for 3 hours after test meal intake. A crossover study design was used with each woman acting as her own control. Iron absorption was measured in each woman from four separate test meals. On the day before the first test meal (day 0) a venous blood sample was drawn after an overnight fast for determination of iron status parameters (hemoglobin (Hb) and plasma ferritin) and body weight and height were measured. The first two test meals
Erythorbic acid enhances iron absorption

(meals 1 and 2) were fed on the following days (days 1 and 2). A second venous blood sample was drawn 14 days after intake of the second test meal (day 16). The second pair of labeled test meals (meals 3 and 4) was fed on days 17 and 18 and a final blood sample was drawn on day 32.

**Test meals**
The test meals consisted of 50 g roller-dried wheat-based infant cereal (Nestlé PTC, Orbe, Switzerland) fed with reconstituted milk (8 g milk powder, Sano Lait, Coop Schweiz, Basel, Switzerland and 75 mL deionized water). The infant cereal was made from 79.7% wheat flour, partially hydrolyzed, 10% sucrose, 4% honey, 3% palm oil, 0.3% calcium carbonate and 3% water. Except for calcium, no minerals or vitamins were added. Each test meal contained 5 mg of added iron, 4 mg iron as $^{58}\text{FeSO}_4$ plus 1 mg iron as $\text{FeSO}_4$ of natural isotopic composition or 5 mg iron as $^{57}\text{FeSO}_4$. Deionized water (200 g) was served as a drink. Erythorbic acid (Merck KGaA, Darmstadt, Germany) was added to test meals 2 and 3 at a molar ratio of erythorbic acid to added iron of 2:1 (31.5 mg erythorbic acid, meal 2) and 4:1 (63 mg erythorbic acid, meal 3). Ascorbic acid (Merck KGaA, Darmstadt, Germany) was added to meal 4 at a molar ratio of ascorbic acid to added iron of 4:1 (63 mg ascorbic acid).

**Stable isotope labels**
[$^{57}\text{Fe}$] and [$^{58}\text{Fe}$] ferrous sulfate was prepared from isotopically enriched elemental iron (Chemgas, Boulogne, France) by dissolution in sulfuric acid and dilution to appropriate concentration.

**Erythorbic acid and ascorbic acid dose**
Aqueous solutions of food grade erythorbic acid and ascorbic acid were prepared freshly on the day of administration and added to the test meals at the time of serving.

**Quantification of iron isotopes in labeled iron fortificants**
Isotope dilution mass spectrometry was used to precisely determine the concentration of $^{57}\text{Fe}$ and $^{58}\text{Fe}$ stable isotopes in the ferrous sulfate solutions. An accurately measured amount of iron of natural isotopic composition was added to aliquots taken from the prepared solutions of labeled iron fortificants. The iron standard was pre-
Erythorbic acid enhances iron absorption

pared gravimetrically from an isotopic reference material (IRMM-014, EU Institute of Reference Materials, Geel, Belgium). Isotopic analysis was performed using negative thermal ionization mass spectrometry (NTI-MS) (12). Iron concentrations in each labeled iron fortificant solution were calculated based on the shift in iron isotopic abundances, the determined isotopic abundances of the isotopic labels and the natural iron isotopic abundances (13).

Iron status measurements
Venous blood samples (7 ml) were drawn in EDTA-treated tubes the day before the first labeled test meal was administered, on day 16 and on day 32. Samples were analyzed for iron status indices (Hb, plasma ferritin) and for incorporation of $^{57}$Fe and $^{58}$Fe into red blood cells (day 16 and day 32). Whole blood samples were aliquoted for analysis of Hb and isotopic composition and plasma was separated, aliquoted and frozen for later analysis of ferritin. Hb was measured by the cyanmethemoglobin method (Sigma kit, Sigma, St. Louis, MO) and plasma ferritin by ELISA (Ramco Laboratories, Houston, Texas). Commercial quality control materials (DiaMed, Cressier sur Morat, Switzerland and Ramco Laboratories) were analyzed in parallel.

Quantification of iron isotope in blood
Each isotopically enriched blood sample was analyzed in duplicate for its iron isotopic composition as previously described by Davidsson et al. (14). The blood samples were mineralized by microwave digestion using a mixture of nitric acid and hydrogen peroxide. The iron was separated from the matrix by anion-exchange chromatography and a solvent-solvent extraction step into diethyl ether. The isotopic analyses were performed by NTI-MS (12).

Calculation of iron absorption
The amounts of $^{57}$Fe and $^{58}$Fe isotopic labels in blood 14 days after test meal administrations were calculated based on the shift in iron isotope ratios and on the amount of iron circulating in the body. The calculations were based on the principles of isotope dilution and took into account the iron isotopic labels were not monoisotopic (13). Circulating iron was calculated based on blood volume and Hb concentration (15). Blood volume calculations were based on height and weight according to Brown et al. (16). For calculations of fractional absorption, 80% incorporation of the absorbed iron into red blood cells was assumed (17).
Erythorbic acid enhances iron absorption

Food analysis

Samples of cereal and milk powder were analyzed for iron and calcium by electro-thermal/flame atomic absorption spectroscopy (SpectrAA 400, Varian, Mulgrave, Australia) after mineralization by microwave digestion (MLS-Ethos plus, Mikrowellen-Labor-System, Leutkirch, Switzerland) in a HNO$_3$/H$_2$O$_2$ mixture, using standard addition technique to minimize matrix effects. Phytic acid in the cereal was determined by a modification of the Makover method (18) in which cerium replaced iron in the precipitation step.

Statistics

Fractional iron absorption values are presented as geometric means (−1SD, +1SD). Student’s paired t-test was used to compare absorption data within each study. Absorption values were logarithmically transformed before statistical analysis (Excel 2002, Microsoft Corporation, Redmond, WA, USA).

Results

None of the women had a hemoglobin concentration <120 g/L. However, four women had no iron stores, indicated by low plasma ferritin values (<12 µg/L).

The test meals contained 0.6 mg native iron (1.1 mg iron/100g cereal, 0.15 mg iron/100 g milk powder), 167 mg calcium (148 mg calcium /100g cereal, 1159 mg calcium/100 g milk powder) and 84 mg phytic acid (168 mg phytic acid/100g cereal). The ascorbic acid content of the cereal and milk powder was not measured as it was assumed to be negligible.

Table 1: Influence of erythorbic acid and ascorbic acid on iron absorption from a cereal fortified with ferrous sulfate (5 mg iron) in ten healthy adult women

<table>
<thead>
<tr>
<th>Plasma ferritin* (µg/L)</th>
<th>Test meal</th>
<th>Molar ratio (erythorbic or ascorbic acid to added iron)</th>
<th>Iron absorption, %*</th>
<th>Absorption ratio* (relative to meal A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.0 (6.0, 28.3)</td>
<td>1 no addition</td>
<td></td>
<td>4.1$^a$ (1.7, 9.5)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2 erythorbic acid</td>
<td>2:1</td>
<td>10.8$^b$ (3.9, 30.2)</td>
<td>2.6 (1.8, 4.0)</td>
</tr>
<tr>
<td></td>
<td>3 erythorbic acid</td>
<td>4:1</td>
<td>18.8$^c$ (7.3, 48.7)</td>
<td>4.6 (2.9, 7.3)</td>
</tr>
<tr>
<td></td>
<td>4 ascorbic acid</td>
<td>4:1</td>
<td>11.7$^b$ (3.8, 36.1)</td>
<td>2.9 (1.6, 5.3)</td>
</tr>
</tbody>
</table>

*Geometric mean (−SD, +SD)

$^a, ^b, ^c$ mean values not sharing a common superscript letter were significantly different (p ≤ 0.01)

Geometric mean iron absorption from the test meal fortified with ferrous sulfate was 4.1% (Table 1). The addition of ascorbic acid at a molar ratio of 4 (relative to added iron) increased iron absorption 2.9-fold (geometric mean 11.7%, p=0.0004). The ad-
Erythorbic acid enhances iron absorption

Addition of erythorbic acid (at molar ratios 2 and 4 relative to added iron) increased iron absorption 2.6- and 4.6-fold, respectively (geometric mean 10.8%, p<0.0001, and 18.8%, p<0.0001, respectively). Iron absorption was significantly enhanced by increasing the molar ratio of erythorbic acid from 2 to 4 (p=0.01). Iron absorption from the test meal with added erythorbic acid (molar ratio 4 relative to added iron) was 1.6-fold higher (p=0.0002) than when fortified with ascorbic acid at the same molar ratio.

