Iron fortification of rice: a potential strategy to counteract iron deficiency?

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IRON FORTIFICATION OF RICE: A POTENTIAL STRATEGY TO COUNTERACT IRON DEFICIENCY?

A dissertation submitted to the

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For the degree of

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2006
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>AOAC</td>
<td>Association of Official Analytical Chemists (AOAC International)</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for disease control and Prevention, Atlanta</td>
</tr>
<tr>
<td>CHr</td>
<td>Hemoglobin content of reticulocytes</td>
</tr>
<tr>
<td>DcytB</td>
<td>Brush border iron reductase</td>
</tr>
<tr>
<td>DMT1</td>
<td>Dimetal iron transporter</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EAR</td>
<td>Estimated average requirement</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and agriculture organization of the United Nations, Rome</td>
</tr>
<tr>
<td>GDP</td>
<td>Gross domestic product</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally recognized as safe</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HFE</td>
<td>Hemochromatosis protein</td>
</tr>
<tr>
<td>HTST</td>
<td>High temperature short time</td>
</tr>
<tr>
<td>HYPO</td>
<td>percentage of hypochromic erythrocytes</td>
</tr>
<tr>
<td>ID</td>
<td>Iron deficiency</td>
</tr>
<tr>
<td>IDA</td>
<td>Iron deficiency anemia</td>
</tr>
<tr>
<td>IoM</td>
<td>Institute of Medicine</td>
</tr>
<tr>
<td>IQ</td>
<td>Intelligence quotient</td>
</tr>
<tr>
<td>IRE</td>
<td>Iron responsive elements</td>
</tr>
<tr>
<td>IREG1</td>
<td>Ferroportin1</td>
</tr>
<tr>
<td>IRP</td>
<td>Iron regulatory proteins</td>
</tr>
<tr>
<td>IRRI</td>
<td>International rice research institute</td>
</tr>
<tr>
<td>JECFA</td>
<td>Joint FAO/WHO expert committee on food additives</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean corpuscular volume</td>
</tr>
<tr>
<td>MDFP</td>
<td>Micronised dispersible ferric pyrophosphate</td>
</tr>
<tr>
<td>MGFP</td>
<td>Micronised ground ferric pyrophosphate</td>
</tr>
<tr>
<td>MPS</td>
<td>Mean particle size</td>
</tr>
<tr>
<td>PATH</td>
<td>Program for appropriate technology in health</td>
</tr>
<tr>
<td>PDS</td>
<td>Public distribution system</td>
</tr>
<tr>
<td>RBV</td>
<td>Relative bioavailability</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended dietary allowance</td>
</tr>
<tr>
<td>SF</td>
<td>Serum ferritin</td>
</tr>
<tr>
<td>sTfR</td>
<td>Soluble transferrin receptor</td>
</tr>
<tr>
<td>TfR</td>
<td>Transferrin receptor 1</td>
</tr>
<tr>
<td>TfR2</td>
<td>Transferrin receptor 2</td>
</tr>
<tr>
<td>TIBC</td>
<td>Total iron binding capacity</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization, Geneva</td>
</tr>
<tr>
<td>ZPP</td>
<td>Zink protoporphyrin</td>
</tr>
</tbody>
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SUMMARY

Background
Rice is a major staple food worldwide but is a poor source of dietary iron when it is milled and refined. Rice-eating populations are therefore at risk of iron deficiency and iron deficiency anemia (IDA). In south East Asia, where rice is a major staple food, prevalence of IDA is estimated to be ≥50% (WHO). Iron fortification of rice is challenging, as rice is consumed as intact grains and iron compounds that typically cause fewer adverse organoleptic changes tend to have reduced bioavailability. To judge the bioavailability of iron compounds, the relative bioavailability is defined as the fractional absorption in relation to ferrous sulfate. Ferric pyrophosphate might be a promising iron fortification compound; however, there are few data on the effect of processing, ascorbic acid addition, food matrix, and iron status on its bioavailability.

Aims
i) The primary aim of this thesis was to develop iron-fortified rice with acceptable sensory characteristics and ii) test its biological efficacy in an iron deficient population.

iii) A further aim was to investigate the effect of processing, food matrix, and ascorbic acid on the bioavailability of ferric pyrophosphate. By comparing its absorption with ferrous sulfate, the relative bioavailability to ferrous sulfate was calculated.

Design
i) Traditional and novel iron fortification compounds were screened as suitable iron fortificants for iron fortified rice produced via an extrusion-premix approach. Iron fortified rice was characterized instrumentally via colorimetric measurements, by measuring the texture of cooked rice grains and by quantification of iron loss by rinsing. Blended with natural rice at a 1:100 or 1:200 ratio, iron fortified rice was compared to unfortified rice in triangle tests in a panel of Caucasian testers (n=18).
Summary

ii) The efficacy of extruded iron fortified rice was tested in a randomized, controlled, double-blind school feeding trial in 6-13 year old children, treated against intestinal parasites in Bangalore, India. Extruded rice was fortified with micronised ground ferric pyrophosphate (mean particle size 2.5 m) to provide 20 mg Fe/day. Iron status (SF, sTfR, Hb, body iron stores) and anthropometric measures were assessed at baseline, midpoint (3.5 months) and at the end of the trial (7 months).

iii) The effect of food matrix, ascorbic acid, processing and iron status on iron absorption and relative bioavailability of micronised dispersible ferric pyrophosphate (MDFP) were investigated. Isotopically labeled $^{57}$Fe-MDFP was produced and extruded into artificial rice grains. Bioavailability of ferric pyrophosphate and ferrous sulfate were measured in a processed and unprocessed rice meal and in an infant cereal with and without ascorbic acid (4:1 ascorbic acid: Fe molar ratio). Iron absorption was measured in 26 subjects based on erythrocyte incorporation of stable isotope labels after 14 days.

Results

i) Ferric pyrophosphate was the only iron fortification compound which resulted in small, but acceptable, color discolorations in extruded iron fortified grains. Ferrous sulfate, elemental iron, encapsulated ferrous sulfate, and NaFeEDTA all resulted in unacceptably colored grains. Cooked rice grains had comparable texture to natural rice and losses after rinsing were low. In a 5 month storage trial, no significant color reactions could be detected. In triangle tests, rice grains fortified with ferric pyrophosphate closely resembled to natural rice in both raw and cooked form.

ii) Extruded iron fortified rice given for 7 months in a school feeding program was efficacious in increasing body iron stores and prevalence of iron deficiency in 6-13 year old children (n=184). In the iron group, there were significant improvements in serum transferrin receptor and ferritin concentrations (p<0.05), and a significant increase in body iron stores (p<0.001) compared to the dewormed control group. There was a significant effect of time (P<0.01) and treatment (P=0.010) on the prevalence of ID,
which fell from 78% to 25% in the iron group and from 79% to 49% in the
dewormed control. IDA decreased from 30% to 15% in the iron group but
remained virtually unchanged in the controls (28% and 27%; N.S; P=0.161).
Compliance to the rice fortified meals was excellent, as the iron treatment
group did not consume less rice than the group receiving the unfortified
control.

iii) In the bioavailability studies, geometric mean iron absorption from the
wheat-based meal fortified with MDFP was 2.0% compared to 3.2% for
ferrous sulfate (RBV=62). In the rice meals, mean iron absorption from MDFP
added to the rice at time of feeding was 1.7% compared to 11.6% for ferrous
sulfate (RBV=15). The mean iron absorption from MDFP extruded into artificial
rice grains was 3.0% compared to 12.6% from ferrous sulfate in unprocessed
rice (RBV=25).

Presence of ascorbic acid (P<0.001), SF (P<0.01) and meal type (P<0.01)
were significant predictors of iron absorption from ferrous sulfate. In contrast,
presence of ascorbic acid (P<0.001) was the only significant predictor of iron
absorption from MDFP; processing (P=0.073), type of meal (P=0.133) and SF
(P=0.225) were not significant determinants. Meal type (P<0.001), SF
(P<0.001) and processing of the MDFP compound (P<0.01) were significant
predictors of RBV of MDFP.
Conclusions

i) Iron fortified rice with excellent sensory characteristics can be produced with an extrusion premix approach using micronised ground ferric pyrophosphate as an iron fortificant.

ii) In a school feeding program, extruded iron fortified rice is efficacious in increasing body iron stores and decreasing iron deficiency in combination with deworming.

iii) The use of a single RBV value to estimate of the potential efficacy of an insoluble iron compound as a food fortificant may be of limited utility. We showed that relative bioavailability of an experimental form of ferric pyrophosphate can be strongly affected by food matrix and iron status of the test subjects.
SOMMARIO

Contesto
Il riso è un importante alimento di base ma contiene scarse quantità di ferro quando raffinato. Popolazioni che consumano grosse quantità di riso sono quindi sottoposte al rischio di sviluppare deficienza di ferro (DF) e anemia sideropenica (AS). Nel sud-est Asiatico, dove il riso è un alimento di base di centrale importanza, la prevalenza di AS nella popolazione comune è stimata al 50% (OMS/WHO).
La difficoltà di fortificare il riso con il ferro risiede in due classi di problemi. Il riso è comunemente consumato in forma di grani, e i composti di ferro che creano minori problemi sensoriali tendono ad essere poco bio-assimilabili. Per valutare l'assorbibilità biologica dei composti di ferro, l'assorbibilità relativa (RBV) è definita come il rapporto tra l'assorbimento della sostanza testata e un'identica dose di solfato di ferro. Il pirofosfato ferrico è una promettente sostanza per la fortificazione alimentare, ma pochi dati sono stati pubblicati sull'influsso sulla bio-assorbibilità di trattamenti termici, dell'aggiunta di acido ascorbico, della natura del cibo fortificato e della condizione del soggetto.

Obiettivi
i) Sviluppare un riso fortificato di ferro con proprietà sensoriali accettabili e ii) verificare la sua efficacia biologica in una popolazione con deficienza di ferro.
iii) Investigare l'effetto di trattamenti termici, dell'aggiunta di acido ascorbico, della natura del cibo fortificato e della condizione del soggetto sull'assorbibilità del pirofosfato ferrico. Paragonando l'assorbimento ottenuto con quello da solfato di ferro è stato calcolato l'assorbimento relativo.
Sommario

Procedure utilizzate
i) Il riso fortificato è stato prodotto tramite estrusione. Una varietà di composti di ferro nuovi e tradizionali sono stati esamitati come potenziali veicoli di fortificazione. Il riso cosi prodotto è stato esaminato tramite misurazioni colorimetriche, misurando le caratteristiche fisiche dei chicchi cotti e quantificando le perdite di ferro dopo il lavaggio. Il riso fortificato, ottenuto mischiando i chicchi con riso naturale (premix, 1:100, 1:200), è stato poi paragonato a riso non fortificato tramite test triangolari in soggetti caucasici (n=18).

ii) L’efficacia del riso estrudato e fortificato è stata poi testata in un programma scolastico a Bangalore, India in soggetti d’età compresa tra i 6-13 anni (n=184), trattati contro parassitosi intestinali, tramite uno studio randomizzato e condotto in modalità doppio cieco (double blind). Il riso è stato fortificato con pirofosfato ferrico micronizzato (grandezza media > 2.5 µm) per fornire aggiuntivi 20 mgFe/ giorno. Misure antropometriche e parametri biochimici (SF, sTfR, Hb, Ferro corporeo) sono stati determinati all’inizio dello studio, dopo 3.5 mesi e dopo 7 mesi.

iii) Sono stati investigati gli effetti di trattamenti termici, dell’aggiunta di acido ascorbico, della natura del cibo fortificato e della condizione del soggetto sull’assorbibilità del pirofosfato ferrico micronizzato e disperdibile (MDFP). Un MDFP isotopicamente marcato ([57Fe]- MDFP) è stato prodotto ed estrudato in chicchi di riso artificiali. La bio-assorbibilità del pirofosfato ferrico è stata misurata in un pasto a base di riso termicamente trattato e non, e in cereale per bebè con e senza l’aggiunta di acido ascorbico (rapporto molare acido ascorbico : ferro di 4:1). L’assorbimento di ferro è stato misurato in 26 soggetti tramite la misurazione dell’incorporazione di marcatori isotopici stabili negli eritrociti dopo 14 giorni.

Risultati
i) Il pirofosfato ferrico è risultato l’unico composto a creare piccoli, ma accettabili cambiamenti di colore nel riso. Solfato di ferro, ferro elementare, ferro solfato incapsulato, NaFeEDTA creano invece differenze di colore inaccettabili. Chicchi estrudati e cotti hanno delle caratteristiche fisiche
paragonabili al riso naturale, e le perdite di ferro durante il lavaggio sono basse. Durante 5 mesi di conservazione, nessun cambiamento di colore significante < stato misurato. Nei test triangolari, con riso crudo e cotto < stata ravvisata una vicina somiglianza del riso fortificato con il riso naturale.

ii) Il riso estrudato e fortificato con ferro < stato efficace nell'aumentare il ferro corporeo e nel ridurre la prevalenza di DF in una popolazione di soggetti trattata contro parassitosi intestinali. Paragonati al gruppo di controllo, il gruppo trattato ha significativamente migliorato le concentrazioni di sTfR, SF (p<0.05) e ferro corporeo (p<0.001). Tempo (p<0.01) e trattamento (p=0.010) sono stati identificati come fattori determinanti la prevalenza di DF, che < diminuita dal 78 al 25% nel gruppo trattato, e da 79% a 49% nel gruppo di controllo. L'AS < diminuita dal 30 al 15% nel gruppo trattato (P=0.161, N.S.), mentre < rimasta in pratica invariata nel gruppo di controllo (da 28 a 27%). L'accettabilit< del riso nel programma scolastico < stata valutata come soddisfacente, visto che il gruppo assegnato al riso fortificato non ha consumato meno riso del gruppo assegnato al riso di controllo (non fortificato).

iii) Negli studi di bio-assorbimento, la media geometrica di assorbimento di ferro dal céréale per beb< fortificato con MDFP < stata 2% paragonata con 3.2% di assorbimento da solfato di ferro (RBV=62). Nei pasti a base di riso fortificati senza trattamento termico, l'assorbimento da MDFP < stato di 1.7% paragonato al 11.6% da solfato di ferro (RBV=15). L'assorbimento da MDFP estrudato in chicchi artificiali di riso < stato del 3.0% paragonato con un assorbimento da solfato di ferro del 12.6% (RBV=25). La presenza d'acido ascorbico (p<0.001) < stato l'unico fattore ad influenzare significativamente l'assorbimento di MDFP; trattamento termico (p=0.073), composizione del cibo (p=0.133) e SF (p=0.225) non hanno influenzato l'assorbimento in modo significativo. Il tipo di cibo (p<0.001), SF (p<0.001) e il trattamento termico (p<0.01) sono invece fattori d'influsso significativi sul RBV del MDFP.

Conclusioni
i) Riso fortificato con ferro con eccellenti caratteristiche sensoriali pu< essere prodotto tramite estrusione e premix utilizzando ferro pirofosfato micronizzato come composto fortificante.
ii) In un programma scolastico, il riso fortificato con ferro ha efficacemente aumentato il ferro corporeo e ha diminuito la prevalenza di deficienza di ferro in combinazione con il trattamento contro parassiti intestinali.

iii) L’utilizzo di un singolo parametro (RBV) per stimare la potenziale efficacia di composti di ferro insolubili in acqua potrebbe essere di poca utilità. I dati ottenuti indicano che il relativo bio-assorbimento da una forma sperimentale di pirofosfato ferrico può essere fortemente influenzato sia dal cibo scelto che dal deficit di ferro nei soggetti studiati.
ZUSAMMENFASSUNG

Hintergrund
Reis ist ein weltweit wichtiges Grundnahrungsmittel, jedoch in polierter Form arm an Eisen. Bevölkerungen, die sich einseitig und hauptsächlich von Reis ernähren, sind deshalb dem Risiko ausgesetzt Eisenmangel (EM) oder Eisenmangelanämie (EMA) zu entwickeln. In Südostasien, wo Reis das wichtigste Grundnahrungsmittel ist, liegt die Prävalenz von EMA bei ca. ≈50% (WHO).


Ziele
i) Das erste Ziel dieser Dissertation war die Entwicklung von eisenfortifiziertem Reis mit akzeptablen sensorischen Eigenschaften, sowie ii) die Bewertung seiner biologischen Wirksamkeit in einer Population mit Eisenmangel.


Aufbau der Studien
i) Eisenangereicherter Reis wurde mittels Extrusion produziert, und traditionelle und neu entwickelte Eisen-Verbindungen wurden als mögliche Fortifizierungsmittel untersucht. Der so hergestellte Reis wurde instrumentell
I risultati:

i) L'eisenpyrophosphat risultò essere l'unica combinazione di ferro che causò colorazioni, anche se accettabili, in riso extrusato. L'eisensulfato, il ferro elementare, il ferro enkapsulato e NaFeEDTA causavano comunque forti cambiamenti colorativi. I grani di riso extrusati ondulati avevano una consistenza analoga al riso naturale, e le perdite di ferro dopo lavaggio erano trascurabili. Non si registrarono cambiamenti colorativi significativi in un esperimento a lungo termine di 5 mesi.
In Dreiecks-Tests war eisen angereicherter Reis mit natürlichen Reis vergleichbar.

ii) Eisenangereicherte Reismahlzeiten wurden über 7 Monate an 6-13 Jahre alte Kinder (n=184) als Teil eines Schulmahlzeit-Programms verteilt. In der Eisen-Gruppe, die wie die Kontrollgruppe gegen Darm-Parasiten behandelt wurde, gab es Signifikante Verbesserungen des Serumferritins, des Transferrin Rezeptors (P<0.05) sowie der Eisenspeicher (P<0.001).