Discussion

The novel results from the present study clearly show that erythorbic acid is a potent enhancer of iron absorption from ferrous sulfate. The dose-dependent effect observed with erythorbic acid is also consistent with the previously reported dose-dependent effect of ascorbic acid (19, 20). The enhancing effect of erythorbic acid on iron absorption was not an unexpected finding since erythorbic acid and ascorbic acid have similar physico-chemical properties. However, our results indicate that the enhancing effect of erythorbic acid on iron absorption from ferrous sulfate exceeds that of ascorbic acid. A possible explanation for this finding could be related to the different absorption kinetics of the two acids. Earlier studies, in humans and guinea pigs, have shown that absorption of erythorbic acid is slower than that of ascorbic acid (11, 21), potentially resulting in a prolonged presence of erythorbic acid in the duodenum. In addition, as erythorbic acid is reported to oxidize more rapidly than ascorbic acid (22), it may reduce ferric iron to ferrous iron more efficiently, thus increasing the amount of readily absorbable ferrous iron in the duodenum. However, the rapid oxidation unfortunately also means that erythorbic acid is somewhat less stable than ascorbic acid during food processing (23). One of the major problems with the use of ascorbic acid as an enhancer of iron absorption is its susceptibility to losses during food storage and food preparation (24) and, although erythorbic acid has not been evaluated in this context, these problems would not be expected to be less for erythorbic acid.

Nonetheless, erythorbic acid is widely consumed as an additive in processed foods and could be expected to positively influence iron absorption when such foods are included in the diet. It is approved as a food additive in most North American and Latin American countries as well as in most Asian, Oceanian and Central European...
Erythorbic acid enhances iron absorption

countries (25). Its advantage over ascorbic acid is its lower price (personal communication, Roquette Frères, Lestrem, France). In the US, erythorbic acid is widely used as a food additive in a large variety of processed food items, including baked goods, fruit and water ices, meat and meat products, vegetables and vegetable juices, candy, and non-alcoholic beverages (4). It has been estimated that the average diet can provide as much as 200 mg erythorbic acid per day (11) which would be expected to markedly influence dietary non-heme iron absorption. Consequently, erythorbic acid could have long-term effects on iron status. Based on the results from the present study, dietary intake of erythorbic acid should be taken into account when estimating dietary iron bioavailability (26).

The influence of erythorbic acid on iron absorption would be expected to be of less importance in the European Union and in Switzerland as legislation in these countries restricts the use of erythorbic acid to semi-preserved and preserved meat, fish and crustacean products (27, 28). Consequently, in Western European countries, intake of erythorbic acid would be associated with animal tissue and can therefore be expected to have very limited influence on dietary non-heme iron absorption as the promotive effects of ascorbic acid and animal tissue on non-heme iron absorption are not additive when consumed in the same meal (29, 30).

In conclusion, the results from the present study demonstrated that erythorbic acid is a potent enhancer of iron absorption from ferrous sulfate. Intake of erythorbic acid can therefore be an important factor enhancing dietary iron bioavailability in population groups consuming foods preserved with this antioxidant. However, although erythorbic acid is less expensive than ascorbic acid it should not be recommended as an enhancer in iron fortification programs as it lacks antiscorbutic activity.

Acknowledgement
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Erythorbic acid enhances iron absorption

References

Erythorbic acid enhances iron absorption


Chapter 6

IRON ABSORPTION FROM FISH SAUCE AND SOY SAUCE FORTIFIED WITH SODIUM IRON EDTA

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Iron absorption from fish and soy sauce

Abstract

Background: Fish- and soy sauce have been suggested as food vehicles for iron fortification in Asia. NaFeEDTA is a potentially useful fortificant as it can be added to these condiments without causing precipitation during storage.

Objectives: 1) To evaluate iron absorption from NaFeEDTA fortified fish- and soy sauce against a reference fortificant (FeSO₄), 2) to compare iron absorption from NaFeEDTA fortified fish- and soy sauce and 3) to evaluate the influence of fish- and soy sauce per se on iron absorption.

Design: Five separate iron absorption studies were made in adult women (10 women/study). Iron absorption was based on erythrocyte incorporation of iron stable isotopes (⁵⁷Fe or ⁵⁸Fe) 14 days after intake of labeled meals of rice or rice and vegetables. Fish- or soy sauce (10g) fortified with 5mg Fe as NaFeEDTA or FeSO₄ were fed with selected meals. Results are presented as geometric means.

Results: Iron absorption was similar from NaFeEDTA and FeSO₄ fortified fish sauce (3.3 versus 3.1%) and soy sauce (6.1 versus 5.6%). No significant difference was observed when NaFeEDTA fortified fish- and soy sauce were compared directly (6.7 and 7.9%). Soy sauce per se was inhibitory to iron absorption from rice-based meals (8.5% without versus 6.0% with soy sauce, p<0.02) while the addition of fish sauce did not affect iron absorption significantly.

Conclusion: The relatively high iron absorption from NaFeEDTA fortified fish- and soy sauce and acceptable organoleptic properties of NaFeEDTA indicate the potential usefulness of this iron fortificant for fish- and soy sauce fortification programs.

Key words: Iron, absorption, stable isotopes, soy sauce, fish sauce, NaFeEDTA, iron fortification
Iron absorption from fish and soy sauce

Introduction

Food fortification programs are usually considered the most cost-effective and sustainable approach to combat iron deficiency (1, 2). In Southeast Asia, where the prevalence of iron deficiency, including the more severe form of iron deficiency, iron deficiency anemia, is high (3), the major staple food, rice, would seem the obvious choice as the iron fortification vehicle. Unfortunately, rice is difficult to fortify as it is not usually consumed as flour. Attempts have been made to fortify rice with iron by preparing rice grains coated with iron (4-6) or by the addition of iron fortified simulated rice grains (7). Further development of these technologies is however needed before any large scale evaluations can be made.

When fortification of the staple food is not feasible, condiments are interesting alternatives as fortification vehicles. Fish sauce is a condiment frequently used in Southeast Asia, for example in Vietnam where 80% of the population regularly consumes fish sauce (8). This clear brown liquid, manufactured by fermenting freshwater or saltwater fish with salt (9, 10), is in the Southeast Asian cuisine the equivalent to salt in the West. Another condiment which is frequently consumed in Asian countries is soy sauce. In China, soy sauce has been used as an all-purpose seasoning for thousands of years. It is produced by yeast fermentation of soybeans and wheat or of soybeans alone (11).

Besides the advantage of a relatively high average daily consumption of soy sauce, this condiment could be especially useful as fortification vehicle since soy sauce per se has been shown to enhance iron absorption from rice (12). The reason for this reported enhancing effect is unclear since soy products are well known to inhibit iron absorption due to their high phytic acid content and due to the peptides formed during digestion of the conglycinin (7S) fraction of soy protein (13, 14). Phytic acid however can be expected to be substantially degraded during the fermentation process and the soy proteins can be expected to be transformed into a mixture of amino acids and peptides which might enhance iron absorption. The effect of fish sauce on iron absorption has so far not been studied.

The aim of the present study was to evaluate iron absorption from NaFeEDTA fortified fish sauce and soy sauce. NaFeEDTA, an iron compound which has recently been approved for use in supervised food fortification programs (15), was evaluated...
as preliminary studies have indicated that it is the most promising iron fortification compound for fish sauce and soy sauce since it produces no off-flavors, off-colors, or precipitation during storage. Iron absorption from rice-based meals fed with NaFeEDTA fortified sauces was compared in the same women with iron absorption from meals fed with sauces fortified with ferrous sulfate. The influence of the sauces per se on iron absorption from rice was evaluated and, lastly, a direct comparison of iron absorption from rice meals fortified with NaFeEDTA fortified fish sauce or with NaFeEDTA fortified soy sauce was included in the study. Erythrocyte incorporation of stable isotopes 14 days after administration was used to estimate iron absorption in young adult women.

**Subjects and Methods**

**Subjects**

Fifty apparently healthy adult women (19-29 years; max body weight 60 kg) were recruited from the student and staff populations at the ETH and the University of Zurich. The subjects were randomly allocated into five separate studies (10 subjects/study). Exclusion criteria included pregnancy or lactation and known gastrointestinal or metabolic disorders. No medication (except oral contraceptives) or vitamin/mineral supplements were allowed during the study. Women regularly taking vitamin/mineral supplements discontinued the supplementation 2 weeks before the start of the study.

The study protocol was reviewed and approved by the Ethical Committee of ETH Zurich, Switzerland. Subjects were informed orally and in writing about the aims and procedures of the study. Written informed consent was obtained from all study subjects.

**Study design**

Iron absorption was determined by a double stable isotope technique. The iron compounds were labeled with $^{57}$Fe or $^{58}$Fe and added to the different test meals as described below. All test meals were fed, after an overnight fast, on two consecutive days under standardized conditions. Iron absorption was based on erythrocyte incorporation of iron stable isotope labels 14 days after intake of labeled test meals. A cross-over study design was used with each woman acting as her own control. On day 1, a venous blood sample was drawn after an overnight fast for determination of
Iron absorption from fish and soy sauce

iron status parameters (hemoglobin (Hb), ferritin and circulating transferrin receptor). Body weight and height were measured and the first labeled test meal was served. The following day, the second test meal was administered. Test meals were fed under strictly standardized conditions under close supervision. No intake of food or fluids was allowed for 3 hours after test meal intake. A second venous blood sample was drawn 14 days after intake of the second test meal (day 16).

**Test meals**

All test meals included boiled white rice (50 g dry weight; Jasmine perfume rice, Dragon Phoenix Brand, Thailand). In studies 1 and 2, the rice was fed with vegetables (44% Chinese cabbage, 22% carrots, 22% zucchini, 13% onions), which were boiled until tender and then stir-fried in vegetable oil before being puréed (25 g fresh weight/serving). The vegetable purée was prepared in bulk and kept frozen until consumed. Fish sauce (10 g, Cat Hai medium quality fish sauce, Hai Phong, Vietnam) or soy sauce (10 g, Wadakan soy sauce, Beijing Hetiankuan Food Company, Xishanqi, Beijing, China) were added to some of the test meals (see below). The sauces were purchased in bulk in Vietnam and China, respectively. Each test meal contained 5 mg added iron; 4 mg as labeled ferrous sulfate or NaFeEDTA, plus 1 mg iron of normal isotopic composition. Deionized water (200 g) was served as a drink.

In study 1, rice plus vegetable purée was served with 10 g fish sauce fortified with Na$_{58}$FeEDTA or $^{57}$Fe ferrous sulfate. In study 2, rice plus vegetable purée was served with 10 g soy sauce fortified with Na$_{58}$FeEDTA or $^{57}$Fe ferrous sulfate. The test meals in study 3 consisted of rice only fortified with $^{57}$Fe or $^{58}$Fe ferrous sulfate; 10 g fish sauce was added to one of the meals. Study 4 was identical to study 3 but served with 10 g soy sauce. In study 5, 10 g fish sauce fortified with Na$_{58}$FeEDTA or soy sauce fortified with Na$_{57}$FeEDTA was served with rice alone.

**Stable isotope labels**

Stable isotope labels ($^{57}$Fe ferrous sulfate and $^{58}$Fe ferrous sulfate) were prepared from isotopically enriched elemental iron (Chemgas, Boulogne, France) by dissolution in sulfuric acid. The solutions were stored in Teflon containers flushed with argon to keep iron in the +II oxidation state. Na$_{58}$FeEDTA and Na$_{57}$FeEDTA were prepared in solution from $^{58}$Fe and $^{57}$Fe enriched elemental iron. The metal was dissolved in 1 mL ($^{58}$Fe) or 2 mL ($^{57}$Fe) HCl, diluted with water and stored in Teflon containers. The
resulting FeCl₃ solution was mixed with a freshly prepared aqueous Na₂EDTA solution (Na₂EDTA·2H₂O, Sigma Chemicals Co, St Louis, MO) at a molar ratio 1:1 (Fe:EDTA) and added to individual servings of fish sauce or soy sauce (10 g) 22-24 h prior to test meal administration. The containers were wrapped in aluminum foil and kept refrigerated overnight.

**Quantification of iron isotopes in labels**
Isotope dilution mass spectrometry was used to determine iron concentrations of the labeled compounds in solution. An accurately measured amount of iron of natural isotopic composition was added to aliquots taken from the prepared isotopic labels. The iron standard was prepared gravimetrically from an isotopic reference material (IRM-014, EU Institute of Reference Materials, Geel, Belgium). Isotopic analysis was performed using negative thermal ionization mass spectrometry (NTI-MS) (16). Iron concentrations in the isotopic labels were calculated based on the shift in iron isotopic abundances, the determined isotopic abundances of the pure isotopic labels and the natural iron isotopic abundances (17).

**Iron status measurements**
Venous blood samples (7 ml) were drawn in EDTA-treated tubes before intake of the first labeled test meal and again on day 16. Samples were analyzed for iron status indices (hemoglobin (Hb), plasma-ferritin, circulating transferrin receptor) and for the incorporation of ⁵⁷Fe and ⁵⁸Fe into red blood cells (day 16). Blood samples were aliquoted for the analysis of Hb and plasma was separated, aliquoted and frozen for later analysis of ferritin and circulating transferrin receptor. Hb was measured by the cyanmethemoglobin method (Sigma kit, Sigma, St. Louis, MO), plasma-ferritin and circulating transferrin receptor by ELISA (Ramco Laboratories, Houston, Texas). Commercial quality control materials (DiaMed, Cressier sur Morat, Switzerland and Ramco Laboratories) were analyzed together with samples analyzed for Hb and plasma-ferritin, respectively.

**Quantification of iron isotope in blood**
Each isotopically enriched blood sample was analyzed in duplicate for its iron isotopic composition as previously described by Davidsson et al. (18). The blood samples were mineralized by using a mixture of nitric acid and hydrogen peroxide and microwave digestion. The iron was separated from the matrix by anion-exchange
chromatography and a solvent-solvent extraction step into diethyl ether. The isotopic analyses were performed by NTI-MS (16).

**Calculation of iron absorption**
The amounts of $^{57}\text{Fe}$ and $^{58}\text{Fe}$ isotopic labels in blood 14 days after test meal administrations were calculated based on the shift in iron isotope ratios and on the amount of iron circulating in the body. The calculations were based on the principles of isotope dilution and took into account that iron isotopic labels were not monoisotopic (17). Circulating iron was calculated based on blood volume and Hb concentration (19). Blood volume calculations were based on height and weight according to Brown et al. (20). For calculations of fractional absorption, 80% incorporation of the absorbed iron into red blood cells was assumed (21).

**Food analysis**
Rice, vegetable purée, fish sauce and soy sauce were analyzed for iron and calcium by electrothermal/flame atomic absorption spectroscopy (SpectrAA 400, Varian, Mulgrave, Australia) after mineralization by microwave digestion (MLS 1200) in an HNO$_3$/H$_2$O$_2$ mixture and using standard addition technique to minimize matrix effects. Phytic acid in rice was determined by HPLC (22, 23) and phytic acid in soy sauce was determined by a modification of the Makower (24) method in which Ce replaced Fe in the precipitation step. Ascorbic acid in the vegetable purée was measured by HPLC using a reversed phase column and photometric detection (Merck-Hitachi System, Merck, Germany) (25, 26). Nitrogen content of the fish sauce and soy sauce was determined by Kjeldahl analysis and the amino acid content was measured by ion exchange chromatography (27).

**Statistics**
Absorption ratios are presented as geometric means ($-1\text{SD}, +1\text{SD}$). Student's paired t-test was used to compare absorption data within each study. Absorption values were logarithmically transformed before statistical analysis (Excel 97, Microsoft Corporation, Redmond, WA, USA).
Results

Subjects
Two of the 50 women were iron deficient anemic (Hb 119 g/l and 118 g/l, TfR >8.5 mg/l and plasma ferritin <12 μg/l). Sixteen women had iron deficiency indicated either by elevated TfR (>8.5 mg/l) and/or by low plasma ferritin (<12 μg/l).

Test meals
The contents of iron, calcium, phytic acid and ascorbic acid in the different test meals are shown in Table 1. The total iron content varied between 5.1 and 5.9 mg per meal. Most iron (5 mg) came from the fortification compound with further small amounts from soy sauce (0.7 mg) and fish sauce (0.2 mg), puréed vegetables (0.1 mg) and rice (0.1 mg). The calcium content was low and varied between 2-16 mg/meal. Nearly all phytic acid came from the rice (25 mg/meal). Soy sauce contained 20 mg phytic acid /100 g but provided only 2 mg phytic acid/meal. The phytic acid content of the vegetable purée was not measured and assumed to be negligible. The phytic acid to iron molar ratio in the test meals was about 0.4:1. There was little ascorbic acid remaining in the cooked and puréed vegetables. The ascorbic acid content of all meals was negligible. The nitrogen content of the fish sauce and soy sauce was 1.3 and 1.1 g/100g and the amino acid content after hydrolysis was 4.0 and 5.3 g/100g, respectively.