Zeit (P<0.01) und Behandlung (P=0.010) hatten einen signifikanten Effekt auf die Prevalenz von EM, die von 78% auf 25% (Eisengruppe) und 79% auf 49% (Kontrollgruppe) abnahm. EMA nahm in der Eisengruppe von 30% auf 15% ab (P=0.161, N.S.) und blieb in der Kontrollgruppe praktisch unverändert (28-27%). Die Befolgung des Studien Protokolls (Compliance) war gut, da die Eisengruppe nicht weniger Reis konsumierte als die Kontrollgruppe.

iii) Der geometrische Mittelwert der Eisenabsorption aus der mit MDFP fortifizierten Weizenmahlzeit war 2.0%, verglichen mit 3.2% von Eisensulfat (RBV=62%). In den Reismahlzeiten war die mittlere Absorption von nicht thermisch behandelter MDFP 1.7%, verglichen mit 11.6 % für Eisensulfat (RBV=15). Die mittlere Absorption aus MDFP, das in Reiskerne extrudiert wurde, war 3.0%, verglichen mit 12.6% Absorption aus Eisensulfat (RBV=25%).

Ascorbinsäure (P<0.001), Serumferritin (P<0.01) und Art der Mahlzeit (P<0.01) waren signifikante Einflussfaktoren hinsichtlich der Absorption von Eisensulfat. Dagegen war Ascorbinsäure der einzige signifikante Parameter, der die Absorption von MDFP beeinflusste (P<0.001); thermische Behandlung (P=0.073), Mahlzeit (P=0.133) und Serumferritin (P=0.225) hatten keinen signifikanten Einfluss. Die relative Verfassbarkeit von MDFP wurde von der Mahlzeit (P<0.001), von Serumferritin (P<0.001) und von der thermischen Behandlung (P<0.01) signifikant beeinflusst.

Schlussfolgerungen

i) Eisen angereicherter Reis mit guten sensorischen Eigenschaften kann mittels eines Extrusion-Premix Ansatzes und mikronisiertem Eisenpyrophosphat produziert werden.
ii) In einem schulbasierten Programm, und in Kombination mit der Entwurmungsbehandlung aller Studienteilnehmer, konnten die Eisenspeicher erhöht werden und die Prävalenz von Eisenmangel verringert werden.

INTRODUCTION

Ancient Greeks and Romans attributed to iron therapeutic properties, and administered it to injured soldiers to treat "muscle weakness", likely caused by hemorrhagic anemia. Hippocrates might have been the first physician to describe chlorosis, or "green sickness", a disease often diagnosed in the 17th-18th centuries. Patients appeared pale, tired and lethargic, and the condition was partly associated with being in love, probably because it mainly affected young unmarried women. Although chlorosis is not anymore diagnosed, it can be considered a form of iron deficiency, as iron supplements were later shown to effectively treat the disease (Beutler, 2002).

Today, iron deficiency (ID) and iron deficiency anemia (IDA) are major public health problems worldwide (WHO, 2001). In India, prevalence of anemia is estimated to be 88% and 74% in pregnant and non pregnant women, respectively (WHO, 2001). IDA can have heavy consequences on the independence and productivity of people affected, as it influences work capacity, school achievement, pregnancy outcome and immune status. It has been estimated that in countries with high IDA prevalence, the economic cost of this disease can be in the range of a percentage point of the gross domestic product (GDP; Horton and Ross, 1992).

Iron fortification of staple foods with iron is considered one of the most promising and sustainable strategies to counteract IDA. Rice is a major staple food in south East Asia, were people typically consume 200-300 g of rice per day. Nineteen percent of the world rice production is consumed in the developing world (FAO, 2003), and a monotonous diet based on milled rice likely contributes to the development of iron deficiency. Iron fortification of rice could therefore help to reduce the burden of ID and IDA in rice eating populations.

This thesis begins with a literature review, where the fields of iron biochemistry, etiology of IDA, food fortification and rice fortification are reviewed.
Introduction

The first manuscript of this thesis describes the development and the evaluation iron fortified rice, with a description of its technological and sensorial proprieties. The second manuscript is an investigation on the effect of iron status and different food matrixes on the relative bioavailability of ferric pyrophosphate, a promising iron fortification compound. In the third manuscript, the biologic efficacy of iron fortified rice is investigated in a randomized, controlled trial in iron deficient, Indian school children.

REFERENCES

CHAPTER 1 - BIOCHEMISTRY OF IRON

1.1 Iron metabolism

Iron is an essential element of all eukaryotes and for nearly all prokaryotes (Kaplan, 2002). As a transition metal, it is an important component of a broad range of physiological oxidation/reduction reactions, and its chemical proprieties have forced living organisms to evolve complex mechanisms of for absorption, transport and delivery.

1.1.1 Chemistry of iron

Iron has several oxidation states, ranging from Fe$^{6+}$ to Fe$^{2+}$ depending on the chemical environment. In aqueous solution ferric (Fe$^{3+}$) and ferrous (Fe$^{2+}$) iron are the only soluble species (Lee et al., 1979). Therefore, in biological systems, ferric and ferrous ions are the predominant iron forms (Emery, 1982). In strong acid solutions ferrous iron exists in a complex with six molecules of water. With increasing pH some protons are lost from the complex generating Fe(OH)$_2$ which has maximal solubility at pH=7 (0.1M). By increasing the pH of a watery solution of ferric ions, Fe(OH)$_3$ is generated, which is virtually insoluble ($10^{-18}$M) at neutral pH (Brody, 1999). The behavior of ionic iron in solution can largely explain the effect of intraluminal factors on iron absorption (Conrad and Umbreit, 2000; Miret et al., 2003).

An additional chemical property of iron is that the ferrous ion can be a potent prooxidant; it can catalyze the reaction of hydrogen peroxide to hydroxyl radical (Brody, 1999; Emery, 1982), or generate the superoxide anion by reacting with oxygen (Kaplan, 2002). All organisms that utilize iron have therefore developed complex mechanisms to deal with iron's toxicity and insolubility. These strategies are, with the exception of Archea, highly conserved through the biological kingdoms (Kaplan, 2002).

In the human cell, iron is stored by ferritin, a ubiquitous protein consisting in 24 subunits surrounding a core of up to 4500 ferric iron atoms. Ferritin
synthesis is tightly regulated by the intercellular iron concentration (Eisenstein, 2000) and it has the function of sequestering the metal from the intracellular labile iron pool to achieve both iron storage and detoxification (Harrison, 1977). From the basolateral membrane of the enterocytes in the duodenum, iron is transported by transferrin, which functions to solubilize Fe$^{3+}$, attenuate its reactivity and facilitate the delivery of iron to the cells (Andrews, 2002).

1.1.2 Molecular processes of iron absorption

The current knowledge on the mechanisms regulating intestinal iron absorption has been dependent on the study of inherited disorders of iron homeostasis in human and animals (Anderson et al., 2005). In man the duodenum is the primary site for the regulation of iron homeostasis, as there is no mechanism to regulate iron loss (Hentze et al., 2004). Iron absorption can be divided in two distinct processes (Miret et al., 2003). First, iron enters the enterocyte from the intestinal lumen through the brush border membrane (uptake); secondly it passes the basolateral membrane of the enterocyte to reach the circulatory system (transport).

Ferric iron is reduced at the enterocyte brush border via iron reductase (DcytB), after which ferrous iron is transported into the enterocyte by the divalent metal transporter (DMT1). DMT1 also transports zinc, copper, cobalt and lead, and is coupled with an energy-dependent proton transport. An additional pathway has been proposed for absorption of ferric iron (Conrad et al., 2000) through mobilferrin and a $\beta_3$ integrin. A large complex is formed containing mobilferrin, integrin, falvin monoxigenase and DMT1 and serves as ferric reductase to make available ferrous iron inside the cell (Conrad and Umbreit, 2002). Absorbed iron in the enterocyte can be then either stored intercellularly as ferritin and be excreted in the feces with enterocyte turnover, or be transported across the basolateral membrane through the likely concerted action of ferroportin 1 (IREG1) and hephaestin (Anderson et al., 2005; Fleming, 2005).
As opposed to ionic iron, heme iron is absorbed intact into the enterocyte (Hallberg, 1981; Uc et al., 2004). Recently an intestinal heme transporter has been identified (Shayeghi et al., 2005). The fact that heme iron is not released from the heme moiety prior to uptake explains why it is less susceptible to the variation in dietary composition and to intraluminal factors than inorganic iron (Hallberg, 1981). After uptake, the transport of heme iron is dependent upon the same regulatory mechanisms than non heme iron (Fleming, 2005), as heme iron joins the same iron pool as non heme iron after uptake (Hallberg and Solvell, 1967). It has been reported, however, that absorption of heme iron is less affected by iron status than absorption from non heme iron (Hallberg et al., 1997; Roughead and Hunt, 2000). Although the reasons for this are unclear, a teleological view would suggest that preferential absorption of heme iron would be energetically advantageous, as less heme neogenesis would be needed for the production of hemoglobin.

1.1.3 Intraluminal factors

The complex processes in the intestinal lumen affecting the solubility and the diffusive proprieties of iron are far from being completely understood (Miret et al., 2003). Iron is released from the food matrix and other ligands in the stomach. This process is essential, as inability to secrete hydrochloric acid (achlorydria) produces iron deficiency (Conrad and Umbreit, 2002). Solubilized ferric iron is reduced or chelated by various food and intestinal derived substances (mucin) to be kept in solution when iron enters the less acidic medium in the duodenum. Mucin has been discussed as an important iron ligand in the duodenum, as iron must traverse the mucus layer to come in contact with the intestinal cell receptors. In a model recently proposed by Conrad, vesicles of iron transport proteins travel out of the enterocyte to the mucin layer in the lumen, where they bind iron and are successively internalized into the cell (Conrad and Umbreit, 2001).
1.1.4 Distribution of iron in the body

The total amount of iron present in a healthy individual can be estimated to be approximately 4 g (Hentze et al., 2004). Most of the iron (about 2 g) is contained in the erythron, the mature erythrocytes and their precursors in the bone marrow. The reticuloendothelial macrophages (0.6 g) retrieve senescent erythrocytes and release iron to transferrin for reutilization. Transferrin bound iron accounts for a minor (3-8 mg) but very dynamic pool of iron, as up to 30-35 mg per day are transported and reutilized (Wessling-Resnick, 2000). The amount of additional iron can vary broadly between individuals, and occurs as stores in the liver (1g) and in other tissues (400 mg; Hentze et al., 2004). At steady state, only approx. 1-2 mg of iron are usually absorbed daily which roughly cover physiological iron losses through urine, sweat, and exfoliation of cells of the skin and gastrointestinal mucosa (Wessling-Resnick, 2000).

1.1.5 Regulation of iron metabolism

Several physiological processes have been found to influence iron absorption. These include the amount of body iron stores, erythropoietic activity, hemoglobin concentration, oxygen content of the blood and chronic inflammation (Fleming, 2005). Intracellular regulation of iron metabolism occurs through iron regulatory proteins (IRP) which interact with noncoding sequences of mRNA, the iron responsive elements (IRE), to modulate the translation of iron transport proteins, sensitively reacting in response to intracellular iron concentration. IRE have been identified for ferritin, transferrin, DMT1 and Ferroportin 1 (Pietrangelo, 2002). Whereas these mechanisms regulate the expression of iron transport proteins at a cellular level, additional mechanisms are likely needed to modulate absorption by sensing systemic iron needs.

Two models are currently proposed to describe the regulation of iron absorption. In the crypt programming model, undifferentiated duodenal crypt cells take up iron from the blood stream with the interaction of HFE
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(hemochomatosis protein) and TfR, two proteins "sensing" the iron concentration in the blood stream. The intracellular iron concentration in the undifferentiated enterocyte programs the absorptive capacity of the mature enterocyte (Pietrangelo, 2004). However, this model has been challenged by the discovery of hepcidin, likely to directly interact with the mature enterocyte (Frazer and Anderson, 2003; Pietrangelo, 2004).

In recent years, the role of the liver as a regulator of iron homeostasis has received increased attention (Fleming, 2005; Frazer and Anderson, 2003). Genes that code for hepcidin (HAMP), TfR2 and hemojuvelin (HFE2) are highly expressed in the liver, and mutations in these genes cause hemochromatosis. The physiological role of hemojuvelin and TfR2 has not been elucidated. Hemojuvelin and TfR2 might modulate hepcidin expression, by sensing the concentration of saturated transferrin in serum (Johnson and Enns, 2004; Robb and Wessling-Resnick, 2004), thus contributing to the postulated liver mediated-regulatory pathway.

Hepcidin is a small molecule which resembles peptides from the innate immunity-system, it has antimicrobial proprieties and increasing evidence is strongly suggesting its role as an iron regulatory hormone (Ganz, 2003). Its name derives from the site of synthesis (hep-) and its antimicrobial proprieties (-cidin).

Hepcidin synthesis can be induced by iron loading (Pigeon et al., 2001). Patients with anemia caused by chronic inflammation have a 100-fold hepcidin concentration in urine, whereas less acute infections produce smaller increases (Nemeth et al., 2003). Iron metabolism would be strongly influenced by anemia and hypoxia, and an effect of these two conditions on hepcidin concentration has been shown (Nicolas et al., 2002).

Hepcidin synthesis inhibits iron absorption in the proximal intestine and iron release from reticuloendothelial macrophages (Ganz, 2005). Two mechanisms of action of hepcidin on the hepatocyte have been proposed: (i) the binding to ferroportin and the successive internalization and inhibition of the iron transporter (Nemeth et al., 2004) or (ii) a direct effect on DMT1 without effect on ferroportin (Laftah et al., 2004; Yamaji et al., 2004). TfR2 and
hemojuvelin were both proposed to constitute an iron sensor in the hepatocytes, where they sense serum transferrin saturation and induce the synthesis of hepcidin (Fleming, 2005).

1.1.6 Iron overload and inherited disorder of iron homeostasis

Inherited disorders of iron metabolism that lead to iron deficiency are extremely rare in humans, and this finding might be a further indication of the essentiality of the metal (Anderson et al., 2005). In the general population, the evidence of a causal relationship between iron intake and coronary heart disease, cancer and diabetes has been matter of debate (Schumann et al., 2002). Evidence that elevated iron status increases the risk of coronary heart disease and diabetes is at present not convincing (Sempos, 2002), as it is very difficult to correct the studies for potential dietary and biochemical confounders (Heath and Fairweather-Tait, 2003).

Classic hemochromatosis is likely to have originated in a single mutation (C282Y) in a common ancestor in northwestern Europe and caused no serious obstacle to reproduction as the disease is mainly of adult onset. It may even have conferred some evolutionary advantages (e.g. resistance to iron deficiency, Pietrangelo, 2004). Homozygosity for C282Y is found in 5/1000 persons of northern European descent, who develop liver cancer, cardiomyopathy and diabetes mellitus considerably more frequently than the normal population (Heath and Fairweather-Tait, 2003). In classic hemochromatosis the mutation induces a conformational change in the HFE protein that impairs its interaction with the TfR receptor. The role of HFE in the normal cell is not completely clear (Pietrangelo, 2002), but it appears to facilitate TfR uptake of transferrin bound iron (Pietrangelo, 2004). In hereditary hemochromatosis, duodenal transfer of iron to the plasma is inappropriately high, reflecting either a pathogenic abnormality in the enterocytes themselves or a disruption of regulatory signals; it appears however, that in hemochromatosis enterocytes have a relatively iron deficient
phenotype, which induces increased expression of iron transport proteins (Pietrangelo, 2004).

C282Y homozygotes have elevated transferrin saturation and a three- to five-fold increase in serum ferritin concentrations compared to their wild type counterparts (Heath and Fairweather-Tait, 2003). Phlebotomy is very effective in treating the symptoms of hemochromatosis, and screening of the overall population has been debated (Pietrangelo, 2004). There is however disagreement on the extent by which the presence of C282Y mutation predicts the disease, as the prevalence of diagnosed hemochromatosis is not as high as the prevalence of C282Y homozygosity, and mass under-diagnosis is not a likely explanation (Heath and Fairweather-Tait, 2003). In studies in Australia and Norway, hepatic fibrosis/cirrhosis was found in 25% and 10%, respectively of the screened homozygote-population (Asberg et al., 2001; Olynyk et al., 1999). Further research is needed to determine the true penetration of the disease in the general population and to determine the factors (genetic or environmental) that might modulate the onset of the disease in homozygotes.

Heterozygosity of the C282Y mutation is frequent (9% of the general population of northern European descent) and is associated with small increases in transferrin saturation and no increase of serum ferritin compared with the normal population (Heath and Fairweather-Tait, 2003). Conflicting results have been reported on the increased risk of coronary hearth disease in C282Y heterozygotes, and current research suggests no increased risk of diabetes (Heath and Fairweather-Tait, 2003). In a study published this year, iron absorption from a complete diet was not different between heterozygote and wild types for the C282Y mutation, and serum ferritin and serum iron (and not genotype) were the only significant determinants of iron absorption. However the authors argued that their data cannot exclude a subtle effect of the heterozygote genotype (Roe et al., 2005).
Other mutations in iron genes coding for iron transport proteins can generate inherited iron overload. These include additional mutation on the HFE gene (H63D and S65C); mutations of the genes coding for TfR2 and HFE2 (juvenile hemochromatosis), HAMP (coding for hepcidin) and mutations in the ferroportin gene. The latter mutation has been reported in different ethnic groups (Pietrangelo, 2004) and in a sub-Saharan population showing symptoms of iron overload previously attributed to dietary excess (Gordeuk et al., 2003).