Table 1: Contents of iron, calcium, phytic acid and ascorbic acid in the test meals

<table>
<thead>
<tr>
<th>Meal</th>
<th>Iron fortification (mg)</th>
<th>Iron native (mg)</th>
<th>Calcium (mg)</th>
<th>Phytic acid* (mg)</th>
<th>Ascorbic acid (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice, vegetables, fish sauce (Study 1)</td>
<td>5</td>
<td>0.4</td>
<td>16.2</td>
<td>25</td>
<td>0.4</td>
</tr>
<tr>
<td>Rice, vegetable, soy sauce (Study 2)</td>
<td>5</td>
<td>0.9</td>
<td>16.0</td>
<td>27</td>
<td>0.4</td>
</tr>
<tr>
<td>Rice, fish sauce (Studies 3, 5)</td>
<td>5</td>
<td>0.2</td>
<td>5.8</td>
<td>25</td>
<td>nd*</td>
</tr>
<tr>
<td>Rice, soy sauce (Studies 4, 5)</td>
<td>5</td>
<td>0.7</td>
<td>5.6</td>
<td>27</td>
<td>nd*</td>
</tr>
<tr>
<td>Rice (Studies 3, 4)</td>
<td>5</td>
<td>0.1</td>
<td>2.0</td>
<td>25</td>
<td>nd*</td>
</tr>
</tbody>
</table>

*the molar ratio of phytic acid to iron was 0.4:1 in all meals
**not determined (nd), assumed to be negligible

Iron absorption
The geometric mean iron absorption from the different test meals varied between 3.1% and 11.6% (Table 2). There was no significant difference in iron absorption from the rice and vegetable meal served with fish sauce fortified with ferrous sulfate
Iron absorption from fish and soy sauce

or NaFeEDTA (geometric mean 3.1% vs. 3.3%, p=0.66, Study 1), or from the same meal served with soy sauce fortified with ferrous sulfate or NaFeEDTA (geometric mean 5.6% vs. 6.1%, p=0.44, Study 2). Fish sauce per se did not influence iron absorption from rice (geometric mean 9.5% vs. 11.6%, with and without sauce, respectively, p=0.14, Study 3), although soy sauce per se decreased iron absorption significantly (geometric mean 6.0% vs. 8.5%, with and without sauce, respectively, p=0.02, Study 4). When compared directly, there was no difference in iron absorption from the rice meal fed with NaFeEDTA fortified fish sauce or soy sauce (geometric mean 6.7% vs. 7.9%, p=0.08, Study 5).

Table 2: Iron absorption by healthy adult women (10 women/study) from rice-based meals served with iron fortified fish sauce, soy sauce or without any added condiment

<table>
<thead>
<tr>
<th>Study</th>
<th>Ferritin (μg/L)*</th>
<th>Transferrin receptor (mg/L)*</th>
<th>Fish Sauce + FeSO4</th>
<th>Fish Sauce + NaFeEDTA</th>
<th>Soy Sauce + FeSO4</th>
<th>Soy Sauce + NaFeEDTA</th>
<th>No Sauce</th>
<th>P value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Rice &amp; vegetables</td>
<td>23 (10, 50)</td>
<td>7.6 (6.2, 9.4)</td>
<td>3.1 (1.2, 7.9)</td>
<td>3.3 (1.6, 6.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.66</td>
</tr>
<tr>
<td>2. Rice &amp; vegetables</td>
<td>14 (9, 23)</td>
<td>8.5 (6.7, 10.8)</td>
<td>-</td>
<td>-</td>
<td>5.6 (3.3, 9.5)</td>
<td>6.1 (3.2, 11.8)</td>
<td>11.6 (5.6, 23.8)</td>
<td>0.46</td>
</tr>
<tr>
<td>3. Rice</td>
<td>16 (7, 34)</td>
<td>7.5 (1.2, 9.2)</td>
<td>9.5 (4.3, 21.0)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.5 (3.8, 19.1)</td>
<td>0.14</td>
</tr>
<tr>
<td>4. Rice</td>
<td>16 (9, 29)</td>
<td>6.1 (5.4, 6.9)</td>
<td>-</td>
<td>-</td>
<td>6.0 (2.3, 15.8)</td>
<td>-</td>
<td>11.6 (5.6, 23.8)</td>
<td>0.02</td>
</tr>
<tr>
<td>5. Rice</td>
<td>17 (8.36)</td>
<td>8.5 (6.0, 12.1)</td>
<td>-</td>
<td>6.7 (3.7, 12.1)</td>
<td>-</td>
<td>7.9 (4.7, 13.5)</td>
<td>-</td>
<td>0.08</td>
</tr>
</tbody>
</table>

*Geometric mean (-SD, +SD)
** p values refer to statistical analysis within each study (paired t-test)

Discussion

Iron absorption from meals fed with NaFeEDTA fortified fish sauce was not statistically different from the identical test meals fed with ferrous sulfate fortified fish sauce. Nor was there a statistical difference between iron absorption from meals fed with NaFeEDTA fortified soy sauce and the identical meals fed with ferrous sulfate fortified soy sauce (Table 2). Although iron absorption was similar from the two iron fortificants evaluated in these studies, it is important to stress that major differences exist in relation to provoking unacceptable organoleptic changes. NaFeEDTA can be added to fish sauce and soy sauce without changing the sensory properties of the
Iron absorption from fish and soy sauce

fortified condiment while the addition of ferrous sulfate causes unacceptable precipitation (unpublished observations, M Fidler, 2002) (28).

Although iron absorption from NaFeEDTA has been demonstrated to be 2-3 times higher than ferrous sulfate from high phytic acid containing meals, no difference in iron absorption from the two iron compounds has been reported when evaluated in moderately inhibitory meals (29). It was therefore not unexpected that in the present study NaFeEDTA had no enhancing effect on iron absorption relative to ferrous sulfate. The phytic acid contents of the rice meals fed in the present study were 25-27 mg at an iron content of 5.1-5.9 mg (Table 1). The phytic acid to iron molar ratio (about 0.4:1) was low and can be considered as only moderately inhibitory to iron absorption (30). The present studies thus demonstrated relatively high iron absorption from rice meals accompanied by NaFeEDTA fortified fish sauce or NaFeEDTA fortified soy sauce, and indicate that these sauces are potentially useful as iron fortification vehicles. By adjusting the iron absorption values (Table 2) to a serum ferritin concentration of 12 μg/L (31) it can be estimated that women with no iron stores would absorb 5.9-15.2% (mean 9.9%) from rice meals accompanied by these sauces fortified with NaFeEDTA. For example, if the sauces were fortified with 0.5 mg Fe/mL and the consumption was 20 mL/day, the sauces would provide 10 mg extra dietary iron. The fortified condiment would thus provide approximately 30% of the daily iron requirements of young women, based on an iron absorption from the diet of 10% (Type II diet) (32).

Based on previous studies, it was somewhat unexpected that soy sauce and fish sauce per se did not enhance iron absorption from the rice meals. Soy sauce has previously been reported to enhance iron absorption from a rice meal (12) and fish and a mixture of amino acids similar to that present in fish protein have been reported to improve iron absorption from a variety of meals (33-35). In the present study, the addition of soy sauce decreased iron absorption by about 30% (p=0.02; Study 4, Table 2), whereas fish sauce had no statistically significant effect on iron absorption (Study 3, Table 2). However, when directly compared within the same women (Study 5), there were no differences (p>0.05) in iron absorption from rice meals fed with NaFeEDTA fortified fish sauce or NaFeEDTA fortified soy sauce.
There are several different types of commercial soy sauce and the contradictory effects of soy sauce on iron absorption between the present study and that of Baynes et al. (12) can presumably be explained by differences in the composition of the sauces, i.e. variations in raw ingredients and differences in methods of soy sauce manufacture. In China, the traditional soy sauce, which is comparable to the Japanese tamari-shoyu, is prepared from soybeans only, whereas the modern type is made from defatted soybean meal and wheat bran (36, 37). The soy sauce used by Baynes et al. was tamari-shoyo type soy sauce whereas the Chinese soy sauce used in the present study was the modern variety, which included wheat bran as a raw ingredient. The tamari-shoyo sauces are subjected to a longer fermentation time than the soy/wheat bran sauces, resulting in a greater extent of hydrolysis and therefore include a larger proportion of amino acids and low-molecular weight peptides in the final product (36). This could be important as the extent of protein hydrolysis might influence iron absorption by decreasing the amount of iron absorption inhibiting soy protein fractions (14). In support of this hypothesis, MacFarlane et al. (38) showed, with a few exceptions, that there is an inverse relationship between iron absorption and the proportion of higher-molecular-weight fraction of the soy proteins. Inhibition of iron absorption by phytic acid seems unlikely as a reason for the inhibitory effects of soy sauce in the present study, since the very small amounts of phytic acid present in the soy sauce used in this study (2 mg per test meal) would not be expected to significantly influence iron absorption (13).