1.1.7 Consequences of iron deficiency

Compiling data about the global prevalence of anemia and iron deficiency can be challenging due to the fact that only a few countries collect data on their anemia prevalence. However, non-representative estimates can be generated from isolated reports and hospital records (Allen and Casterline-Sabel, 2001). The World Health Organization estimates that about 2 billion individuals or about 40% of the world’s population suffer from anemia. Anemia affects roughly 3 to 4 times more people in non-industrialized regions than in developed countries, afflicting principally pregnant women and preschool children (52% and 39% prevalence of anemia, respectively), but also men (30%), elderly people (45%) and pregnant and non pregnant women (42%; WHO, 2001). It has been estimated that 75% of anemia is due to iron deficiency (Allen and Casterline-Sabel, 2001). In Southeast Asia, prevalence of anemia is particularly high with an estimate of 53% among all population groups (WHO, 2001).

Iron deficiency anemia produces nonspecific symptoms such as tiredness and lack of energy but can also have far-reaching consequences on pregnancy outcome (Ramakrishnan, 2001), child growth (Angeles et al., 1993; Chwang et al., 1988), immune status and resistance to infections (de Silva et al., 2003), work capacity (Haas and Brownlie, 2001) and cognitive development (Lozoff and Wachs, 2001). These symptoms are mainly associated with iron deficiency anemia, but there is evidence that iron deficiency without anemia
Iron deficiency decreases work capacity in women (Brownlie et al., 2004; Haas and Brownlie, 2001) and affects cognition (Bruner et al., 1996; Stoltzfus et al., 2001). It has been suggested that tissue iron deficiency rather than anemia affect psychomotor development in children (Beard, 2001), however this has not been firmly established (Lynch, 2005).

Two additional functional consequences of iron deficiency are its effects on thyroid metabolism and chronic lead intoxication. Iron deficiency has been shown to negatively affect thyroid metabolism in children with goiter receiving iodine supplements (Zimmermann et al., 2000). Children receiving double fortified salt with iron and iodine had significantly lower thyroid volumes compared to children receiving iodized salt alone in a 40 week fortification trial in northern Morocco (Zimmermann et al., 2003). The interaction between iron and iodine metabolism shown in these studies could be due to a lower thyroidperoxidase activity in iron deficiency (Hess et al., 2002).

Elevated blood lead levels impair neurological development in children (Baghurst et al., 1992; Bellinger, 2004) and no discernible blood lead threshold concentration can be defined for its adverse effects (CDC, 1991; WHO, 1995). Adverse effects of blood lead on IQ are likely to occur even at lower concentrations than the accepted cutoff for lead poisoning, i.e. 10 μg/dl (Lanphear et al., 2005). Low iron intake (Hammad et al., 1996) and low iron stores are associated with increased lead load in a recent population studies (Wright et al., 2003). However, both conditions share many of the same social risk factors (Kwong et al., 2004). Although strong epidemiologic indications suggest a biologic interaction between iron deficiency and lead absorption, until recently, no convincing evidence from randomized trials had been generated (Kwong et al., 2004). However, a recent, 4 month randomized controlled trial in school aged children, iron fortification of rice in combination with deworming significantly decreased blood lead levels. In the group receiving iron fortified rice, prevalence of elevated blood lead levels (>10 μg/dl) decreased form 70% to 25%, whereas in the non fortified, dewormed
control prevalence of elevated blood lead levels decreased from 76% to 55% (Zimmermann et al., 2006).

Iron deficient rats absorb a larger fraction of lead (Robertson and Worwood, 1978). In the only human study examining lead absorption and iron absorption in the same subjects, a significant correlation between iron absorption and lead absorption was found (Watson et al., 1986). A synergistic effect of lead and iron deficiency has been proposed on ferrochelatase and δ-aminolevulinic acid dehydratase, two enzymes essential for heme synthesis. Higher ZPP levels (in blood) and aminolevulinic acid concentrations (in urine) are found with combined conditions than with the presence of one condition alone (Kwong et al., 2004). Preventing and/or counteracting elevated blood levels might thus be an additional advantage of iron supplementation and fortification in school aged children.
1.2 Assessment of iron absorption in humans

1.2.1 Chemical balance methods

Chemical balance methods were the first methods available to measure iron absorption. The estimation is based on measuring the difference between iron intake and fecal excretion using metabolic ward conditions for prolonged periods (Hunt et al., 1990). Due to the small difference between intake and excretion, iron measurements have to be performed at highest precision, and extreme care has to be taken in complete collection of feces (Wienk et al., 1999). An additional problem in this methodology can be the occasional iron loss from the intestine occurring even in healthy individuals, as fecal blood markers are not useful in individuals consuming meat-containing diets (Hallberg and Hüllten, 1996). In general these methodologies have been abandoned in man, as other more accurate techniques became available (Hallberg and Hüllten, 1996).

1.2.2 Assessment of absorption using isotopic labels

A large part of the knowledge accumulated on iron absorption originates from studies using radioactive and stable isotopes of iron ($^{54}$Fe, $^{55}$Fe, $^{57}$Fe, $^{58}$Fe, $^{59}$Fe).

In early studies, it was observed that if radioactive iron was administered along with biosynthetically labeled foods, the absorption from the two isotopic species was identical (Hallberg and Hüllten, 1996). This finding has lead to the seminal intuition that an added radio iron tracer rapidly exchanges with the non heme iron in the food. This technique, termed the extrinsic tag technique, made it possible to study influences of different food components on non heme iron absorption.

Iron labeled test meals are fed after an overnight fast to subjects instructed not to consume any food in the subsequent 2-3 hours after test meal consumption. Iron absorption can then be assessed by measuring the
retained radioactivity after 10-14 days incorporation/wash out period after correction for radioactive decay. In case of $^{59}$Fe whole body counters can be used; for $^{55}$Fe, which emits less radiation, a blood sample has to be evaluated (Wienk et al., 1999), similarly as with stable isotope methods. There are exceptions for the uniform labeling proprieties of extrinsic tracers (Hallberg and Hullten, 1996). For example, insoluble, nonhomogenenously mixed iron does not readily exchange with the non heme iron pool (Consaul and Lee, 1983). Further exceptions are ferritin iron, iron from soil and unpolished grains. In the latter, the husk has been reported to form a barrier for isotopical exchange (Bjorn-Rasmussen et al., 1972; Bjorn-Rasmussen et al., 1973). The bioavailability of insoluble iron compounds, as they are often used in food enrichment or fortification, can therefore not be established with extrinsic tag technique (Hoppe et al., 2004). An additional disadvantage of the extrinsic tag radioiron technique is that it cannot be safely used to assess iron absorption in pregnant women, infants and children, the most vulnerable groups for iron deficiency.

To obviate to this limitation, methods using stable isotopes of iron as $^{57}$Fe, $^{58}$Fe have been developed (Fomon et al., 1988; Kastenmayer et al., 1994; Walczyk et al., 1997). With this technique the shift from natural isotopic abundances are measured in whole blood after an incorporation period of 14 days. Iron absorption is then calculated by assuming that absorbed iron is incorporated in erythrocytes at a fixed rate, 80% in healthy adults (Wienk et al., 1996), and by estimating blood volume (Brown et al., 1962). The advantages of stable isotopic methods are counterbalanced by the high cost of the isotopic labels and by the limited access to sophisticated analytical equipment (Turnlund, 1989). A further limitation of stable isotope methods is that a relative high dose of tracer (3 mg) is necessary for a measurement, a fact which limits the measurement of native iron bioavailability.

Isotopic methods offer very high sensitivity, so that biological variations are often larger than analytical error. Absorption data has high inter-individual variation, making it difficult to compare groups given different test meals
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(Hallberg and Hulten, 1996). Several study designs have been proposed to obviate to this problem. A reference dose of iron given at fasting state can correct for individual variations in iron absorption. Another proposed method is to correct the absorption for the serum ferritin concentration (Cook et al., 1991), but this procedure has the disadvantage that even a small infection, as a common cold, can falsely increase serum ferritin levels in the test subjects (Hallberg and Hulten, 1996). Using double isotopic techniques, multiple meals can be tested in the same subject, thus making every subject its own control (Kastenmayer et al., 1994) and increasing the power of statistical tests for detecting differences. This design does however not necessarily obviate for day to day variations in the same individual. This challenge can be overcome by feeding the same test meal over multiple days (Hallberg and Hulten, 1996).

1.2.3 Relative Bioavailability

Isotopically labeled iron fortification compounds can be produced by downscaling the production procedures and assessing their bioavailability in humans in selected food matrices (Davidsson et al., 2000). To compare different iron compounds, the concept of relative bioavailability was introduced. With this methodology ferrous sulfate is used as an internal standard, so that absorption data can be easily compared across different subjects, test meals and iron compounds. This procedure has been widely used to compare the bioavailability of different potential iron fortification compounds in animals and humans (Hurrell, 2002). Hallberg and Hulten suggest that the selection of subjects for iron absorption studies is of high importance, as in individuals with high serum ferritin values (50-60 μg/L) iron absorption differences between different test meals may become negligible or statistically undetectable, as subjects would only absorb enough iron to cover their physiological losses (Hallberg and Hulten, 1996). It is well established that there is a close relationship between iron status and iron absorption (Finch, 1994; Gavin et al., 1994). The authors suggest that differences in iron
absorption would be difficult to detect within individuals with high serum ferritin levels.

1.2.4 Relative bioavailability in animal studies

The large majority of animal studies of iron absorption were done in rats (Wienk et al., 1996). The Hb repletion method, recommended by the Association of Official Analytical Chemists (AOAC), measures the efficiency of Hb repletion in rats previously rendered anemic by an iron deficient diet. This method was originally proposed to compare iron fortification compounds. The curve which describes the repletion is of sigmoid shape, and to reliably compare different fortification compounds, comparison must be made within the linear part. Modified versions of the method correct the Hb increase for the iron intake, resulting in greater power and accuracy (Wienk et al., 1996). An alternative method is the prophylactic method, where iron-sufficient rats are used. Some authors did not find any differences between the two methods (Motzok et al., 1975) whereas other authors suggest that the length of the prophylactic test is critical. Miller reported that a repletion test with egg yolk resulted in higher RBV's than a prophylactic method (Miller, 1982), suggesting that, with this approach, rats might utilize iron more efficiently. In a large collaborative study, the AOAC method was found to be the most predictive of RBV in humans for electrolytic Fe and ferric orthophosphate (Forbes et al., 1989).

Dietary determinants of iron absorption have a profound effect on bioavailability in man, but only marginally affect non heme iron absorption in rats (Schricker et al., 1983). To eliminate the potential methodological influences of different absorption estimating-methods, an extrinsic tag technique was used in both man and rats given the same meals (Reddy and Cook, 1991). Rats had 10-fold higher non heme iron absorption from control meals than man, and absorptive response to enhancers (meat, ascorbic acid) and inhibitors (phytic acid, tea) was far less than in human subjects (Reddy and Cook, 1991). Possible explanation for this species differences could be
related to the fact that rats have a high content of ascorbic acid in the gastric lumen and that they do not absorb ferrous iron preferentially (Reddy and Cook, 1991).

The rat model should therefore not be used to estimate the effect of enhancers and inhibitors on iron absorption. Despite the limitations of rats as an experimental model for iron absorption, other animal models are likely to bear similar unknowns (Wienk et al., 1996).

1.2.5 Measurement of serum iron increase

The difficulty of labeling fortification iron compounds with radiotracers or stable isotopes has increased the interest in alternative methods to measure iron absorption. The measurement of the increase of serum iron after an oral iron dose has been discussed as a possible method to estimate absorption. An additional advantage of this method is that it may also allow direct measurement of heme iron absorption (Dainty et al., 2003). The principal limitation for this technique is that a high dose of iron must be administered to obtain a measurable signal in serum, so that fortification concentrations cannot be used. Recently, Hoppe et al reported good correlation between the area under the curve of serum iron increase and the absorption from a radiotracer at a dose of 100 mg Fe as FeSO₄. The authors also claim a good correlation between the absorption of 3 mg Fe and 100 mg Fe both as FeSO₄, suggesting the possible use of this method to evaluate the bioavailability of elemental iron powders (Hoppe et al., 2004). This method has been recently used to estimate the relative bioavailability from elemental iron powders (Hoppe et al., 2005). Another approach was reported using the serum iron method combined with a kinetic compartment model. By comparing this technique with absorption from a labeled dose an agreement of 95% was found (Sarria et al., 2005). However, the authors claim that further studies are needed before the technique can be applied to studies of elemental iron powders (Sarria et al., 2005).
Chapter 1

1.3 Assessment of iron deficiency in humans

Iron deficiency without anemia is present when body iron stores are fully exhausted and some degree of tissue iron deficiency is present (Cook, 2005). Iron deficient erythropoiesis indicates an insufficient supply of iron to the erythroid marrow, whereas iron deficiency anemia is the more severe form of iron deficiency. In this state, tissue iron deficiency is concomitant with a decreased hemoglobin concentration (Bridges and Seligman, 1995).

1.3.1 Hemoglobin

Hemoglobin is widely used as a screening parameter for anemia, but cannot be used as a single parameter to assess iron deficiency. It has low sensitivity in assessing ID because individuals must lose large amounts of iron before they develop anemia (Cook, 2005). In addition, its specificity is impaired by the numerous other causes, including different hemoglobinopathies and thalassemia (Thurlow et al., 2005), folic acid and vitamin B12 deficiency, protein energy malnutrition, chronic infection/inflammation, cigarette smoking and dehydration (Beard et al., 1996).

1.3.2 Serum ferritin

Serum ferritin offers an ideal tool to estimate iron stores in healthy individuals, as 1 g/L roughly corresponds to 8-10 mg storage iron (Cook and Skikne, 1982). A low serum ferritin concentration is diagnostic for iron deficiency (Baynes and Bothwell, 1990). Cutoffs for serum ferritin were defined with comparative measurements of the stainable iron in the bone marrow, which is considered as the gold standard for the assessment of iron stores if performed rigorously (Cook, 2005). In studies performed in healthy subjects, a cutoff value of 15 g/L was proposed (Hallberg et al., 1993), whereas earlier studies on iron replete individuals suggest a 95% confidence range between 12-300 g/L, thus indicating 12 g/L as a cutoff (Cook et al., 1974). Currently the WHO guidelines indicate 15 μg/L as the cutoff to be used for diagnosis of...
iron deficiency (WHO, 2001). A strong limitation of serum ferritin is its elevation in values independently from iron status during acute or chronic infection/inflammation (Cook et al., 2005).

1.3.3 Circulating, soluble transferrin receptor

First discovered in 1986 (Kohgo et al., 1986), the soluble transferrin receptor (sTfR) consists of the extracellular domain of the intact transferrin receptor, and is directly proportional to the erythroid precursor mass and tissue iron need (Skikne et al., 1990). The soluble transferrin receptor is not affected by acute inflammation, or liver disease (Punnonen et al., 1997). However, in situations with concomitant iron deficiency and changes in erythropoietic activity, sTfR values have to be interpreted with caution. In infants affected by malaria, increased sTfR levels have been reported (Menendez et al., 2001). Pregnancy can also affect sTfR concentration, with increased levels in the third trimester due to increased erythropoiesis (Béguin, 2003). Children have been reported to have higher concentrations of sTfR than adults (Suominen et al., 2001), and recently published study has investigated sensitivity and specificity of different diagnostic cutoffs in African children (Zimmermann et al., 2005). Despite the usefulness of sTfR in diagnosing ID and IDA, a major limitation for its general use has been the lack of an international standard to define an internationally recognized cutoff value (Béguin, 2003; Cook, 2005).

1.3.4 Body iron stores

In healthy individuals serum ferritin concentration has a linear relationship with body iron stores, whereas serum transferrin receptor reflects tissue iron status. The sTfR/SF ratio has been proposed as a tool to quantitatively estimate body iron stores in a study with healthy volunteers undergoing repeated phlebotomies until iron depletion (Cook et al., 2003). Using the extrapolated formula, the authors reported increased sensitivity in differentiating between groups receiving different doses of supplemental iron. In a study in Finland using bone marrow aspiration as a standard, the use of the sTfR/SF ratio
increased specificity and sensitivity compared to the use of both parameters alone (Punnonen et al., 1997). The limitations for the use of the sTfR/SF ratio to quantify body iron stores are similar as the limitations for serum ferritin. In addition, as sTfR assays are hardly standardized, every assay would need to be calibrated in repeated phlebotomies before it can be used to estimate body iron (Cook et al., 2003).

1.3.5 Zinc protoporphyrin

In iron sufficiency, ferrochelatase catalyses the reaction that leads to the chelation of Fe by protoporphyrin in the last step of heme synthesis. Zinc incorporation occurs to a trace extent in the bone marrow in normal conditions, but iron deficient erythropoiesis exacerbates this process (Labbe et al., 1999). Hastka et al reported that ZPP can be a reliable tool to differentiate between iron depletion, iron deficient erythropoiesis and iron deficiency anemia (Hastka et al., 1994). When washed cells are used to eliminate interference from plasma constituents the cutoff value for ZPP 40 μmol/mol heme has been proposed (Hastka et al., 1992). In a recent study in Germany comparing the efficiency of sTfR and ZPP in detecting ID patients a good diagnostic agreement between ZPP and sTfR could be found (in 148/174 patients, r=0.86, p<0.001; Metzgeroth et al., 2005). However, before comparing the parameters in a "competitive" fashion a clearer definition of the reference ranges for sTfR with different biochemical assays is necessary. One important limitation of the ZPP measurement is that ZPP levels are increased with lead exposure (Parsons et al., 1991). The mechanism of this interaction has not been clearly elucidated, but lead appears to impair the uptake or the utilization of iron by the maturing erythrocytes (Labbe et al., 1999).

1.3.6 Other biochemical parameters used to assess iron status

Transferrin saturation is defined as the ratio between plasma iron and the total iron binding capacity (TIBC). Plasma iron is measured colorimetrically in
acidified serum, and total iron binding capacity is determined as the amount of iron that can be specifically bound to plasma. An important limitation is the wide diurnal variation in plasma iron, as well as the susceptibility to infection and inflammation of TIBC (Cook et al., 1992). However, the long experience in interpreting transferrin saturation values accumulated during the years and the relative low cost maintain this indicator in use in large laboratories and hospitals (Cook, 2005). Transferrin saturation values below 16% and above 55% indicate iron deficiency and iron overload, respectively.

The mean corpuscular volume (MCV) of erythrocytes can be useful to determine iron deficient erythropoiesis, but its late onset makes it less useful for patients who are not actively bleeding (Cook, 2005). Recently, two additional erythroid indices for iron deficient erythropoiesis have been proposed: (i) the percentage of hypochromic erythrocytes (HYPO) and (ii) the hemoglobin content of reticulocytes (CHr). Due to the lifespan of erythrocytes, HYPO is a late indicator if iron deficient erythropoiesis. CHr on the contrary is a short term marker, as reticulocytes exist in the blood stream for 1-2 days (Thomas and Thomas, 2002). The available data suggest the potential utility of these parameters (Brugnara, 2003), and a recent study comparing bone marrow aspirates with ferritin, transferrin saturation, CHr and MCV, found sensitivity and specificity for CHr of 76.0 and 60.7%, respectively, compared with 93.6 and 42.3%, for serum ferritin and 73.8 and 62.5%, for transferrin saturation, respectively (Mast et al., 2002). However, 66% of the patients described had either hematological disorders or some form of malignancy, so that the applicability of these measurements to the general population for screening purposes still needs to be demonstrated.
CHAPTER 2- ETIOLOGY OF IRON DEFICIENCY AND IRON DEFICIENCY ANEMIA

Nutritional iron deficiency develops when iron requirements cannot be covered with the amount of absorbed iron from the diet.

2.1 Iron requirements

2.1.1 Basal iron losses

Basal iron losses are mainly due to desquamation of cells from the skin and from the gastrointestinal and renal tract. Additional losses can be due to small blood losses into the gastrointestinal lumen, whereas sweat contains negligible amounts of iron (Hallberg, 2001). Iron losses have been estimated to amount to roughly 14 g per kg BW/day. Children appear likely to have proportionately more losses than adults due to their greater surface area relative to body weight (Bothwell et al., 1989). Estimated daily basal iron losses are 0.9-1 mg and 0.8 mg for a 70 kg man and a 55 kg woman, respectively. The range of individual variation is estimated to be around 15% (Hallberg, 2001).

2.1.1 Iron requirements due to growth, menstruation, pregnancy

The iron requirement is proportional to the growth rate of the individual. The iron need for growth can be estimated by adding obligatory basal iron losses, the increase in Hb mass, the increase in tissue iron and the increase in body iron stores (IoM, 2001). Term infants between 0-6 months of age cover their iron requirements through the replacement of fetal hemoglobin with adult hemoglobin, and a successive depletion of body iron stores. In infants between 7 to 12 months of age, the median total requirement for absorbed iron was estimated with 0.69 mg/day (IoM, 2001). Between 1 and 3 years of age and 4 to 8 years of age, children have a median requirement of 0.54 and 0.74 mg absorbed Fe, respectively. Estimation of absorbed iron requirements
for children between 8 and 18 years is more difficult due to the non normality in the distribution of iron need, the strong individual differences in growth rates, and the variable onset of menstruation in adolescent girls (IoM, 2001). However, for non menstruating girls older than 11 years a median requirement of 1.2 mg absorbed Fe/day was estimated, with the requirement increasing with menarche (1.6 mg abs. Fe/day between 11-17 years old), and a slight decrease in requirement with completed growth (1.46 mg abs. Fe for women older than 18 years). The growth spurt in boys strongly increases the iron requirement between 15-17 years (1.5 mg abs.Fe/day), whereas the median iron requirement decreases after 18 years of age (1.05 mg abs.Fe/day; Hallberg, 2001).

Menstrual iron loss is individually constant, but marked variations between individuals exist (Hallberg, 2001), adding an average iron loss of 0.5 mg Fe/day to basal and growth iron requirements. The distribution of menstrual iron loss has been found to be highly skewed, increasing overall needs from 0.8 to 2.3 mg of absorbed Fe in the upper 90th percentile (Bothwell et al., 1989). Contraceptive methods can markedly affect menstrual iron losses (Hallberg and Rossander-Hulten, 1991). During pregnancy approximately 1000 mg of iron are needed by a 55 kg woman. This amount accounts for roughly 230 mg Fe for basal losses, the increased red cell mass (450 mg), the iron need of the fetus (270-300 mg) and the placenta (50-90 mg). The greatest increase in fetal and erythrocytes requirements is during the second and third trimester, where 5-6 mg absorbed Fe/day are needed (Bothwell et al., 1989). This iron requirement must be either covered by iron stores or supplementation.

Lactating women do not have increased iron requirements compared to non pregnant, menstruating women. As menstruation is often absent during exclusive breastfeeding iron requirements are calculated by the sum of basal iron needs and iron secretion in human milk (between 0.15 and 0.3 mg/day), resulting in an estimated requirement of absorbed iron of 1.26 mg/day (Bothwell et al., 1989; IoM, 2001).
2.2 Iron intake and absorption

In developed countries, RDA and EAR values for iron are defined assuming an average western diet with high intakes of absorption enhancers and low intakes of inhibitors, and iron intakes ranging between 12-18 mg Fe/day. Based on the general properties of the major enhancers and inhibitors of iron absorption the FAO/WHO identified three levels of bioavailability, as summarized by Table 1. Typical diversified diets containing generous quantities of meat and ascorbic acid have been suggested to be 15% bioavailable (WHO, 2001). It has been suggested that the bioavailability of iron in the US diet might be somewhat higher (IoM, 2001). A self selected diet assessed over a period of two weeks had an absorption of 16.7% when adjusted for marginal iron stores (15μg/l SF; Cook et al., 1991). In subjects with borderline iron deficiency, a range of bioavailability between 14 and 17% has been suggested (Hallberg and Rossander-Hulten, 1991), whereas diets with large amounts of meat and vegetables might reach bioavailability of 22-23%. The estimated overall iron bioavailability in the mixed American or Canadian diet has been approximated to 18% (IoM, 2001).
Table 1- Iron requirements and recommended intakes in different population and age groups (WHO, 2001).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body Weight (Kg)</th>
<th>Required intake for growth¹ (mg/day)</th>
<th>Iron losses¹ (mg/day)</th>
<th>Total Requirements¹ (mg/day)</th>
<th>Recommended intakes¹,² (mg/day)</th>
<th>Iron absorption 5% 10% 15%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Menstrual</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Children</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5-1</td>
<td>9.0</td>
<td>0.55</td>
<td>0.17</td>
<td>0.72</td>
<td>18.6</td>
<td>9.3 6.2</td>
</tr>
<tr>
<td>1-3</td>
<td>13.3</td>
<td>0.27</td>
<td>0.19</td>
<td>0.46</td>
<td>11.6</td>
<td>5.8 3.9</td>
</tr>
<tr>
<td>4-6</td>
<td>19.2</td>
<td>0.23</td>
<td>0.27</td>
<td>0.50</td>
<td>12.6</td>
<td>6.3 4.2</td>
</tr>
<tr>
<td>7-10</td>
<td>28.1</td>
<td>0.32</td>
<td>0.39</td>
<td>0.71</td>
<td>17.8</td>
<td>8.9 5.9</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11-14</td>
<td>45.0</td>
<td>0.55</td>
<td>0.62</td>
<td>1.17</td>
<td>29.2</td>
<td>14.6 9.7</td>
</tr>
<tr>
<td>15-17</td>
<td>64.4</td>
<td>0.60</td>
<td>0.90</td>
<td>1.50</td>
<td>37.6</td>
<td>18.8 12.5</td>
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<tr>
<td>18+</td>
<td>75.0</td>
<td>1.05</td>
<td>1.05</td>
<td>2.05</td>
<td>47.4</td>
<td>23.7 13.7</td>
</tr>
<tr>
<td>Females</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>11-14</td>
<td>46.1</td>
<td>0.55</td>
<td>0.65</td>
<td>1.20</td>
<td>28.0</td>
<td>14.0 9.3</td>
</tr>
<tr>
<td>11-14</td>
<td>46.1</td>
<td>0.55</td>
<td>0.65</td>
<td>0.48</td>
<td>1.68</td>
<td>65.4 32.7 21.8</td>
</tr>
<tr>
<td>15-17</td>
<td>56.4</td>
<td>0.35</td>
<td>0.79</td>
<td>0.48</td>
<td>1.62</td>
<td>62.0 31.0 20.7</td>
</tr>
<tr>
<td>18+</td>
<td>62.0</td>
<td>0.87</td>
<td>0.48</td>
<td>1.46</td>
<td>58.8</td>
<td>29.4 19.6</td>
</tr>
<tr>
<td>Post-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>menopause</td>
<td>62.0</td>
<td>0.87</td>
<td></td>
<td>0.87</td>
<td>22.6</td>
<td>11.3 7.5</td>
</tr>
<tr>
<td>Lactating</td>
<td>62.0</td>
<td>1.15</td>
<td></td>
<td>1.15</td>
<td>30.0</td>
<td>15.0 10.0</td>
</tr>
</tbody>
</table>

¹Expressed as median values
²Estimated to cover requirements for 97.5% of the population for diets of different bioavailability

2.2.2 Factors in the diet that affect iron absorption

2.2.2.1 Phytic acid

Phytic acid, myo-inositol hexaphosphate, is a major inhibitor of non heme iron absorption (Hallberg et al., 1989; Hallberg et al., 1987; Hurrell et al., 2002; Tuntawiroon et al., 1990). It constitutes 1-2% of many cereals, legumes and seeds, and has the function of a phosphorous store for the germinating plant. Phytate inhibition of iron absorption is dose dependent (Hallberg et al., 1989), and is assumed to be due to the complexation and precipitation of ferric-phytate salts in the small intestine with increasing pH (Conrad and Umbreit, 2000). In the duodenum, phytate can form complexes with cations and proteins (Cheryan, 1980), but as yet no evidence has been found on a
differentiated effect of phytic acid with different types of proteins (Reddy et al., 1996).

Extraction grade at milling strongly decreases both phytic acid and iron content in cereals. Iron absorption from different cereal grains with or without food processing can be predicted by the phytate content (Cook et al., 1997; Hurrell et al., 2002).

Studies in single meals indicate that phytic acid must be almost entirely removed from meals to eliminate its inhibiting effect on iron absorption. It has been suggested that the phytic acid to iron molar ratio must be reduced to <0.7:1 to achieve at least a two-fold increase in iron absorption (Hurrell, 2002).

Phytic acid can be degraded by native or fungal phytase (Oatway et al., 2001). Phytases are active at slightly acid pH (5.1) and are inactive in dry cereals. Normal bread fermentation reduces phytate content up to 50%, but an optimal pH is never reached during fermentation (Lopez et al., 2001). However, lowering the pH in bread, for example in sourdough fermentation, has been reported to decrease phytate content up to 90% (Lopez et al., 2001).

2.2.2.2 Polyphenols

Polyphenols can be classified in phenolic acids, flavonoids and complex polyphenols. All classes have been shown to inhibit iron absorption in a dose dependent fashion, depending on their structure, whereas the presence and number of orthodihydroxy-groups has been reported to be critical for inhibition (Bothwell et al., 1989; Brune et al., 1989). Tannins of black tea, for example, are the most potent iron absorption inhibitors (Brune et al., 1989).

Phenolic compounds in red wine inhibit iron absorption compared to water and white wine (Cook et al., 1995), and polyphenols are the absorption inhibiting factor in coffee (Morck et al., 1983), black tea (Disler et al., 1975), herbal tea (Hurrell et al., 1999) and cocoa (Gillooly et al., 1984; Hurrell et al., 1999). It has been shown that in beverages containing 20-50 mg polyphenols per serving, iron absorption was reduced by 50-70% (Hurrell et al., 1999).
Polyphenols-containing vegetables have been shown to inhibit iron absorption (Gillooly et al., 1983), and the authors report a strong correlation ($R=0.86$, $P<0.001$) between the total polyphenols content and the iron absorption form different vegetables. Iron absorption from Sorghum, an important food crop in Central America, Africa and South Asia, is dependent on its polyphenols content, and it has been shown that low polyphenols-containing varieties are better sources of bioavailable iron (Gillooly et al., 1984).

2.2.2.3 Ascorbic acid

Ascorbic acid has repeatedly been shown to enhance non heme iron absorption in a dose dependent fashion in different foods, on both native iron (Hallberg et al., 1986; Hallberg et al., 1989), as well as on fortification iron from different iron compounds (Fidler et al., 2003; Forbes et al., 1989; Hallberg et al., 1986; Hallberg et al., 1989). Ascorbic acid addition can overcome the inhibitory effect of absorption inhibitors as phytate (Davidsson et al., 1994; Hallberg et al., 1989) and polyphenols (Derman et al., 1977; Siegenberg et al., 1991). In foods rich in inhibitors, higher amounts of ascorbic acid are necessary to increase iron absorption, whereas 25 mg ascorbic acid were shown to significantly increase iron absorption in test meals containing 5.9 mg Fe (1.34:1 ascorbic acid to iron molar ratio), 58 mg phytic acid, 158 mg polyphenols, and 156 mg Ca (Davidsson et al., 1998). The mechanism by which ascorbic acid enhances iron absorption is related to its chemical proprieties. Ascorbic acid reduces ferric iron into ferrous iron and forms a chelate with ferric iron preventing its binding by absorption inhibiting substances (Bothwell et al., 1989; Conrad and Umbreit, 2000). Both these mechanisms prevent iron to be made unavailable to the intestinal mucosa at neutral pH in the duodenum.

Ascorbic acid is routinely added to infant formulas and infant cereals to improve iron absorption, and might be added to chocolate drink powders and other beverages. In dry state, ascorbic acid is stable and can therefore be used in dry blended foods such as infant formulas, dry milk, and precooked
cereal legume blends. It is not stable in baked cereal products and in liquid milk. Ascorbic acid is generally sensitive to heat water and oxygen (Hurrell et al., 2004).

2.2.2.4 Meat and muscle tissue

Meat is a good source of heme iron, which is less affected than non heme iron by inhibitors of iron absorption, with the only exception of calcium and muscle tissue (Hallberg, 2001). Iron absorption from heme iron ranges between 15-45% depending on iron status (Hallberg, 2001). The average heme iron absorption in meat containing meals can be estimated to be 25% (Hallberg, 2001). Muscle protein has been repeatedly shown to increase non heme iron absorption (Bjorn-Rasmussen and Hallberg, 1979; Cook and Monsen, 1976; Hallberg and Rossander, 1984). This effect has been associated to the reducing amino acid cysteine (Taylor et al., 1986). However, a mechanism that explains the enhancement of non heme iron absorption by meat, the long sought “meat factor” has still to be described.

2.2.2.5 Other factors affecting iron absorption

Calcium supplementation and dietary calcium has been shown to decrease both non heme iron absorption (Cook et al., 1991) and heme iron absorption (Hallberg et al., 1993; Roughead et al., 2005). Organic acids as tartaric, citric, lactic and malic acid have been found to increase non heme iron absorption (Ballot et al., 1987), whereas soy protein (Lynch et al., 1994) and casein (Hurrell et al., 1989) have been shown to decrease iron absorption. Some authors have suggested that the high iron content of soy protein in infant cereals is likely to counterbalance its lower absorption (Hallberg, 2001).

2.2.3 Epidemiological evidence and studies from complete diets

Several population surveys have associated iron intake and iron bioavailability with iron status. In a recent, cross sectional study in Kazakhstan, iron
deficiency anemia was associated with low iron and high phytic acid intakes and with diets of low iron bioavailability, as assessed by 24-hour recalls (Hashizume et al., 2004).

In a longitudinal, cohort study in northern Morocco, free living, iron replete, school aged children experienced a drastic decrease in body iron and Hb by consuming their habitual diet over 15 months. The diet was relatively rich in iron (10 mg Fe/day), with high concentrations of phytate and low amounts on absorption enhancers (Zimmermann et al., 2005). In a study in Mexico, non heme iron and ascorbic acid were associated with serum ferritin, and higher intakes of ascorbic acid predicted a lower risk of decreased hemoglobin values (Backstrand et al., 2002).