Several different qualities of fish sauce are produced which differ in nitrogen content. The supernatant collected after the first fermentation is referred to as high-quality fish sauce. Lower quality fish sauces are produced by extracting the fermented fish residue with hot brine. These sauces contain less nitrogen and consequently smaller amounts of amino acids and peptides (10). In the present study, a medium quality fish sauce was evaluated since this quality level is frequently consumed in Vietnam. A possible reason therefore why the Vietnamese fish sauce did not enhance iron absorption in the present study could be the low content of amino acids and peptides. The amount of fish sauce (10 g) added to the test meals contributed only 0.4 g amino acids and peptides. This amount is considerably lower than the amounts of fish protein (approximately 10-20 g) which have been shown to enhance iron absorption (33-35) in a dose dependent manner (35). Finally, although fish sauce is made by fer-
Iron absorption from fish and soy sauce

mentation of whole fish, including bones, the calcium content of the Vietnamese fish sauce was low (4 mg/10g) and would not be expected to influence iron absorption (39, 40).

In conclusion, these studies demonstrate that iron absorption by young women consuming rice meals served with NaFeEDTA fortified fish sauce or NaFeEDTA fortified soy sauce is relatively high and that both NaFeEDTA fortified sauces appear to be useful vehicles for iron fortification.

Acknowledgement
MCF, LD, TW and RFH contributed to the study design. MCF and LD were responsible for the implementation of the study. TW was responsible for the preparation of the stable isotope labels and the analytical work. The statistical analysis was done by LD. The manuscript was written by MCF and LD and edited by TW and RFH.

RFH is a member of the Technical Advisory Board of ILSI Project IDEA (Iron Deficiency Elimination Action). At the time of the study, LD was a member of the Scientific Advisory Committee, ILSI Project IDEA. None of the other co-authors reported any conflict of interest.

References
Iron absorption from fish and soy sauce

32. WHO/FAO. Recommended Nutrient Intakes. in press. (as referenced in Appendix 2, Nutr Rev 2002;60:S60) 2002.
Chapter 7

PHOTOSTABILITY OF NaFeEDTA IN STORED FISH SAUCE AND SOY SAUCE

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Photostability of NaFeEDTA

Abstract
NaFeEDTA has been suggested as a fortification compound for fish sauce and soy sauce. Its susceptibility to photodegradation in aqueous solutions however is a potential disadvantage. We determined the photostability of NaFeEDTA in fish and soy sauce stored under well defined conditions. No degradation of NaFeEDTA was observed during storage of fortified soy sauce. Losses of up to 35% NaFeEDTA, however, occurred within 2-6 weeks in fortified fish sauce stored in clear bottles exposed to direct sunlight. Losses were prevented by storage in amber bottles or by storing the clear bottles under indirect sunlight or in the dark.

Keywords: NaFeEDTA, photostability, fish sauce, soy sauce, iron fortification

Introduction
Sodium iron ethylenediaminetetraacetic acid (NaFeEDTA) is a promising iron compound for food fortification programs. The major advantage of this compound is that it can overcome the inhibitory effect on iron absorption of phytic acid, which is present in cereal and legume grains. Iron absorption from fortified cereals and legumes is 2 to 3-fold higher when fortified with NaFeEDTA than with ferrous sulfate (INACG 1993; Hurrell and others 2000). In addition, NaFeEDTA can be added to soy sauce and fish sauce without causing peptide precipitation which occurs with ferrous sulfate and other soluble iron compounds. NaFeEDTA however has been reported to be sensitive to photodegradation in aqueous solutions (Jones and Long 1952; Hill-Cottingham 1955; Kruger and Agterdenbosch 1957).

In addition to its potential use in food fortification programs, EDTA is widely used in industrial, pharmaceutical, and agricultural applications. With these practices, a major concern has been its possible presence in natural water supplies (Nowack and other 1996; Sorensen and Frimmel 1997) and studies on the photodegradability of EDTA have been focused on the development of techniques to eliminate EDTA from drinking water. The aqueous photolysis of EDTA, Fe(III)EDTA, and other EDTA chelates has been examined in several studies (Carey and Langford 1973; Lockhart and Blakeley 1975a; Nowack and others 1996; Sörensen and Frimmel 1997; Nowack and Baumann 1998; Sörensen and others 1998) and the half life for Fe(III)EDTA in a sunlit river in summer has been reported as being 2 hours (Kari and Giger 1995). Although a distinct advantage environmentally, the photodegradation of FeEDTA in
Photostability of NaFeEDTA aqueous solution is of concern when this compound is used for fortification of water-based condiments, such as soy sauce and fish sauce, which are potential vehicles for food fortification (Fidler and others 2001, Thuy and others 2001).

The aim of this study was to evaluate the stability of NaFeEDTA in fish sauce and soy sauce when stored under clearly defined conditions. The sauces were filled into clear and amber glass bottles, and into polyethylene terephthalate (PET) bottles, and stored under indirect sunlight/fluorescent light, artificial and natural sunlight, and in the dark. NaFeEDTA concentrations were assessed by measuring Fe(III)EDTA concentrations in regular intervals for up to 1 year using a HPLC technique.

Materials and Methods

Test materials

The stability of NaFeEDTA was assessed in a Vietnamese medium quality fish sauce (Cat Hai, Hai Phong, Vietnam), a Thai fish sauce (Rayong Fish Sauce Industry Co. Ltd, Thailand), and a European soy sauce (fancy grade, Kikkoman Food Europe B.V., The Netherlands). NaFeEDTA (food grade, Dr. Paul Lohmann Ltd., Emmerthal, Germany) was added at a concentration of 500 mg Fe/L sauce. For each storage condition, 1 batch of fish sauce or soy sauce was fortified by adding the NaFeEDTA to the sauces and shaking for 3 d until the NaFeEDTA had dissolved completely. As a control, NaFeEDTA was dissolved in ultrapure (18 MΩ) water (500 mg Fe/L) and one batch of bottles were filled per storage condition. No shaking was required with water as the NaFeEDTA dissolved readily.

The clear and amber glass bottles (100 mL, Faust, Schaffhausen, CH) as well as PET bottles (100 mL, Semadeni, Ostermundigen, CH) were manually filled with 100 mL fortified sauce or 100 mL NaFeEDTA solution. Two bottles were filled per time point for any given storage condition.

Storage conditions

Artificial sunlight

The bottles were stored under a halide lamp (135 V, 3.25 A, 400 W, 29000 lm; MT400DL/BH-E40, Iwasaki Electric Co., Ltd., Tokyo, Japan) in a 30 °C climate chamber. The wavelengths emitted by the lamp (310-800 nm) were similar to the
Photostability of NaFeEDTA

wavelengths emitted by sunlight (290-800 nm). The filled bottles were illuminated 24 h/d for 56 d and their NaFeEDTA concentration was assessed twice weekly.

**Natural sunlight**
The bottles were stored outside in the garden of the Laboratory for Human Nutrition, Rueschlikon, Switzerland, from the 3rd of July until the 14th of August 2000 under direct sunlight with no shade. Approximate length of the days during the storage period was between 16 h (03.07.2000) and 14.5 h (14.08.2000). The fortified sauces and the aqueous solutions were stored for up to 42 d, and their NaFeEDTA concentration was assessed every 14 d.

**Florescent light/indirect sunlight**
The bottles were stored for 364 d on a shelf next to a window. They received both florescent light (Lumilux de Luxe, L36 W/22-940, white, Osram, Winterthur, Switzerland, wavelengths 400-700 nm) and indirect sunlight passing though a glass window. The NaFeEDTA concentration in the sauces stored in glass bottles was assessed weekly for the first 112 d and then at 182 d and at 364 d. The NaFeEDTA concentrations of the sauces stored in PET bottles, and the aqueous solutions stored in glass and PET bottles, were determined after 14, 28, 56, 112, 182, and 364 d.