It has been reported that adaptive regulation of iron absorption can be effective with diets rich in bioavailable iron, but is likely to be less efficient in diets of poor iron bioavailability (Hunt, 2003; Hunt and Roughead, 2000). This indicates that the diet can strongly affect the etiology of iron deficiency. In a study in free living subjects, the effect of ascorbic acid on iron absorption from a complete diet was assessed. The two groups consumed different amounts of ascorbic acid (250 mg compared to 50 mg /day), and only marginal associations between of ascorbic acid and animal tissue on iron absorption could be found after correcting for a serum ferritin level of 30 g/L. This study has been often cited to question the reliability of iron absorption studies based on single meals. However, subjects in the low ascorbic acid group did consume a moderate amount of ascorbic acid, and, although marginal, a significant effect of ascorbic acid on iron absorption could be found. Additionally, subjects studied had relatively high body iron stores, making it more difficult to detect differences in iron absorption (Cook and Reddy, 2001). In a more controlled study, Gleerup et al. reported a significant increase in iron absorption when subjects were fed a high iron bioavailability diet compared with a diet of low bioavailability (Gleerup et al., 1995). Hunt suggested that the apparent discrepancy between single meal studies and studies in complete diets can be at least partly explained by the adaptation of
iron absorption to high bioavailability diets and to the difficulty in implementing same experimental conditions (Hunt, 2001).

2.3 Pathological causes of ID/IDA

2.3.1 Infection with parasites

Hookworm infections (Necator americanus and Ancylostoma duodenale) are believed to affect over 1 billion people worldwide (Stoltzfus et al., 1997), whereas ascariasis and Trichuris trichiura were reported to affect 1.4 billion and 1 billion people worldwide, respectively (Crompton and Nesheim, 2002). Schistosomiasis is estimated to affect more than 200 million people worldwide (Friedman et al., 2005). Infection with parasitic helminths can strongly affect iron balance. Moderate-intensity hookworm infection with N. americanus increases the iron requirement in women by 1.1 mg/day, whereas infections with A. duodenale and Schistosoma hematobium increase iron requirements up to 2.1-2.3 mg/day (Stoltzfus et al., 1997). In most infections by parasites, iron loss is proportional to blood loss from the gastrointestinal tract, which in turn is related to parasite load (Stoltzfus et al., 1997).

In the case of hookworm infections, studies have found a significant relationship between egg counts in the faeces and hemoglobin level. Stoltzfus et al report that a threshold is present between egg counts and hemoglobin concentration. This is likely to depend on the poor sensitivity of Hb as an iron status parameter, as measured iron loss in the stool is in linear relationship with parasite load (Stoltzfus et al., 1997). The intensity of infection with Trichuris Trichura has been associated with blood loss and infection of damaged intestinal wall, and is associated with iron deficiency, impaired growth, protein loss and reduced food intake (Crompton and Nesheim, 2002). Anemia in T.trichiura infection might be caused by blood loss or chronic inflammation. Even if iron loss due to T.trichura with moderate parasite load is lower than with other parasites (Stoltzfus et al., 1997) blood losses are considered sufficient to cause anemia (Layrisse et al., 1967).
Although the magnitude and mechanism of the relationship between schistosomiasis and anemia is not completely elucidated, several cross sectional and longitudinal studies have associated schistosomiasis with iron status (Friedman et al., 2005). A quantitative relationship is however difficult to establish (Friedman et al., 2005). The main difficulty in the quantification of the effect is the presence of multiple confounders (concurrent parasitic infection, malaria, dietary iron deficiency, anemia of inflammation) in the affected populations (Friedman et al., 2005).

2.3.2 Malaria

Malaria causes acute and chronic hemolysis and is an important cause of anemia in endemic areas. There is no evidence that malaria causes iron loss. Rather, body iron is dramatically redistributed towards storage forms (Stoltzfus et al., 1997). Coexisting malaria and iron deficiency will exacerbate anemia, and iron supplementation can counteract anemia in regions of endemic malaria. The advantages seem to outweigh the risks (Mebrahtu et al., 2004; Verhoef et al., 2002), in anemic and iron deficient anemic children (Crawley, 2004; Oppenheimer, 2001). Supplementation or fortification might be important in iron deficient, anemic infants living in endemic regions for malaria, especially considering the negative effects of anemia on the developing brain.

A recently completed randomized, double blind trial of iron supplementation in Zanzibar (a malaria endemic area) added considerable weight to the suggestions that routine iron prophylaxis can be deleterious to iron sufficient children in areas of endemic malaria. Supplementation was found to significantly increase hospitalizations, and was non-significantly associated with mortality.

In a study subgroup, iron supplementation was not associated with increased morbidity in subjects with ID/IDA. In iron sufficient subjects, however, supplementation had deleterious effects on morbidity, and this finding has led the investigators to interrupt the trial. An identical study in Nepal (a
nonmalarial region) found no overall impact of iron supplementation on childhood morbidity and mortality (Sommer, 2005). The findings of the trial are not yet published but were recently discussed in international conferences (Sommer, 2005).

2.3.2 Helicobacter pylori

Prevalence of *Helicobacter pylori* infection in the developing world was reported to range between 65 and 95% in adults and between 22 and 80% in children (Frenck and Clemens, 2003). Infection with *H. pylori* has been associated with gastritis and impaired gastric acid secretion (DuBois and Kearney, 2005), and in epidemiological studies *H. pylori* infection and lowered serum ferritin concentration have been associated (DuBois and Kearney, 2005). In a study in Bangladesh, *H. Pylori* infection significantly affected gastric acid secretion, and treatment with antibiotics increased Hb concentration in the study subjects. However, iron absorption from ferrous sulfate and ferrous fumarate were not significantly affected by *H. Pylori* (Sarker et al., 2004). In a small randomized trial in adolescents suffering from IDA, treatment the *H. Pylori* infection had a stronger effect on iron status parameters than treatment with iron alone (Choe et al., 1999). Despite the high prevalence of infection with *H. Pylori* in both the developed and the developing world, it is unclear why only a small number of subjects develop clinical symptoms and IDA. It is likely that persons at higher risk for IDA are more likely to develop IDA following an infection with *H. Pylori* (DuBois and Kearney, 2005).

2.4 Other nutrients affecting iron metabolism

2.4.1 Riboflavin Deficiency

Riboflavin is a constituent of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN has been found to mobilize intercellular iron by reducing ferric iron bound to ferritin in vitro (Sirivech et al., 1974) and from
various tissues in rats. In animal studies, it was found that riboflavin deficiency can impair iron absorption and increase iron loss (Powers, 2003). In weanling rats, riboflavin deficiency causes irreversible morphologic and cell kinetic changes in the gastrointestinal tract. In the only human absorption study carried out at present, no effect of riboflavin status on iron absorption could be found, even if an increase in hemoglobin was measured in the riboflavin supplemented group (Fairweather-Tait et al., 1992). In general, correcting riboflavin deficiency in humans improves their response to iron supplements (Powers, 2003).

2.4.2 Folic acid and vitamin B\textsubscript{12} deficiency

Folate and vitamin B\textsubscript{12} are essential cofactors in the synthesis of DNA. Folate has the function to provide the methyl groups for purine and pyrimidine synthesis, whereas vitamin B\textsubscript{12} is the coenzyme involved in the regeneration of methionine and tetrahydrofolate, that are both essential for methylation reactions. Both folic acid and vitamin B\textsubscript{12} deficiency cause ineffective erythropoiesis, which leads to anemia (Koury and Ponka, 2004). The high proliferation rates in erythropoiesis make erythroid precursors mostly affected by impaired DNA synthesis (Koury and Ponka, 2004). The quantitative contribution of folate deficiency to the global burden of anemia is unclear, but it has been suggested that its effect might be rather limited compared to iron deficiency (Allen and Casterline-Sabel, 2001).

Data on the prevalence of vitamin B\textsubscript{12} deficiency and on its contribution to anemia are scarce. In a worldwide survey, prevalence of vitamin B\textsubscript{12} deficiency was low in all the countries studied except for rural India, where animal product intake is especially low (Allen and Casterline-Sabel, 2001). High prevalence of vitamin B\textsubscript{12} can be found in the elderly population due to gastric atrophy. Additional studies are necessary to establish prevalence, causes and consequences of vitamin B\textsubscript{12} deficiency (Allen and Casterline-Sabel, 2001).
2.4.3 Vitamin A deficiency

The main causes of vitamin A deficiency are nutritional; a diet poor in fruits, vegetables and animal products causes vitamin A deficiency (Allen and Casterline-Sabel, 2001). Infections with *Ascaris lumbricoides* and diarrhea can impair fat absorption and the utilization of the vitamin. Ascaris infections are associated with vitamin A deficiency (Crompton and Nesheim, 2002). Vitamin A deficiency can be a cause of anemia (Allen and Casterline-Sabel, 2001; Semba and Bloem, 2002). Vitamin A supplementation increases hemoglobin concentration independently from iron supplementation (Mejia and Chew, 1988). In another study in children given an iron fortified soup during a period of 15 weeks, significant improvements in serum iron levels and transferrin saturation could be seen only in children with a serum retinol level >40 g/l, compared with no significant effect in children with a serum retinol level <20 g/l (van Stuijvenberg et al., 1997).

Several possible mechanisms for the effect of vitamin A on iron status have been proposed. It has been found that vitamin A can partly enhance erythroid precursor cell maturation in vitro (Correa and Axelrad, 1992). In addition the enhancer region of the erythropoietin gene contains a responsive element that appears to be regulated by retinoic acid, and both animal studies and cell culture studies support the idea that vitamin A enhances erythropoietin production in vitamin A deficiency (Okano et al., 1994). However, a link between vitamin A and erythropoietin could not be confirmed in human studies (Semba and Bloem, 2002; Semba et al., 2001).

In rats, vitamin A deficiency has been reported to reduce iron incorporation in erythrocytes (Mejia et al., 1979) and was associated with iron accumulation in the liver and spleen (Mejia et al., 1979; Roodenburg et al., 1996; Roodenburg et al., 1994). In the study by Van Stuijvenberg et al., a 15 week feeding trial in children, the highest increase in SF was seen in the group having a mild Vit. A deficiency (<40 µg/l). Vitamin A status was negatively correlated with SF but positively with serum iron and transferrin saturation (van Stuijvenberg et al., 1997).
This data might support the hypothesis that vitamin A plays a role in iron mobilization from the liver and spleen in humans.

Vitamin A deficiency impairs immune function and it is possible that deficiency enhances anemia of inflammation. However, although this hypothesis might appear logical, little data has been generated to support it (Semba and Bloem, 2002).
CHAPTER 3 - STRATEGIES TO COUNTERACT IRON DEFICIENCY

The four widely recognized strategies to reduce micronutrient malnutrition and iron deficiency worldwide are dietary diversification, supplementation, fortification and disease reduction (Maberly et al., 1994; Trowbridge et al., 1993). In this chapter, the focus is the first three strategies.

3.1 Diet modification and diversification

Diet modification and diversification is considered “highly desirable” by the WHO in communities where nutrition is monotonous, and diet is low in iron and rich in absorption inhibitors (WHO, 2001). Epidemiologically, heme iron intake correlates with iron status (Takkunen and Seppanen, 1975). However, increasing meat availability not always increases its consumption in poor communities, as it was reported that study participants preferably increased their income rather than consume the self produced animal products (Ruel and Levin, 2001). The impact of well targeted interventions (e.g. the promotion of small animals) still needs to be assessed.

Diet diversity is usually associated with nutrient adequacy in studies performing dietary assessments in both developed and developing countries (Ruel, 2003). In cross sectional studies, anthropometric assessments in children were significantly correlated with measures of diet diversity. It is however possible that diet diversity is simply a proxy for better socioeconomic status (Ruel, 2003). Nutritional education and community nutrition campaigns (e.g. home gardening) have been shown to increase Vitamin A intake in targeted populations (Ruel, 2003; Ruel and Levin, 2001), but data on nutritional education trials on iron is scanty.

A study in Peru using a non equivalent control group, nutritional education of community kitchen workers and participating subjects increased hemoglobin concentration in the treatment group (Carrasco-Sanez et al., 1998). The intervention was targeted to increase ascorbic acid, heme and non heme iron
intake and to reduce inhibitors of non heme iron absorption, but was not published on the peer reviewed literature. In a community based trial in Mexico, providing 25 mg ascorbic acid twice a day along with regularly consumed meals drastically (but non statistically significantly) decreased the serum transferrin receptor/ferritin ratio in the treatment group. The study had however limitations, as it was underpowered (with only 18 iron deficient women per group) and not randomized (Garcia et al., 2003). It is thus very likely that such an intervention would have a positive impact on iron status in a larger population sample.

Diet modifications are likely to be the most sustainable measures to combat iron and other micronutrient deficiencies; however, economical constraints and the need for behavioral changes make them particularly difficult to implement and to monitor.

3.2 Supplementation

Supplementation has repeatedly demonstrated to be effective in targeted interventions. In pregnant women supplementation is widely used, whereas different approaches are used depending on the available resources (Ekström, 2001). The WHO recommends universal iron supplementation for all pregnant women (WHO, 2001). In children not having access to iron fortified complementary foods, iron supplementation with 12.5 mg Fe/day is recommended for the first year of life (Stoltzfus and Dreyfuss, 1998). Logistics in the delivery of supplements has been reported to be a major constraint in the implementation of supplemental programs (Ekström, 2001), and the delivery system for supplements is a central factor in ensuring effectiveness. A major reason for ineffective supplemental programs is limited compliance due to adverse side effects, and to the understanding and motivation to follow the program (Ekström, 2001).

The efficacy of supplementation programs might be improved if supplements would be taken weekly and not daily. Both options have been reported to
efficiently counteract iron deficiency anemia, although daily supplementation appeared to be more efficacious (Beaton and McCabe, 1999). However, weekly iron supplementations might decrease side effects, lower cost and improve compliance (Allen, 2002). Although pregnant women are the most often targeted group, children, women, elderly people and men with parasite infections or living in malaria endemic regions might need iron supplements, but the beneficial effects of iron supplementation are likely not to be long lasting once supplementation is stopped (Allen, 2002). The feasibility of large scale supplementation programs as a public health measure has been questioned (Allen, 2002). However, until either drastic improvements are made in diet composition at community level or food fortification is achieved, supplementation is likely to remain the most common strategy used to combat iron deficiency in developing countries (WHO, 2001).

3.3 Bio-Fortification

Limitations in micronutrient uptake in plants are likely to be of biological nature, as soil trace element content is not the limiting factor for micronutrient uptake. In this respect selenium and iodine seem to be the only exceptions (Lonnerdal, 2003). Native micronutrient concentration in rice and other cereal grains and pulses have been reported to vary widely, and this variation is mainly genetic, and not environmentally determined (Frossard et al., 2000). Therefore efforts to bio-fortify plants can be accomplished either by traditional breeding or by the use of molecular biology and genetic engineering (Lonnerdal, 2003). Biofortification has been defined as the breeding of plants to produce staple food crops with increased vitamin and mineral concentrations (AAv, 2005; Bouis, 1996; Bouis, 2003).

Raw polished rice has an iron content of 4-8 mg/kg. Iron concentration in different rice cultivars can however show broad variation, and has been reported to range between 4-29 mg/kg dry weight (Frossard et al., 2000). Therefore, there seems to be a large potential in increasing the iron content of rice by breeding or genetic modification. A conventionally bred, iron dense
Strategies to counteract iron deficiency

A rice variety containing 7-13.4 mg/kg Fe when milled has been developed by the International Rice Research Institute (IRRI) in the Philippines (Gregoirio et al., 2000). This rice named IR 68144 was tested in weaning piglets, where it slightly but not significantly increased hemoglobin concentration in the group receiving iron fortified rice (Schaffer et al., 2004). The authors suggest that the study design was likely not to be adequate to detect a relatively small difference in iron intake of 10 mg Fe/kg feed in the control compared to 20 mg Fe/Kg feed in the treatment group. In a human efficacy trial in the Philippines, the same rice variety was tested in a 9 month long trial in 317 religious sisters. The rice provided extra 3 mg of Fe/day and significantly increased body iron in nonanemic women with body iron in the lowest quartile. However, no significant effect could be detected in the remaining two thirds of the study sample and in anemic women participating in the study (Haas et al., 2005).

Transgenic rice varieties containing iron have been developed (Goto et al., 1999; Lucca et al., 2001; Nandi et al., 2002; Vasconcelos et al., 2003). The transgenic rice produced by Goto was tested in an hemoglobin repletion test in rats, but due to the fact that iron was not expressed in the endosperm, iron concentration in the dehusked rice was relatively low (e.g. 2.7-6.7 mg Fe/kg). In the repletion test the same iron concentration provided by the transgenic rice grains was given to a control group in form of FeSO₄, and no significant difference in the efficiency of hemoglobin repletion between endogenous iron in rice and FeSO₄ could be found, indicating that ferritin iron fed in these conditions was as well absorbed as ferrous sulfate. However all the rats remained anemic after the repletion period, as the iron concentrations were too low to replete the animals (Murray-Kolb et al., 2002).

Later work has focused to express ferritin under the endosperm specific glutelin promoter so to increase iron content in polished rice and not only in brown rice (Lucca et al., 2001; Vasconcelos et al., 2003). Lucca et al achieved an iron concentration of 22 mg/kg dehusked rice by inserting a genetic construct containing a ferritin gene from Phaseolus vulgaris. Additionally, to increase iron bioavailability, a thermostable phytase from Aspergillus niger
and a methallothienin-like protein rich in cysteine were inserted. Unfortunately, once the phytase was expressed in the rice it was not as thermostable as expected, and was destroyed after cooking (Lucca et al., 2001). The bioavailability of iron in the genetically modified rice was however not investigated.