**Dark**
Clear glass bottles were stored in a cardboard box in the same climate chamber (30 °C) as the bottles stored under artificial sunlight. The bottles were stored for 112 d and the NaFeEDTA concentrations were determined after 14, 28, 56, and 112 d.

**Analytical Methods**
NaFeEDTA concentrations were assessed by determining Fe(III)EDTA concentrations using the HPLC method as described by Yamaguchi and others (1983) with small modifications to the mobile phase, injection volume and detection wavelength. The HPLC system consisted of a Merck-Hitachi-system L-6200A intelligent pump, equipped with a D-6000 interface, a diode array detector, a column thermostat and an intelligent auto sampler (Merck (Schweiz) AG, Dietikon, Switzerland). The column used was an ODS RP-18 (Hypersil, ODS, 200 x 4.6mm, 3 µm, Grom Analytic & HPLC GmbH, Herrenberg, Germany). The mobile phase consisted of a 0.01M am-
Photostability of NaFeEDTA

monium dihydrogen phosphate buffer (puriss., Fluka Chemie AG, Switzerland) with 10% acetonitrile (hypergrade for liquid chromatography, Merck AG, Germany) and 0.2% of a 40% tetrabutylammonium hydroxide solution (TBA, puriss., Fluka Chemie AG, Switzerland). The pH was adjusted to 2.42 with 85% ortho-phosphoric acid (puriss, Fluka Chemie AG, Switzerland). Before use the solution was filtered though a 0.45 μm nylon filter (Alltech Associates, Inc., Deerfield, Ill., U.S.A.) and degassed with helium.

The fortified sauces and NaFeEDTA in aqueous solution were diluted 1:200 with ultrapure (18 MΩ) water, filtered though a 0.45 μm filter (Chromafil® filter, Type A 45/25) and transferred into HPLC vials (500 x 2 SV vials, 8 mm screw cap, 8 mm silicon/Teflon septum, Infochroma AG, Switzerland). From each of the 2 bottles of fortified sauce and aqueous NaFeEDTA solution, 1 dilution was made and analyzed by HPLC in duplicate under the following conditions: flow rate 1 mL/min; injection volume 40 μL; column temperature 25 °C; detection wavelength 257 nm, elution time 15 min. Retention time of Fe(III)EDTA in fish sauce was 5.78 ±0.11 min, 5.73 ±0.06 min in soy sauce, and 5.99 ±0.05 min in aqueous solution. Calculations were made using the D-6500 DAD software from Merck, Darmstadt, Germany. Fe(III)EDTA concentrations were quantified by a 4 point calibration curve ranging from 2 to 20 mg EDTA/mL. Calibration solutions were prepared by dissolving Na₂EDTA·2H₂O (Sigma Chemical Co., St Louis, Mo., U.S.A) and FeCl₃·6H₂O (puriss., Fluka Chemie AG, Switzerland) in ultrapure (18 MΩ) water.

Statistics

LSD-test was used to calculated significant differences between the NaFeEDTA concentration before and after exposure to light (Köhler and others 1995).

Results and Discussion

Mean values for the percentage of NaFeEDTA remaining in water, fish sauce, and soy sauce stored under different conditions are shown in Tables 1-5. The values given are mean values from duplicate analyses of 2 separate samples at each time point. Within-run precision of the HPLC technique used to quantify Fe(III)EDTA was 2-5% based on 10 repeated measures of 3 different Fe(III)EDTA concentrations (2, 5 and 10 mg EDTA/mL) in ultrapure (18 MΩ) water.
Photostability of NaFeEDTA

NaFeEDTA in aqueous solution

After approximately 2 weeks storage under artificial and natural sunlight, the NaFeEDTA concentrations in aqueous NaFeEDTA solutions stored in clear glass and PET bottles had decreased by 50-60% (p<0.01; Table 1); little further degradation occurred during the continued storage. When stored in amber bottles for 51 d under artificial sunlight NaFeEDTA was stable, however, the exposure to natural sunlight resulted in a 20% decreased in NaFeEDTA concentration after 42 d (p<0.01). NaFeEDTA degradation in PET and clear glass bottles could not be prevented by storing the aqueous NaFeEDTA solutions under indirect sunlight/fluorescent light (Table 1). However, when stored in amber bottles under indirect sunlight/fluorescent light for up to one year (Table 1) and in the dark for 112 d (Table 5), NaFeEDTA was stable.

Table 1: Percentage of NaFeEDTA remaining* in aqueous NaFeEDTA solutions stored under different conditions.

<table>
<thead>
<tr>
<th>days</th>
<th>clear glass</th>
<th>amber glass</th>
<th>PET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>artificial sunlight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>41** (0.6)</td>
<td>95 (1.0)</td>
<td>44*** (2.0)</td>
</tr>
<tr>
<td>31</td>
<td>44** (10.7)</td>
<td>101 (0.3)</td>
<td>50*** (1.2)</td>
</tr>
<tr>
<td>51</td>
<td>30*** (0.3)</td>
<td>94 (1.2)</td>
<td>42*** (3.0)</td>
</tr>
<tr>
<td></td>
<td>natural sunlight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>52** (2.6)</td>
<td>95 (1.1)</td>
<td>56** (2.2)</td>
</tr>
<tr>
<td>28</td>
<td>59** (0.5)</td>
<td>90 (1.7)</td>
<td>57** (1.2)</td>
</tr>
<tr>
<td>42</td>
<td>48** (1.8)</td>
<td>78** (3.9)</td>
<td>44*** (2.6)</td>
</tr>
<tr>
<td></td>
<td>indirect sunlight/fluorescent light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>92 (0.4)</td>
<td>97 (0.8)</td>
<td>97 (1.5)</td>
</tr>
<tr>
<td>28</td>
<td>82 (6.1)</td>
<td>97 (1.6)</td>
<td>84 (1.5)</td>
</tr>
<tr>
<td>56</td>
<td>58** (3.1)</td>
<td>93 (0.4)</td>
<td>68** (1.2)</td>
</tr>
<tr>
<td>112</td>
<td>49** (0.4)</td>
<td>95 (0.4)</td>
<td>58*** (0.4)</td>
</tr>
<tr>
<td>182</td>
<td>51** (1.2)</td>
<td>89 (0.4)</td>
<td>57*** (3.5)</td>
</tr>
<tr>
<td>364</td>
<td>51** (0.4)</td>
<td>89 (1.5)</td>
<td>54*** (1.2)</td>
</tr>
</tbody>
</table>

*mean (+/-SD), concentration of NaFeEDTA at baseline was 3.09 (mg/mL) under artificial light, 3.09 (mg/mL) under natural sunlight and 3.31 (mg/mL) under indirect sunlight/fluorescent light. Mean values were significantly different from NaFeEDTA concentrations at baseline **p<0.01, ***p<0.001

The observed NaFeEDTA degradation of aqueous NaFeEDTA solutions stored in clear glass and PET bottles can be explained by the known sensitivity of aqueous NaFeEDTA solutions to photodegradation in sunlight and the light transmission proper-
ties of the bottles used. The wavelengths emitted by the sun include those wavelengths (UVA: 315-400 nm and UVB 280-315 nm) which have a strong influence on Fe(III)EDTA degradation (Kari 1994). Amber glass reduces the UVA and UVB radiation by 90% (Buchner 1999) thus explaining the stability of NaFeEDTA solutions stored in amber glass bottles. Clear glass on the other hand only reduces UVB radiation substantially (by 90%). Similarities between NaFeEDTA degradation in clear glass bottles and PET bottles can be explained by the similar light transmission properties of these materials (Buchner 1999).

**NaFeEDTA Fortified Soy Sauce**

No significant degradation (p>0.05) of NaFeEDTA was detected in the fortified soy sauce under the tested storage conditions (Tables 2 and 5), indicating that there should be little or no degradation of NaFeEDTA during storage. The stability of NaFeEDTA in soy sauce is probably due to the dark brown pigments of the sauce which presumably act in a similar way to the amber glass, reducing the transmission of the wavelengths responsible for photodegradation of NaFeEDTA.