Nandi et al. achieved the cloning of human recombinant lactoferrin into the rice endosperm at the remarkable concentration of 5 g of lactoferrin/kg rice, resulting in an iron concentration of 19.3 mg Fe/kg in dehusked seeds (Nandi et al., 2002). Lonnerdal, one of the coauthors of the study, suggested that this iron concentration might not be sufficient to substantially increase the iron content of an iron deficient diet. Even with a relatively high rice intake of 250-300 g rice/day, the additional amount of iron consumed compared to conventional rice would only be around 3-4 mg/day (Lonnerdal, 2003).

Plants having a low phytic acid (Ipa) mutation cause the seed to store most of the phosphorous in inorganic form (Frossard et al., 2000). In maize, an (Ipa) mutant was produced with 65% decrease in phytic acid content compared with the wild type (348 mg phytate/100g Maize; Mendoza et al., 1998). When baked into tortillas, iron absorption from the low phytate maize was 49% higher than in the wild type (Mendoza et al., 1998). Concern has however been raised on the seedling vigor of low phytate species in low fertility soils (Frossard et al., 2000). In a follow up study with the addition of FeSO₄ and NaFeEDTA as iron fortificants, no significant difference in iron absorption between the two fortified maize cultivars could be found (Mendoza et al., 2001). The reasons for this discrepancy are unclear.

In the past, crops have been selected for pest resistance and high yield, and micronutrient content has only recently received attention. Micronutrient enrichment is likely to have also agronomical advantages, as micronutrient rich seedlings help plants resist diseases, increase seedling survival and seedling growth (Bouis, 2003). Bio-fortification would also seem the ideal approach to reach the unprivileged rural population living from self sustaining agriculture (Bouis, 2003). Plant breeding holds great promise in addressing
problems of micronutrient deficiency in the developing world. However, several questions need to be addressed before bio-fortification can lead to a sustainable improvement in trace element nutrition. Nutritional fortification of cereals is still in its infancy, and additional research is needed in the mechanisms of plant cation uptake and transport (Poletti et al., 2004). In addition, acceptability of genetically modified plants and the nutritional impact of micronutrient dense crops need to be established in animal and human studies (Lonnerdal, 2003; Zimmermann and Hurrell, 2002).

3.4 Food Fortification

There is a consensus that food fortification can be an effective long term approach to increase the iron status of a population (Cook and Reusser, 1983; Mannar and Sankar, 2004; WHO, 2001). Ideally, fortification reaches all segments of the population and does not require the constant cooperation from the individual or drastic changes in food habits (Cook and Reusser, 1983). The cost effectiveness of food fortification is higher than supplementation, and due to the lower dosage, the gastrointestinal side effects of iron supplementation are avoided.

Four types of food fortification are recognized by the WHO: mass or universal fortification refers to foods consumed by the entire population, it is regulated by the government and is encouraged in countries where several population segments are at risk of deficiency; open market fortification is practiced mainly in developed countries (e.g. breakfast cereals and functional foods), it is done by the private sector with the aim of increasing the public appeal and the added value of food products; targeted fortification is directed specifically to high risk groups (e.g. infants and pregnant women), whereas household fortification nutrients are added immediately before consumption (Lynch, 2005).

Several factors are critical for a successful fortification program. The fortificant food must be commonly consumed in constant patterns with low risk of over-
consumption; the food vehicle should be centrally produced and fortification must be possible at relatively low cost (FAO, 1996). A fortification program must be adapted to the local food consumption patterns, to the prevalence of deficiency, and, in the case of iron, to the bioavailability from the local diet (Lofti M. et al., 1996). Even if very cost effective (Darnton-Hill, 1998) fortification will marginally increase prices (Underwood and Smitasiri, 1999). Country level experiences for long term success with fortification show that political will, involvement of the private sector at early stages, willingness to enforce quality standards and consumer awareness are important in the implementation of a fortification program (Darnton-Hill, 1998; Underwood and Smitasiri, 1999). Ideally, food fortification should be embedded in an overall strategy to promote nutritional health that includes diet diversification, fortification and supplementation (Mannar and Sankar, 2004).

The major technical challenge in iron fortification is to identify a bioavailable iron fortification compound which does not induce unacceptable sensory changes in the selected food vehicles. In general, highly water-soluble iron fortification compounds cause unacceptable sensory changes in food vehicles, whereas poorly soluble iron fortification compounds do not react with the food matrix but are less bioavailable (Hurrell, 2002).
3.4.1 Iron fortification compounds

In order to enter the common non heme iron pool, iron has to be soluble in the gastric juice. The solubility of iron fortification compounds is a primary determinant of their bioavailability (Forbes et al., 1989; Motzok et al., 1975; Shah et al., 1977; Swain et al., 2003). For this reason iron fortification compounds are often classified according to their solubility in water (Hurrell, 1999). A further critical measure used to judge an iron fortification compound is the relative bioavailability (RBV), which is defined as the relative absorption of a certain iron compound in comparison to the same dose of ferrous sulfate, which per definition has a relative bioavailability of 100% (Hurrell, 1999).

Ferrous sulfate is the cheapest iron salt. To avoid unacceptable color changes and oxidative reactions it can only be used in cereal flours that are used within one month from production and in low moisture foods as noodles and pasta (Hurrell et al., 2002). Readily water soluble iron compounds like ferric lactate, ferrous gluconate, ferric ammonium citrate and ferrous ammonium citrate have been reported to have bioavailability close to that of ferrous sulfate, when tested in human subjects at high, pharmacological doses (Hurrell, 1999). In general these compounds have a higher price compared to ferrous sulfate.

In a recently published study using iron fortified Thai fish sauce as a fortification vehicle, the relative bioavailability of ferrous lactate and ferric ammonium citrate compared to ferrous sulfate was 67 and 51% respectively (Walczyk et al., 2005), indicating that in fortified foods these compound might be less bioavailable than ferrous sulfate.

Iron compounds that are only partially soluble in water include ferrous fumarate, ferrous succinate, ferrous citrate and ferrous tartrate. Ferrous fumarate has been extensively investigated in animal and human bioavailability studies due to its low relative cost to ferrous sulfate (~1.3 more expensive). Ferrous fumarate is not completely free from sensory problems, but interacts to a smaller extent with the food matrix than ferrous sulfate. Ferrous fumarate is widely used as iron fortificant in infant cereals in Europe.
and in chocolate drink powders (Hurrell et al., 1999). It has also been used in precooked corn flour and wheat flour fortification in Venezuela (Garcia-Casal and Layrisse, 2002).

In adults, the relative bioavailability of ferrous fumarate is comparable to that form ferrous sulfate (Hurrell et al., 1989; Hurrell et al., 1991). There is, however little information on the bioavailability of ferrous fumarate in children and infants. A study in Bangladesh showed that iron absorption from ferrous fumarate was roughly 30% that of ferrous sulfate in treated and untreated children with *Helicobacter pylori* infection. Although gastric acid secretion in treated children was comparable to uninfected controls, it might be possible that the infection depressed iron absorption for other unknown reasons (Sarker et al., 2004). Similarly, in a study in Mexican infants, iron absorption from milk based weaning food was only 30% that of ferrous sulfate (2.4% absorption from ferrous fumarate compared to 7.9% absorption from ferrous sulfate). Iron status was difficult to estimate due to the presence of infection as reported by the elevated CRP levels (Perez-Exposito et al., 2005).

In a further study, isotopically labeled microencapsulated ferrous fumarate was given to rural African infants during three consecutive days at a dose of 16.5 mg Fe with 50 mg ascorbic acid (1:1 molar ratio) and 300 g retinol equivalents. Iron absorption ranged between 8.25 and 4.48% depending on the iron status of the infant (Tondeur et al., 2004), resulting in a high amount of iron incorporated into erythrocytes (1.5-2.5 mg Fe). The obtained absorptions are in the same range as reported in European infants (Davidsson et al., 2000), where iron absorption from nonencapsulated ferrous fumarate was 4.1%. Absorption could not be significantly improved with ascorbic acid at an ascorbic acid: Fe ratio of 3:1 and 6:1 (Davidsson et al., 2000). It is however difficult to compare the iron status in the populations studied, as the studies were partly performed in population with high prevalence chronic infections.
3.4.1.1 Poorly soluble iron fortification compounds

Poorly soluble iron fortification compounds have lower bioavailability compared to ferrous sulfate, but are nonetheless widely used in food enrichment and fortification due to the negligible sensory problems they cause in food vehicles (Hurrell, 2002).

*Elemental iron powders*

The main determinants of solubility of elemental iron powders in the gastric juice are particle size distribution, surface area, purity and solubility in acid (Hurrell, 2002). There are five production processes for electrolytic iron: H-reduction, CO-reduction, electrolytic and carbonyl processes, and the process for the production of atomized iron. Every process results in products with distinct physical characteristics. Therefore, it is not possible to give a single recommendation on the use of elemental iron powders in cereal fortification (Hurrell et al., 2002).

The difficulty in judging elemental iron powders is related to the challenge in manufacturing isotopically labeled elemental iron compounds that are physically comparable to the commercial products. The bioavailability of elemental iron powders was extensively studied and recently reviewed by an expert panel to evaluate their usefulness for food fortification (Hurrell et al., 2002). The only form explicitly recommended based on the evidence available in 2002 was electrolytic iron which has been shown to have relative bioavailability of 75% in an human absorption study using a similar but not identical radiolabeled iron than the commercial compound (Forbes et al., 1989), in contrast to that, animal studies suggested an average relative bioavailability of 44% with a range between 16 and 77%.

Inconsistent results between animal and human studies were found for carbonyl iron. In a large human study using different food matrixes, Hallberg found RBV's between 5 and 33% (Hallberg et al., 1986) whereas in rat studies an average bioavailability of 47% with a range between 27 and 66% was
found. Lower RBV were found for H-reduced iron and CO-reduced iron with a mean RBV 30% (range 13-54%) and 19% (range 12-32), respectively (Hurrell et al., 2002).

Two recent studies have shown a similar pattern of bioavailability from elemental iron powders. In a rat Hb repletion study, carbonyl (RBV=64%) and electrolytic iron (RBV= 54-46%) were found to be the most readily available compounds, followed by H-reduced (RBV= 42%), atomized (RBV= 24%) and CO-reduced iron (RBV=21%; Swain et al., 2003). Serum iron increase was measured to judge iron bioavailability from iron fortified bread rolls. All elemental iron forms were significantly different in their response relative to ferrous sulfate, with electrolytic iron having a RBV between 65 and 59%, depending on the supplier of the metal. H-reduced iron had an RBV situated between 56-58%, whereas carbonyl iron showed larger variation with RBV between 58 and 36% depending on the supplier. Atomized iron had a lower RBV of 36% (Hoppe et al., 2005).

A randomized, controlled efficacy trial showed that electrolytic iron has a relative efficacy to ferrous sulfate of 77% in humans. In contrast, H-reduced iron had ≈50% of relative efficacy. This study was performed in Thai-women which were supplied a mid day cookie fortified with 12 mg Fe as either electrolytic Fe, H-reduced Fe, ferrous sulfate or a unfortified control (Zimmermann et al., 2005). This study showed for the first time in humans that electrolytic iron powders and H-reduced iron powders are efficacious in preventing and treating iron deficiency. There is a further need in evaluating the usefulness of elemental iron powders in controlled settings in iron deficient populations. Ultimately, the true test of iron compounds for fortification is their ability to reduce iron deficiency in population groups at risk (Hurrell, 2002; Zimmermann et al., 2005).
Strategies to counteract iron deficiency

Ferric pyrophosphate

Ferric pyrophosphate or ferric diphosphate \([\text{Fe}_4 \left(\text{P}_2\text{O}_7\right)_3]\) is a water insoluble iron salt in amorphous state (Tsuchita et al., 1991). Its white color makes it a compound of choice for iron fortification of rice (Zilberboim, 1994) and it was shown to have excellent sensory characteristics in salt (Wegmuller et al., 2003). It has been reported to be used as an iron fortification compound in chocolate drink powders and in infant cereals (where it has been partly replaced by ferrous fumarate; Hurrell, 1999). In hemoglobin repletion test in rats, ferric pyrophosphate of regular particle size has a relative bioavailability to ferrous sulfate between 45-60% (Fritz et al., 1970; Hurrell et al., 1989; Sakaguchi et al., 2004; Wegmuller et al., 2004).

In a study in infants, ferric pyrophosphate was absorbed at 1.3% compared to absorption from ferrous fumarate of 4.1% (Davidsson et al., 2000), whereas in a study in adult women using an infant cereal as a food vehicle, the relative bioavailability of ferric pyrophosphate of regular particle size was 36% (Fidler et al., 2004). In a group of mainly iron-replete subjects, a study in chocolate drink powder showed a relative bioavailability of ferric pyrophosphate of 75% in the unprocessed test meal (2.11% absorption compared to 2.82% absorption from ferrous sulfate). The same compound was evaluated several years later in infant cereals and was found to be absorbed only at 0.26%, compared an absorption for ferrous sulfate of 1.76% (Hurrell et al., 2000). It would therefore appear that storage time could affect the bioavailability of ferric pyrophosphate. This could be explained by changes in the amorphous structure of the compound (Tsuchita et al., 1991), as shown by Hallberg for a complex ferric orthophosphate (Hallberg et al., 1989), or by the different solubility proprieties in different food matrixes.

Being a ionic species, ferric pyrophosphate can be ground to fine powders to increase its particle size without the disadvantage of pyrophoric activity (the incandescent reaction with oxygen when in contact with air), which might affect electrolytic iron (Hallberg et al., 1986). Ferric pyrophosphate can be produced by the chemical reaction between ferric chloride and sodium...
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pyrophosphate in watery solution. Under specific conditions and with the use of stabilizing emulsifiers micronised dispersible ferric pyrophosphate can be produced with a mean particle size of 0.3 μm (Nambu et al., 1998).

3.4.1.2 Novel compounds and strategies to increase their bioavailability

Reduction of the particle size

It has been suggested that the reduction of particle size and surface area of elemental iron powders improves iron solubility and bioavailability (Bjorn-Rasmussen et al., 1977; Swain et al., 2003; Verma et al., 1977). There are strong indications that reducing the particle size of ferric pyrophosphate increases bioavailability in rats (Sakaguchi et al., 2004; Wegmuller et al., 2004) and in humans (Fidler et al., 2004), and micronised dispersible ferric pyrophosphate with mean particle size (MPS) of 0.3 μm has been shown to have high relative bioavailability in an infant cereal and in a yoghurt drink (Fidler et al., 2004).

Micronized ground ferric pyrophosphate (MPS=2.5 μm) has been shown to have an RBV of 72% in a recent hemoglobin repletion test in rats, whereas the same compound of larger particle size had a RBV of 60%, however the two results were not significantly different (Wegmuller et al., 2004). In a recent double blind randomized trial in northern Morocco, micronized ground ferric pyrophosphate (MPS=2.5 μm) significantly decreased IDA and ID in school aged children when used to fortify salt at a concentration of 20 mg/day (Zimmermann et al., 2004). The compound was acceptable and did not cause any sensory changes when used in the regular diet. Ferric pyrophosphate of reduced particle size can therefore be an efficacious iron fortification compound in salt.

Encapsulation

Encapsulation of soluble iron fortification compounds is a possible way to reduce sensory interactions with the food vehicle and maintain high bioavailability. The effect of capsule material on the bioavailability from
Strategies to counteract iron deficiency

Encapsulated iron compounds was recently reviewed (Zimmermann, 2004). A capsule: substrate ratio of 50:50 does not appear to affect RBV of encapsulated ferrous sulfate, but there are indications that ratios > 60:40 decrease RBV by 20% (Zimmermann, 2004). Three field studies showed good efficacy in treating IDA in children with encapsulated iron compounds. The first study was carried out in Morocco in a double blind randomized controlled trial (Zimmermann et al., 2003). The second study was done in Ghana comparing the efficacy of ferrous sulfate drops with that of microencapsulated ferrous fumarate sprinkles (Zlotkin et al., 2001). The third study compared the efficacy of ferrous fumarate capsules with and without zinc in treating IDA in infants and young children (Zlotkin et al., 2003). In study published recently, absorption of encapsulated ferrous fumarate in infants was shown to range between 8.25-4.65%, depending on iron status when given at high dose of 16.5 mg Fe on three days (Tondeur et al., 2004).

In salt, encapsulated ferrous sulfate may cause sensory changes in moist climates and damp seasons. Better encapsulation techniques that do not impair bioavailability are needed for food fortification (Zimmermann, 2004). In general a limitation for the use of encapsulated iron compounds in food fortification is that capsules are not heat stable and that colour oxidation reactions can take place with food processing or simple addition of hot water or milk. An additional concern in salt fortification was the abrasion of capsules during mixing (Wegmuller et al., 2003; Zimmermann et al., 2003). The possibility to use microencapsulated, soluble iron compounds in cereal products needs to be explored, as flours are consumed at larger quantities than salt, making color problems less urgent. However, possible oxidation reactions need to be carefully assessed in the food vehicles.

NaFeEDTA

Has been recently reviewed and approved by the JECFA (Joint FAO/WHO expert committee of food additives) for government supervised fortification programs, but has not been yet permitted widely on a country level (Hurrell, 2002). NaFeEDTA has been shown to be two to three times better absorbed
than ferrous sulfate in cereal flours rich in phytic acid (Hurrell et al., 2000). Interestingly, NaFeEDTA does only slightly improve iron absorption in foods rich in polyphenols, compared to ferrous sulfate (Hurrell et al., 2000), probably because polyphenols have greater affinity to non heme iron than EDTA (Bothwell and MacPhail, 2004). NaFeEDTA has been recommended for use in soy and fish sauces and proposed for high phytate flours and other condiments (Hurrell et al., 2004).