**Table 2: Percentage of NaFeEDTA remaining\(^{a}\) in NaFeEDTA fortified soy sauce stored under different conditions**

<table>
<thead>
<tr>
<th>days</th>
<th>clear glass</th>
<th>amber glass</th>
<th>PET</th>
</tr>
</thead>
<tbody>
<tr>
<td>artificial sunlight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>97 (2.7)</td>
<td>105 (2.5)</td>
<td>103 (2.9)</td>
</tr>
<tr>
<td>31</td>
<td>107 (1.6)</td>
<td>104 (2.8)</td>
<td>103 (1.5)</td>
</tr>
<tr>
<td>51</td>
<td>100 (0.4)</td>
<td>100 (1.2)</td>
<td>98 (3.8)</td>
</tr>
<tr>
<td>natural sunlight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>93 (0.2)</td>
<td>85 (7.4)</td>
<td>95 (1.7)</td>
</tr>
<tr>
<td>28</td>
<td>99 (0.7)</td>
<td>99 (0.2)</td>
<td>100 (1.1)</td>
</tr>
<tr>
<td>42</td>
<td>104 (1.1)</td>
<td>103 (1.7)</td>
<td>97 (2.5)</td>
</tr>
<tr>
<td>indirect sunlight/fluorescent light</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>99 (3.5)</td>
<td>98 (2.7)</td>
<td>99 (3.1)</td>
</tr>
<tr>
<td>28</td>
<td>94 (0.4)</td>
<td>93 (0.7)</td>
<td>94 (0.1)</td>
</tr>
<tr>
<td>56</td>
<td>98 (1.4)</td>
<td>102 (0.9)</td>
<td>96 (1.9)</td>
</tr>
<tr>
<td>112</td>
<td>94 (6.6)</td>
<td>100 (0.3)</td>
<td>102 (0.6)</td>
</tr>
<tr>
<td>182</td>
<td>98 (0.2)</td>
<td>94 (1.5)</td>
<td>95 (0.2)</td>
</tr>
<tr>
<td>364</td>
<td>98 (0.2)</td>
<td>94 (4.6)</td>
<td>95 (3.5)</td>
</tr>
</tbody>
</table>

\(^{a}\)mean (+/-SD), concentration of NaFeEDTA at baseline was 3.10 (mg/mL) under artificial light, 3.10 (mg/mL) under natural sunlight and 3.20 (mg/mL) under indirect sunlight/fluorescent light.
Photostability of NaFeEDTA

**NaFeEDTA Fortified Fish Sauce (Vietnamese and Thai)**

Degradation of NaFeEDTA in the 2 fortified fish sauces is shown in Tables 3, 4, and 5. In clear glass bottles and PET bottles stored under artificial sunlight, a degradation of NaFeEDTA between 16-26% (p<0.01) was measured after 14 d (Tables 3, 4). The NaFeEDTA concentration was monitored regularly over a period of 56 d but little further degradation was observed. Degradation of NaFeEDTA in Vietnamese fish sauce stored in clear glass bottles and PET bottles under natural sunlight was similar to that observed for both fish sauces stored under artificial sunlight. However, a slightly higher degradation of NaFeEDTA was observed in the fortified Thai fish sauce when stored in clear glass bottles and PET bottles under natural sunlight (up to 36%). No difference was found between clear glass bottles and PET bottles (p>0.05). In the amber bottles there was no significant degradation (p>0.05) of NaFeEDTA in the fortified fish sauce when stored under artificial or natural sunlight.

<table>
<thead>
<tr>
<th>days</th>
<th>clear glass</th>
<th>amber glass</th>
<th>PET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>artificial sunlight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>76** (5.3)</td>
<td>97 (1.1)</td>
<td>74** (2.0)</td>
</tr>
<tr>
<td>34</td>
<td>73** (0.0)</td>
<td>93 (0.4)</td>
<td>89 (5.6)</td>
</tr>
<tr>
<td>56</td>
<td>75** (0.4)</td>
<td>95 (1.1)</td>
<td>75** (2.7)</td>
</tr>
<tr>
<td></td>
<td>natural sunlight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>91 (2.8)</td>
<td>92 (0.2)</td>
<td>71** (2.0)</td>
</tr>
<tr>
<td>28</td>
<td>78** (0.0)</td>
<td>95 (2.7)</td>
<td>77** (5.6)</td>
</tr>
<tr>
<td>42</td>
<td>81** (0.8)</td>
<td>91 (2.0)</td>
<td>80** (2.7)</td>
</tr>
<tr>
<td></td>
<td>indirect sunlight/fluorescent light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>99 (0.7)</td>
<td>101 (1.0)</td>
<td>102 (2.7)</td>
</tr>
<tr>
<td>28</td>
<td>91 (0.9)</td>
<td>94 (0.5)</td>
<td>93 (2.7)</td>
</tr>
<tr>
<td>56</td>
<td>101 (2.2)</td>
<td>103 (0.9)</td>
<td>101 (0.2)</td>
</tr>
<tr>
<td>112</td>
<td>98 (1.7)</td>
<td>105 (0.6)</td>
<td>103 (1.2)</td>
</tr>
<tr>
<td>182</td>
<td>91 (2.4)</td>
<td>101 (3.5)</td>
<td>99 (1.5)</td>
</tr>
<tr>
<td>364</td>
<td>90 (4.2)</td>
<td>101 (1.2)</td>
<td>98 (1.9)</td>
</tr>
</tbody>
</table>

*Mean (+/-SD), concentration of NaFeEDTA at baseline was 3.38 mg/mL under artificial light, 3.03 mg/mL under natural sunlight and 2.93 mg/mL under indirect sunlight/fluorescent light. Mean values were significantly different from NaFeEDTA concentrations at baseline **p<0.01, ***p<0.001.

NaFeEDTA losses were also insignificant in both the Vietnamese and Thai fish sauces stored for up to 364 d on a shelf under indirect sunlight/fluorescent light, irre-
Photostability of NaFeEDTA

respectively of the bottle material (Table 3, 4). Further, no degradation of NaFeEDTA could be detected in the fish sauces stored in the dark for up to 112 d (Table 5).

These results show that NaFeEDTA losses of up to 35% can occur in NaFeEDTA fortified fish sauce stored in clear bottles, if the bottles are exposed to direct sunlight for long periods. This lower stability of NaFeEDTA in fish sauce in comparison to soy sauce may be related to the lower concentration of pigments in fish sauce and therefore higher light transmission.

Table 4: Percentage of NaFeEDTA remaining\(^a\) in NaFeEDTA fortified Thai fish sauce stored under different conditions

<table>
<thead>
<tr>
<th>days</th>
<th>clear glass</th>
<th>amber glass</th>
<th>PET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>artificial sunlight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>84** (0.8)</td>
<td>97 (0.1)</td>
<td>74** (2.4)</td>
</tr>
<tr>
<td>31</td>
<td>90 (2.2)</td>
<td>105 (2.0)</td>
<td>83* (7.5)</td>
</tr>
<tr>
<td>51</td>
<td>87** (2.0)</td>
<td>93 (2.3)</td>
<td>92 (0.6)</td>
</tr>
<tr>
<td></td>
<td>natural sunlight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>57** (1.3)</td>
<td>94 (1.1)</td>
<td>57** (4.1)</td>
</tr>
<tr>
<td>28</td>
<td>68** (1.5)</td>
<td>86 (1.5)</td>
<td>66** (0.6)</td>
</tr>
<tr>
<td>42</td>
<td>64** (7.7)</td>
<td>94 (0.2)</td>
<td>69** (0.8)</td>
</tr>
<tr>
<td></td>
<td>indirect sunlight/fluorescent light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>96 (3.0)</td>
<td>96 (0.5)</td>
<td>98 (2.9)</td>
</tr>
<tr>
<td>28</td>
<td>95 (1.3)</td>
<td>97 (2.0)</td>
<td>91 (2.2)</td>
</tr>
<tr>
<td>56</td>
<td>88 (2.3)</td>
<td>89 (2.3)</td>
<td>87 (1.4)</td>
</tr>
<tr>
<td>112</td>
<td>84 (0.6)</td>
<td>88 (0.7)</td>
<td>84 (0.5)</td>
</tr>
<tr>
<td>182</td>
<td>87 (2.2)</td>
<td>92 (0.8)</td>
<td>88 (0.5)</td>
</tr>
<tr>
<td>364</td>
<td>89 (0.7)</td>
<td>92 (0.8)</td>
<td>91 (1.4)</td>
</tr>
</tbody>
</table>

\(^a\)mean (+/-SD), concentration of NaFeEDTA at baseline was 3.25 mg/mL under artificial light, 3.25 mg/mL under natural sunlight and 3.34 mg/mL under indirect sunlight/fluorescent light.