EDTA has the highest affinity for iron in the acid environment of the stomach. It can however be exchanged with other metals in the duodenum. On molar basis, copper and zinc could be affected by the addition of EDTA. Other minerals as calcium and magnesium would be not affected by the low amounts of EDTA when given at quantities to supply the RDA for iron. There are indications that EDTA increases the absorption from copper and zinc in meals rich in phytic acid (Bothwell and MacPhail, 2004). Concern has been expressed on the possible effect of EDTA on the absorption of toxic metals. Although not many studies have been performed on the subject, in a human study with isotopically labeled Pb, simultaneous ingestion of EDTA markedly decreased lead retention (Flanagan et al., 1982). This would therefore indicate that EDTA does not increase the absorption of lead. An additional argument leaning in this direction is that a small amount of food EDTA is absorbed in the gut, reaches the blood stream is and successively excreted in the urine (Bothwell and MacPhail, 2004). EDTA is normally used for complexation therapy in cases of acute heavy metal poisoning. This would therefore suggest that food EDTA might lower blood lead levels. This question merits further attention because of the widespread coexistence of elevated blood lead levels and iron deficiency in urban environments (Kwong et al., 2004).

Several successful iron fortification trials with NaFeEDTA have been performed, including one in an Indian community in South Africa receiving fortified curry powder (Ballot et al., 1989). Fish sauce and soy sauce also can be fortified with NaFeEDTA. A randomized controlled trial has shown that fish
sauce fortified to provide 10 mg Fe/day significantly increased hemoglobin and significantly decreased prevalence of anemia in garment factory workers in Thailand (Van Thuy et al., 2003). This findings were confirmed in a recently completed effectiveness trial (Van Thuy et al., 2005). NaFeEDTA has also been reported to be a suitable fortificant for sugar (Viteri et al., 1995), although the successful controlled study was difficult to interpret due to logistic and acceptability problems (Bothwell and MacPhail, 2004).

The disadvantages of NaFeEDTA are its high cost, and the lack of systematic information on its sensory proprieties. When sugar fortified with NaFeEDTA was added to coffee and tea, sensory changes were visible (Viteri et al., 1995). Additionally, milk and salt cannot be fortified with NaFeEDTA due to adverse sensory changes (Bothwell and MacPhail, 2004). In cereal products no rancidity in flour could be detected after 6 months storage at 37 degrees (Hurrell, 1997), but there are reports of color development in cereal based foods (Viteri et al., 1995). Additional research is however needed on the stability of NaFeEDTA in a wider range of products and during processing and cooking, especially in cereal based products (Hurrell et al., 2004). In condiments such as curry powder, soy and fish sauce NaFeEDTA appears nevertheless to be well tolerated (Bothwell and MacPhail, 2004).

Amino acid chelates
Ferrous bisglycinate is an additive generally recognized as safe (GRAS), but produces fat oxidation in maize porridge to a larger extent than ferrous sulfate. Tris glycine chelate, however, was reported to generate less oxidation products than ferrous sulfate in the same maize porridge model food (Bovell-Benjamin et al., 1999), but is less bioavailable than bis glycine chelate in maize (Bovell-Benjamin et al., 2000). In milk and reconstituted dairy beverages, glycine chelates appear to have good sensory qualities. More data would however be useful on the stability and acceptability of amino acid chelates in different food products (Hurrell et al., 2004). Absorption studies with ferrous bisglycine chelates show absorptions three to four times higher.
than ferrous sulfate (Bovell-Benjamin et al., 2000; Layrisse et al., 2000), due to the likely protective effects of glycine against inhibiting luminal factors.

3.4.2 Sensory aspects of iron fortification

Do to the vast array of iron compounds and of potential fortification vehicles, a systematic methodology in the identification of the most promising approaches was proposed (Bovell-Benjamin and Guinard, 2003). Among sensory attributes, taste is the most important reason for selecting a food product, and sensory proprieties are generally critical in determining consumer’s acceptance to the food.

Food products and fortificants should be first screened in informal bench top tests. Additionally, storage stability trails should be performed. If no evident difference can be found, difference testing should be performed with a sensory panel using simple difference testing as paired comparisons and triangle tests (Meilgaard et al., 1999). Finally, consumer testing should be carried out in large panels of frequent users (Bovell-Benjamin and Guinard, 2003).

3.4.2.1 Color changes

The most visible effect of soluble iron compounds on the fortified food is its change in color (Mellican et al., 2003). Ferrous sulfate rapidly discolors in fortified salt (Wegmuller et al., 2003), it turns fortified extruded grains brown (Kapanidis and Lee, 1996) and reacts with a range of other foods, as reported in bananas (Hurrell, 1999), gingerbread, wheat flour (Hallberg et al., 1989), infant foods (Hurrell et al., 1989), chocolate drinks (Hurrell et al., 1991) and milk (Gaucheron, 2000). The iron induced discoloration in foods can be either due to the direct reaction of soluble ferric iron with oxygen to form iron oxides or to the reaction with other food components resulting in the formation of color active substances.
Polyphenols have been reported to contribute to the color development in foods fortified with soluble iron (Brune et al., 1989). Only certain structures of polyphenols seem to interact with iron to form discoloration. Brune et al. reported that color formation and iron absorption inhibition was dependent on the presence of galloyl groups (as found in o,m-hydroxyphenol; Brune et al., 1989). A recent study done in model solutions and in foods rich of polyphenols confirmed this finding by showing that ortho-hydroxy groups as found in gallic acid (chocolate), catechin (green tea), chlorogenic acid (coffee) cause off color developments with iron (Mellican et al., 2003). Additionally, fruits and vegetables contain polymerized polyphenols of high molecular weight, likely to include many ortho-hydroxy groups (Mellican et al., 2003).

The neutral pH in the food matrix and the presence of oxygen can accelerate the oxidation of iron to its ferric form, which serves as a substrate in the color formation reaction with polyphenols. Lower pH and the addition of reducing and chelating agents (ascorbic acid or EDTA) stabilized the ferrous ions reducing or inhibiting the color formation. It has been suggested that the reduction/oxidation reaction that oxidizes polyphenols and reduces ferric iron back to its ferrous form might induce structural changes and polymerization in the polyphenols influencing their light absorption pattern (Mellican et al., 2003).

The redox activity of soluble iron ions does not affect only the color of the food. Soluble iron itself can have metallic or astringent taste, especially in beverages (Hurrell, 1999). In cereal flours, iron can catalyze the oxidation of unsaturated fatty acids and accelerate the formation of rancidity. Oxidation products as hexanal or pentane can be determined to investigate the extent of oxidation occurred (Bovell-Benjamin et al., 1999; Hurrell et al., 1989). In milk, soluble iron compounds induce unpleasant odors and rancidity and increase the TBA number (thiobarbituric acid test), an oxidation marker which in milk products has been reported to correlate well with organoleptic evaluation (Gaucheron, 2000). Homogenization, deareation or pasteurization at more than 81 C have been suggested to reduce milk off flavor or metallic taste (Gaucheron, 2000).
3.4.3 Iron fortification of rice

Biological approaches for rice fortification are discussed in the chapter 3.3 on bio-fortification. In this section food technological approaches for rice fortification are reviewed.

Fortification is defined as the addition of nutrients based on nutritional needs, whereas enrichment restores the original nutrient content present in the rice grain before milling (Hoffpauer, 1992). Several technological difficulties are encountered in rice enrichment or fortification. Rice is consumed as intact grains and it has poor color masking proprieties, so even a few discolored grains may make the product unacceptable (Cook and Reusser, 1983). Also, fortified rice must be rinse resistant, as in most developing countries rice is washed to remove grits, sand and other impurities. The rice must also show minimal nutrient loss after cooking, in case it is cooked in excess water (Zilberboim, 1994).

Parboiling
Traditionally, parboiling is done by heating a jar of water and rough rice, which is then dried in the sun and milled. As a result, vitamins from the embryo and the bran are transferred into the endosperm. Industrially, this process is performed by applying vacuum on rough and brown rice. After soaking, pressure is applied on the grains to facilitate the transfer of nutrients (Lofti and Britton, 1998). Parboiling can achieve iron concentrations of roughly 15 mg Fe/kg (USDA, 2005) and can result in a golden color that might not always be acceptable to consumers (Dexter, 1998)

Powder enrichment
In powder enrichment a mixture of vitamins and minerals is added to the rice soon after milling, when the powder adheres well to grains due to the moisture and heat on the grain surface. However, aspiration or sifting, as they are common in mills, will remove the nutrients. Another disadvantage is that 20-
100% of the enrichment will wash off the rice if it is rinsed before cooking (Hoffpauer, 1992).

**Coating-premix approach**

In this method, rice grains are first coated with a concentrated nutrient blend. Successively a layer of water insoluble food grade material is added to protect from cooking and rinse losses. The coated grains are then blended with natural rice at a 1:200 concentration. To prevent clumping of the grains talc and ferric pyrophosphate can be applied on the grains (Dexter, 1998). This method was originally marketed by Hoffmann-La Roche (Hunnell et al., 1985).

In Japan enriched rice has been manufactured with a two step coating technology. Rice is soaked in a solution containing water soluble vitamins, dried, coated with vitamin E, calcium and iron, and finally coated with a protective coating material and a coloring agent. This rice called Shingen is then blended with natural rice at a 1:200 ratio. Retention of nutrients was reported to be 90% and was claimed to lower blood pressure and improve hemoglobin in rural elderly Japanese women, but the study was never published in the peer reviewed literature (Hunnell et al., 1985). Peil et al. have tested different coating materials for rice fortification, and found that a blend of hydroxypropyl-methylcellulose and methylcellulose gave the best retention results after cooking, with a 100% retention of iron, 70% for Vitamin A and 18%, 18% and 21% for niacin, thiamin and riboflavin, respectively (Peil et al., 1982). A recent study tested the suitability of different edible coating materials for folic acid fortification of rice, and found the lowest losses with coatings of ethyl cellulose (Shrestha et al., 2003).

Another method has been reported for the fortification of iron in the Philippines, a county that pioneered rice fortification. Rice containing thiamin, niacin and iron marketed by Hoffmann-La Roche was tested in a large effectiveness trial in the province of Bataan in 1946. Half of the province received fortified rice and the other half served as a control. Beriberi prevalence was drastically reduced, and mortality form beriberi was virtually
Chapter 3

eliminated in the experimental area. A rice enrichment act passed in 1952, but was soon withdrawn due to several logistic and economical constraints.

A new technology was developed in 1982: polished rice is coated in a large rotating cylinder and a solution containing ferrous sulfate is sprayed onto the grains. After drying, the rice is coated with a water insoluble coating material with color masking proprieties to minimize washing off of nutrients (Lofti and Britton, 1998). In a trial in 9-12 year old children, iron fortified rice was prepared using a 1:100 ratio. The authors claim that a lower ratio of 1:50 or 1:75 would have been unacceptable in color or odor. The rice provides 5.33 mg Fe/100g as ferrous sulfate, and has been reported to improve iron status (Florentino, 2001).

In the USA, most of the marketed rice is enriched either by powder enrichment or by coating technology. According to the regulations thiamin, niacin, riboflavin, vitamin D, calcium, folic acid, iron (between 28 and 57 mg Fe/Kg) are added. If less than 85% of the nutrients are retained after rinsing, the packaged product has to be labeled with the statement “to retain vitamins, do not wash or rinse before or drain after cooking” (FDA, 1990). Other countries where enriched rice products have been marketed are Canada, Japan, Argentina and Chile (Lofti and Britton, 1998).

Extrusion-premix approach

An advantage of the extrusion approach is the potential utilization of rice kernels that have been broken during milling. These broken grains have been traditionally discarded and/or used for animal feed (Lofti and Britton, 1998). The program for appropriate technology in health (PATH) has promoted a rice premix produced with pasta-like cold extrusion technique using a binder to cross link the rice flour. The rice has been reported to be an effective vehicle for vitamin A (Flores et al., 1994; Murphy et al., 1992).
Rice fortification with iron through extrusion and premix was done with hot extrusion without the use of cross-linking agents (Zilberboim, 1994). The same group used an innovative approach to fortify rice with ferrous sulfate with strong acidification of the rice flour. The acidified products were color compatible after extrusion, but extended storage resulted in color discolorations. Additionally, after cooking, overall acceptability of the rice was significantly lower than unfortified rice (Kapanidis and Lee, 1996).

3.4.4 Effect of food processing on iron bioavailability

Food processing can have an effect on the iron content of foods and their bioavailability either by influencing the food matrix or by directly interacting with intrinsic or fortification iron. The different operations of food processing and their possible effects are summarized in Table 2. In general there is no simple relation between food processing and iron bioavailability (Watzke, 1998). In a recent study the effect of different heating techniques on iron absorption was investigated. No evident differences between extrusion, roller drying or home cooking could be found. Rather, iron absorption was dependent on the phytate content (Hurrell et al., 2002).
Table 2- Possible and estimated effects of different unit operations in food processing on mineral bioavailability, adapted from (Watzke, 1998)

<table>
<thead>
<tr>
<th>Operation</th>
<th>Effect(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soaking</td>
<td>↓ Ash values decreased by leaching, ↓ Bran separation, minerals lost in milling</td>
</tr>
<tr>
<td>Milling</td>
<td>↓</td>
</tr>
<tr>
<td>Boiling/Cooking</td>
<td>↓ Leaching, phytate retention, oxidative losses</td>
</tr>
<tr>
<td>Pasteurisation</td>
<td>↓ Few losses</td>
</tr>
<tr>
<td>Sterilisation</td>
<td>↓ Loss into fluid</td>
</tr>
<tr>
<td>Baking</td>
<td>↑ Phytate hydrolysis</td>
</tr>
<tr>
<td>Freezing</td>
<td>↓ Losses in blanching and thawing</td>
</tr>
<tr>
<td>Fermentation</td>
<td>↑ Phytate destruction</td>
</tr>
<tr>
<td>Germination</td>
<td>↑ Phytate destruction</td>
</tr>
<tr>
<td>Extrusion</td>
<td>↓ Phytase deactivation</td>
</tr>
<tr>
<td>Packaging</td>
<td>↓ Possible reactions in tin cans</td>
</tr>
<tr>
<td>Storage</td>
<td>↓ Oxidation reaction</td>
</tr>
</tbody>
</table>

3.4.4.1 Effect of thermal and extrusion processing on iron bioavailability

Extrusion has been extensively investigated as it represents a combination of different food processing operations, where heat treatment, working, forming and feed transport all happen at the same time (Watzke, 1998). Extrusion cooking has been reported to decrease the phytic acid content in bran products (Sandberg et al., 1987) and in rice legume blends (Chauhan et al., 1988). Sandberg et al reported a 25% decrease in inositol hexaphosphate by extrusion. However, the decrease in hexaphosphates was compensated by an increase of penta and teraphosphates. In the same study, the extrudates were fed to ileostomy patients, and it was found that phytate in unextruded bran did undergo further hydrolysis, whereas the extruded phytate was not further digested. The authors explained this significant effect either by the inactivation of the food phytase by extrusion or by the formation of insoluble phytate complexes (Sandberg et al., 1987). Other authors have confirmed the finding that high temperature short time (HTST) extrusion increases the conversion of phytate to its lower phosphate forms without changes in total phytate content (Ummadi et al., 1995).
In a study in rats, maize and potato extrudates were extruded at high temperature (120-140 C) and pressure (35-62 atm) and iron absorption from food iron was measured with $^{59}$Fe as extrinsic label. Whereas the iron content in extruded products increased by leaching iron out of the extruder (5.9 and 13.3 mg Fe/kg for unextruded and extruded maize, respectively), fractional absorption did not change between extruded and unextruded maize and potatoes (Fairweather-Tait et al., 1987). In a human absorption study estimating iron absorption from fecal excretion of stable isotopes, a similar picture was found in extruded and non extruded wheat bran: although processing did slightly decrease phytic acid content as inositol hexaphosphate, no effect of extrusion on the iron and zinc bioavailability could be measured (Fairweather-Tait et al., 1989).

In most of the studies on the effect of processing on iron bioavailability, model calculations and dialysis experiments were performed to evaluate possible effects of processing on iron valence and solubility (Hazell and Johnson, 1989; Kadan and Ziegler, 1987). Extrusion cooking showed slight increase in iron dialysability, which the authors explained with the depolymerisation under shear forces and temperature that might render iron more soluble (Hazell and Johnson, 1989).

The few animal or human studies on the effect of food processing on iron fortified foods found conflicting results. In a series of bioavailability studies in rats, liquid infant formulas fortified before sterilization, showed better hemoglobin regeneration than when fortified after sterilisation. Ferric pyrophosphate had however a more pronounced increase than ferrous sulfate, which resulted in increased relative bioavailability with sterilization (Theuer et al., 1971; Theuer et al., 1973). In a human study in a chocolate milk which underwent vacuum-drying for 3 hours at 95 C, ferrous sulfate was unaffected by food processing whereas bioavailability from ferric pyrophosphate decreased from 2.11% to 0.21%, resulting in a decreased relative bioavailability after processing (Hurrell et al., 1991). However, the group receiving the unprocessed food had a better iron status than the group
receiving the processed food, and it is unclear if this may help explain the observed difference. An additional possible explanation for this discrepancy could lie in the two different forms of processing, as it is possible that in the heat-sterilized liquid infant formula the ferric pyrophosphate dissolved in the liquid and was therefore made more bioavailable.