Mean values were significantly different from NaFeEDTA concentrations at baseline **p<0.01, ***p<0.001

Most NaFeEDTA degradation in fish sauce occurred within the first 14 d of sunlight exposure. Such exposure might occur in Vietnam, Thailand, or other Asian countries if fish sauces were sold at an outdoor market or by street vendors, or if the sauce remained on the table in an outdoor restaurant. Storage of the fish sauce in amber bottles prevented the sunlight degradation almost completely and storage of fish sauce for up to 1 year in glass or PET bottles under fluorescent light behind glass windows also resulted in little or no degradation. In practical terms, the storage of NaFeEDTA fortified fish sauce should be planned so that degradation of NaFeEDTA
Photostability of NaFeEDTA

is kept to a minimum. The use of amber bottles would be the best approach, otherwise direct sunlight should be avoided by storing the bottles under fluorescent light or in the dark. A large paper label on clear glass or PET bottles might also be an effective barrier to sunlight. However, a thorough evaluation of storage conditions at the wholesale and retail level in Vietnam, Thailand, and other Asian countries is necessary before deciding on the most appropriate bottles.

Table 5: Percentage of NaFeEDTA remaining\(^a\) in aqueous NaFeEDTA solution and in NaFeEDTA fortified soy sauce and Vietnamese and Thai fish sauce stored in the dark

<table>
<thead>
<tr>
<th>days</th>
<th>NaFeEDTA solution</th>
<th>soy sauce</th>
<th>Vietnamese fish sauce</th>
<th>Thai fish sauce</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>100 (0.1)</td>
<td>100 (0.8)</td>
<td>100 (1.6)</td>
<td>100 (1.2)</td>
</tr>
<tr>
<td>28</td>
<td>106 (0.0)</td>
<td>104 (1.2)</td>
<td>100 (0.4)</td>
<td>101 (0.3)</td>
</tr>
<tr>
<td>56</td>
<td>106 (0.5)</td>
<td>100 (1.1)</td>
<td>100 (0.5)</td>
<td>97 (0.5)</td>
</tr>
<tr>
<td>112</td>
<td>107 (1.4)</td>
<td>103 (1.4)</td>
<td>98 (0.8)</td>
<td>98 (0.3)</td>
</tr>
</tbody>
</table>

\(^a\)mean (+/-SD), concentration of NaFeEDTA at baseline was 3.09 mg/mL in aqueous NaFeEDTA solution, 3.10 mg/mL in soy sauce, 3.23 mg/mL in Vietnamese fish sauce and 3.25 mg/mL in Thai fish sauce.

There are 4 potential concerns related to the degradation of NaFeEDTA in fish sauce. These are the loss of mineral binding properties leading to a lower absorption of iron, the precipitation of peptides in the fish sauce, possible flavor changes, and lastly the formation of compounds which could be harmful to human health. The degradation products of EDTA in aqueous solutions are reported to be ethylenediaminetriacetate (ED3A), ethylenediaminediacetate (EDDA), and ethylenediamine monoacetate (EDMA) (Lockhart and Blakeley 1975b). EDMA can further degrade to iminodiacetate (IMDA), glycine, carbon dioxide, and formaldehyde, although the final product of photodegradation is reported to be EDMA (Norwack and Baumann 1998). The progressive loss of carboxylic acid groups from EDTA to give ED3A, EDDA, and EDMA would presumably reduce its mineral binding capacity. However, no precipitation in the stored fish sauce was observed, which would be expected to occur if unbound iron was present. It is also unlikely that 35% degradation of NaFeEDTA would have a major impact on iron absorption since EDTA to Fe molar ratios of 0.5-0.7:1 have been reported to be as effective as a 1:1 molar ratio in enhancing iron absorption from moderately inhibitory meals (MacPhail and others 1994; Hurrell and others 2000). There is no evidence to suspect ED3A, EDDA, EDMA or IMDA have any antiphysiological action. Formaldehyde on the other hand has been reported to be poten-
Photostability of NaFeEDTA

tially carcinogenic (Ash & Ash, 1995). However, based on the evidence that formalde¬
hyde is formed naturally in foods, that it is a normal mammalian metabolite, and that a relatively high threshold exists for carcinogenicity after oral administration the current view is that formaldehyde is not a toxicological problem at daily intakes below 20 mg (Restani & Galli, 1991). Nevertheless, it would seem wise to check formalde¬
hyde levels as well as possible flavor changes in stored NaFeEDTA fortified fish sauce.

Conclusion
NaFeEDTA in soy sauce is photostable during storage and a special packaging does not seem necessary. In fish sauces, however, NaFeEDTA is not stable when the sauces are stored in clear glass or PET bottles exposed to direct sunlight. It may therefore be necessary to protect fortified fish sauces from sunlight by using amber bottles or large labels covering most of the bottle.

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Photostability of NaFeEDTA


Conclusions and Perspectives

The main aim of the present studies was to determine strategies which can potentially be used to optimize iron bioavailability from iron fortificants currently used in iron fortification programs. The results showed that it is possible to achieve this aim by adding ascorbic acid to ferrous fumarate or ferric pyrophosphate fortified foods. Food manufacturers should therefore be encouraged to add adequate amounts of ascorbic acid to foods fortified with these compounds, especially when fortifying with ferric pyrophosphate as this compound is of low bioavailability. Another strategy evaluated to overcome the low bioavailability of ferric pyrophosphate was the optimization of its physico-chemical properties, in this case the particle size. Although decreasing particle size by 25% did not increase iron absorption from ferric pyrophosphate significantly, the 1.4-fold increase in RBV suggested that particle size may influence bioavailability of ferric pyrophosphate. This was further confirmed by the results showing that micronized, dispersible ferric pyrophosphate (Sunactive Fe™) was as available as ferrous sulfate. These results emphasize the importance of a physico-chemical property (particle size) of water-insoluble iron compounds for iron bioavailability and attention should be called towards this fact with the objective of promoting the use of iron fortificants with improved physico-chemical properties.

The addition of Na₂EDTA to ferrous fumarate fortified cereal did not affect iron absorption. Besides showing that Na₂EDTA can not be used as an enhancer in foods fortified with ferrous fumarate, these results demonstrate that finding made with water-soluble iron compounds can not be extrapolated to iron fortificants which have different solubility properties. This later finding was confirmed by the results showing that the magnitude of the enhancing effect of ascorbic acid was significantly lower on iron absorption from ferric pyrophosphate than from ferrous sulfate. These results emphasize the need to evaluate the effect of enhancers and inhibitors on iron absorption from water-insoluble iron compounds.

The results of the present studies further identified erythorbic acid as a potent enhancer of non-heme iron absorption. However, providing cost reduction is the only advantage of this compound over ascorbic acid, its use as enhancer in iron fortified foods should not be recommended as erythorbic acid has no antiscorbutic activity.
Conclusions and Perspectives

Nonetheless, this finding is of great importance as erythorbic acid is used as an anti-oxidant in many processed foods and is therefore present in the diet, especially in the US. Erythorbic acid may therefore already be influencing iron bioavailability. It is further conceivable that erythorbic acid may in the past have influenced results from iron absorption studies and therefore this new knowledge should be taken into account when reviewing previous findings and planning future studies.

Further, the relatively high iron absorption from NaFeEDTA fortified fish sauce and soy sauce indicates the potential usefulness of this iron fortificant for fish sauce and soy sauce fortification programs. The study however also highlights the usefulness of condiments as food fortification vehicles and it would seem of interest to evaluate the suitability of other condiments, for example bullion cubes, as iron fortification vehicles.

Lastly, the clarification whether light affects the stability of NaFeEDTA in fish sauce and soy sauce was important as awareness has be drawn to the fact that FeEDTA-complexes are photolabile. The evaluation showed that NaFeEDTA is not stable in fish sauce when stored in clear glass or PET bottles exposed to direct sunlight. However, the study also demonstrated that NaFeEDTA photodegradation can easily be prevented by using amber colored bottles. Nonetheless, it would seem wise to evaluate whether the degradation of NaFeEDTA in fortified fish sauce affects its quality. For the future it would also seem important to consider the possible photodegradation of NaFeEDTA in all liquid foods which are fortified with this compound.

The results of these studies have identified possible strategies to increase iron absorption from fortification iron. However, these strategies now need to be evaluated in efficacy trials to determine whether iron bioavailability is adequate.
Curriculum Vitae

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