3.4.6 Experiences with food fortification on a country level

In this section, published studies of the effect of food fortification on iron status in countries where food fortification was initiated (or abruptly discontinued) are reviewed. However, these studies do not include a control group and are often not corrected for potential confounders.

3.4.6.1 Targeted iron fortification

The decrease in iron deficiency prevalence in infants in industrialized countries has been suggested to be due to the introduction of iron fortified formulas and iron fortified weaning foods (Fomon, 2001; Lynch, 2005). An example of targeted iron fortification on national scale has been reported in Chile, where the ministry of health provided infants with 2 kg of powdered milk fortified with ferrous sulfate (10 mg Fe/100 g) and vitamin C (70 mg/100g) every month for 18 months. Iron status was measured in a non-representative random sample of free living infants attending a health centre for vaccination (n=129) and the prevalence of anemia among infants decreased from 27 to 9% (Hertrampf et al., 2001).
3.4.6.2 Universal iron fortification

In 1994, Sweden discontinued its universal wheat flour fortification program (65 mg Fe/kg). In a study in 2 random samples of 600 female adolescents taken in 1994 and 2000 (before and after discontinuation) prevalence of iron deficiency (SF<16 µg/l) increased from 39 to 50%, after correcting for potential confounders as intake of supplements, contraceptive pills, dietary factors and others. In this population group, estimated iron intake decreased by 39% after suspension of flour fortification (Hallberg and Hulthen, 2002).

Reports on the situation in Denmark, where flour was fortified with 30 mg Fe/kg, show a slightly different picture. No significant changes in prevalence of iron deficiency and serum ferritin concentration were found in premenopausal women, and a significant increase in serum ferritin was found in postmenopausal women (Milman et al., 2003). In blood donors, however, a tendency towards lower serum ferritin concentrations could be assessed, indicating than in vulnerable population groups discontinuation of food fortification might have decreased iron stores (Lynch, 2005). The authors report a significant effect of various lifestyle factors as BMI, alcohol consumption and smoking on serum ferritin concentration (Lynch, 2005), but did not adjust the generated data for these effects. The reported increase in serum ferritin in postmenopausal women could thus be ascribed rather on the increase in BMI and alcohol consumption or other factors than to changes in iron status (Milman et al., 2003).

In Chile wheat flour is fortified with 30 mg Fe/kg, and although no prevalence data has been published about the period before fortification, prevalence of anemia in children and pregnant adolescents is very low (<5%; Hertrampf, 2002).

A wheat and corn flour fortification program using ferrous fumarate as iron vehicle has been implemented in Venezuela. Every two years the prevalence ID/IDA was assessed in random samples of children from lower
socioeconomic strata in Caracas. Mean serum ferritin levels increased significantly after introduction of the program (13 to 21 g/l) and remained stable in the following years. Anemia prevalence however, decreased from 19 to 9% after introduction of the program but subsequently increased back to its original level in the following years. One of the possible reasons suggested by the authors is that the living conditions in the country continued to deteriorate during the monitored period (Beard, 1996; Garcia-Casal and Layrisse, 2002; Layrisse et al., 1996).

3.4.6.3 Household fortification

Infants might have limited access to industrially processed foods and only partially benefit from the availability of fortified staples (Davidsson, 2003). Household food fortification has therefore been suggested as a way to improve the nutritional quality of infant complementary foods (Zlotkin et al., 2005). The use of micronutrient sprinkles providing 80 mg Fe as encapsulated ferrous sulfate and 50 mg ascorbic acid has been shown to improve hemoglobin and serum ferritin in randomized trials in rural Ghana (Zlotkin et al., 2003; Zlotkin et al., 2001). Iron absorption from sprinkles is high in infants: between 8.25% to 4.65% depending on iron status (Tondeur et al., 2004). Sprinkles are usually added to the complementary food directly by the mother before feeding, and a possible advantage of sprinkles is the lower risk of over dosage compared to ferrous sulfate drops (Zlotkin et al., 2001).

Another approach in home fortification that has been explored is the use of micronutrient fortified energy spreads. This approach was initially introduced for famine alleviation in relief operations (Collins and Sadler, 2002), where the use of dried products to be reconstitute in water is not recommended. The use of fat as a vehicle has the advantage to be hygienically safe, to have relatively long shelf life, and offer the possibility to have taste masking proprieties (Briend, 2002). Spread development is not as advanced as the development
of sprinkles (Nestel et al., 2003), and this vehicle has not been tested in field trials.

The use of iron pots has been discussed as a potentially low cost intervention to counteract iron deficiency. In a randomized trial, the use of iron pots for cooking has been shown to improve iron status, hemoglobin and linear growth after six months of use (Adish et al., 1999). The food consumed from iron pots contained approximately double the amount of iron than the food cooked in aluminum pots, in preliminary laboratory tests (Adish et al., 1999). In a recent study in rural Malawi however, the acceptability of the use of a specific iron pot was significantly lower than for an aluminum pots, and the main complaints were the high weight and the rusting surface of the iron pot (Prinsen Geerligs et al., 2002). Acceptability and the sustainability might therefore be limiting factors for the use of non-steel iron pots in iron deficient communities.
CHAPTER 4- STUDY SITE

Bangalore is situated at approx. 900 meters above sea level in central south India, in the state of Karnataka, India. The climate can be divided in four distinct seasons: the relatively dry, cool winter from December through February; the dry, hot summer from March through May; the southwest monsoon from June through September and the northeast, or retreating, monsoon of October and November. The Deccan region, bounded by the Western and Eastern Ghats, receives most of its annual rainfall during the summer monsoon season, with relatively lower rainfalls compared to more coastal regions of India. The temperature range in Bangalore is 14.5-34.7 C (Heitzman and Worden, 1996).

Bangalore is the economical centre of Karnataka. Figures form the 1991 and 2001 censuses show a strong increase in population, from ≈2 900 000 in 1991 to ≈4 300 000 in 2001 (Helders, 2005), whereas half of this increase is reported to be due to rural to urban migration. Projections indicate that by 2020 half of the Indian population will live in urban environment and that one third of the urban population will be urban slum dwellers (Ghosh and Shah, 2004).

Although the nutritional situation in urban slums may be peculiar, it has not been investigated extensively. Nationwide anthropometric data suggest that roughly half of the children under three years of age are underweight and stunted, and that 70-75% of children up to the age of three have anemia (Ghosh and Shah, 2004). South east Asia is reported to be the region in the world with the highest anemia prevalence (WHO, 2001). In slum populations, prevalence of IDA/ID can only be estimated from individual studies. In children living in the slums of New Delhi prevalence of anemia in less than three year olds was 67%, whereas iron deficiency defined as SF<12 μg/l was 88% (Kapur et al., 2002).
Malaria is not endemic in south India (Sitalakshmi et al., 2003), and dietary factors are suggested to take a large part in the etiology of iron deficiency and iron deficiency anemia (Ahluwalia, 2002). However, prevalence of intestinal parasites is high in the urban slum population (Sur et al., 2005).

The statewide programs to supply the unprivileged population with subsidized foods could potentially offer means to fortify staple foods with iron (Mannar and Sankar, 2004). The Public Distribution System (PDS) supplies 330 million people, and some states like Gujarat currently subsidy the use of iodized salt through the PDS system. The midday meal scheme could also constitute a possible vehicle for fortification. It is aimed to supply school aged children with a simple dish and thus encourage school attendance. The national government provides the rice, whereas the local governments provide the local infrastructure to supply the meals to the schools. It has been implemented in several Indian states, including Andhra Pradesh, Tamil Nadu and Kanataka.
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CHAPTER 5

DEVELOPMENT AND EVALUATION OF IRON FORTIFIED EXTRUDED RICE GRAINS

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Abstract

Although rice can be fortified with iron by producing fortified extruded grains, achieving good sensory properties and high iron bioavailability is difficult. Our study aim was to develop iron-fortified rice with comparable sensory characteristics to natural rice using iron compounds of high bioavailability. We tested ferrous sulfate, NaFeEDTA, ferric pyrophosphate of different particle sizes (mean particle sizes: 20 μm, 2.5 μm, 0.5 μm) and electrolytic iron, as well as encapsulated forms of iron. Extruded rice grains containing 0.5 and 1 g Fe/100 g were produced using a single screw extruder and blended, respectively, with natural rice at a 1:100 or 1:200 ratio. Extruded rice grains were evaluated by color measurements and texture profile analysis, and iron loss during rinsing was measured. The sensory comparison between fortified and unfortified rice was performed using triangle tests.

Color scores in a similar range to natural rice were obtained using ferric pyrophosphate as iron fortification compound. The cooked extruded grains had comparable texture to cooked natural grains, and losses during rinsing were <3%. Fortification with all other compounds resulted in strong color changes. In the triangle tests, rice grains fortified with either of the 2 forms of micronized ferric pyrophosphate closely resembled unfortified rice in both uncooked and cooked form. Iron fortified extruded rice grains with excellent sensory characteristics and potential high bioavailability can be produced using micronized ferric pyrophosphate.

Key words: rice, iron, fortification, bioavailability, color, extrusion.
Introduction

Iron deficiency anemia (IDA) is common in children and young women in the developing world as well as in developed countries (WHO, 2001). IDA has adverse health effects on pregnancy outcome, infant growth, cognitive performance, immune status and work capacity (WHO, 2001) and iron fortification of staple foods is considered a sustainable approach to combat iron deficiency (WHO, 2001).

Rice is the leading staple food in the developing world, where 95% of the world production of rice is harvested and approximately 90% of the world production is consumed (FAO, 2003). Although rice fortification with iron could be a promising strategy to combat iron deficiency, it is challenging because rice is usually consumed as grains and because compounds that cause fewer sensory changes tend to be poorly bioavailable. Water soluble compounds such as ferrous sulfate are more bioavailable, but also highly reactive with the food matrix, often producing oxidation and unacceptable color changes (Hurrell, 2002).

In industrialized countries, rice is often enriched with iron to restore the iron content found in the unmilled grains (Hunnell et al., 1985). In rice fortification, several techniques been reported (Hoffpauer 1992), including coating of a grain premix with a solution of ferrous sulfate (Florentino, 2001) ferric orthophosphate, or elemental iron (Peil et al., 1982). Cold extrusion of simulated rice grains enriched with Vitamin A (Cox and Cox, 1997) has also been reported using a binding and cross-linking agent. None of these techniques has been used extensively for iron fortification in developing countries, due to technical problems. A promising extrusion technology has been reported for making iron fortified rice grains (Zilberboim, 1994; Zilberboim et al., 1993; Kapanidis and Lee, 1996). Kapanidis and Lee (1996) showed that through acidification of the rice flour to a pH of 0.5, overall acceptably colored iron fortified rice grains could be produced with ferrous sulfate. After cooking, however, the acidulated extruded rice grains had lower overall acceptability than Jasmine rice possibly due to a metallic aftertaste (Kapanidis and Lee 1996).
In the past years, novel iron compounds have been developed which have good potential for rice fortification. Encapsulated ferrous sulfate has a physical barrier (hydrogenated palm oil or soy bean oil) that can prevent reactions between the food matrix and the iron if the capsule is not damaged during food processing (Harrison et al., 1976). Encapsulated ferrous sulfate has comparable bioavailability to ferrous sulfate in rat studies (Hurrell et al., 1989), and an efficacy trial with salt demonstrated the efficacy of encapsulated ferrous sulfate in humans (Zimmermann et al., 2003).

Another promising approach is the reduction of the particle size of water insoluble iron compounds to increase their surface area and rate of solubility in the gastric juice (Bjorn-Rasmussen et al., 1977; Swain et al., 2003). In rats, ferric pyrophosphate with mean particle size of 2.5 μm has a relative bioavailability to ferrous sulfate of ≈70% (Wegmuller et al., 2004). In a recent salt fortification trial, this compound was highly effective in reducing the prevalence of iron deficiency in children (Zimmermann et al. 2004). A dispersible ferric pyrophosphate with mean particle size of 0.5μm (commercial name Sunactive Fe™), has been developed for iron enrichment of milk products (Nambu et al., 1998). In a recent human study, this compound had similar bioavailability to ferrous sulfate in a yogurt drink and in an infant cereal (Fidler et al., 2004), indicating its high bioavailability in humans.

Similarly, the iron in NaFeEDTA is highly bioavailable (Hurrell et al., 2000; INACG, 2002) and causes few sensory problems in maize flour (Bovell-Benjamin et al., 1999).

Because of its low reactivity with food matrices and its low price, electrolytic iron is often chosen for fortification of wheat flour (Hurrell et al., 2002).

In the present study the method of (Kapanidis and Lee, 1996) without acidification was used to produce extruded fortified rice grains, containing 0.5g and 1g Fe/100g using the different iron fortification compounds. Appearance of the extruded products was characterized with color.
measurements. Iron loss was determined after rinsing, and texture measurements were done on cooked rice grains. Triangle tests were performed to test sensory differences between fortified and unfortified rice (Bovell-Benjamin and Guinard, 2003). Fortified rice and unfortified rice were compared in both raw and cooked forms. The overall aim of this study was to develop iron-fortified rice containing a bioavailable form of fortification iron, and with similar sensory characteristics to natural rice.

**Material and Methods**

*Iron compounds*

The iron compounds tested were:

- micronized dispersible ferric pyrophosphate with mean particle size \( \approx 0.5\mu \text{m} \) (composition containing dextrin, partly hydrolyzed lecithin, polyglycerol esters of fatty acids and ferric pyrophosphate: Sunactive Fe\(^{\text{TM}}\), Taiyo Kagaku, Yokkaichi-Mie, Japan)
- reagent grade ferric pyrophosphate (Sigma-Aldrich, St.Louis, MO)
- ferric pyrophosphate with mean particle size \( \approx 20 \mu \text{m} \) (FCC grade, Lohmann GmbH, Emmerthal, Germany)
- ferric pyrophosphate with a mean particle size \( \approx 2.5\mu \text{m} \) (FCC grade, Lohmann GmbH, Emmerthal, Germany)
- electrolytic Fe (FCC grade, Lohmann GmbH, Emmerthal, Germany)
- encapsulated FeSO\(_4\) in hydrogenated palm oil (approx. melting point 60 C, Lohmann GmbH, Emmerthal, Germany)
- NaFeEDTA (FCC grade, Lohmann GmbH, Emmerthal, Germany)
- encapsulated FeSO\(_4\) in liposome (composition containing ferrous sulfate stabilized with ascorbic acid and microencapsulated in phospholipids; Biofer\(^{\text{TM}}\), Lipotech SA, Buenos Aires, Argentina)
- FeSO\(_4\) as a positive control (Lohmann GmbH, Emmerthal, Germany)

All compounds were food grade except for reagent grade ferric pyrophosphate.
Iron fortification level

The target iron concentration of the extruded product was set at 0.5g and 1 g Fe/100g of rice. The concentration in the final product, mixed at a ratio of 1:100 or 1:200 with natural rice grains, would be 5 mg Fe/100g. This amount would provide 15 mg of additional iron to a person consuming 300 g of rice per day. Weekly per capita rice consumption in Bangladesh can be up to 3 kg (Torlesse et al., 2003), whereas in China (Chen et al., 2002; Torlesse et al., 2003) reported a consumption of 300-500 g of rice per day in adults. Iron bioavailability from rice depends mostly from phytate content of the grains and reflects the degree of milling (Tuntawiroon et al., 1990). Assuming 5% absorption in iron deficient subjects, this fortification level would potentially provide additional 0.75 mg of absorbed Fe per day, 50% and 75% of the estimated median requirement for women and men, respectively (Hallberg et al., 2000).

Extrusion conditions

Long grain rice flour (22% amylose, RF-L00030-12, Sage Foods, Los Angeles, CA) was mixed for 30 min. in a Hobart mixer (Hobart Corporation, Troy, OH) and the water content was adjusted to 25% by slowly adding distilled water to the rice flour previously fortified with iron. All extrusion experiments were conducted with a Brabender single screw extruder (Model 2003, C.W. Brabender Instruments Inc, S. Hackensack, NJ) with a 19 mm diameter opening, and a 20:1 L/D ratio. A screw with a 3:1 compression ratio was used for hot extrusion experiments, whereas a compression ratio of 1:1 was chosen for the screw for cold extrusion. All fortified rice grains except those fortified with encapsulated iron compounds (cold extrusion) were produced with hot extrusion using 50 C and 70 C in zone 1 and 2 of the barrel, respectively. Cold extrusion was performed at 30 and 40 C in Zone 1 and 2, respectively. A special cutter and a die made of brass (dimensions 9.76x1.62 mm both manufactured at Rutgers University) were used for shaping of the rice grains. Extruded rice grains were air dried over night after extrusion to reach water content of approximately 10g/100g weight. Commercially available rice grains for comparison (Basmati rice and Jasmine rice) were purchased in a local store (Coop, Switzerland).
Color measurements

Color measurements were performed with a Minolta Chroma meter CR-210 (Minolta Camera Co., Osaka, Japan) on extruded simulated rice grains. The camera was calibrated using a standardized white plate (L=97.76, a=-0.51, b=2.26). A granular material attachment (Minolta Camera Co., Osaka, Japan) equipped with a low reflectance glass (Minolta Camera Co., Osaka, Japan) was used to compare color scores. All samples were measured in six replicates. Overall color difference was evaluated with the parameter $\Delta E = ((L_1 - L_2)^2 + (a_1 - a_2)^2 + (b_1 - b_2)^2)^{1/2}$.

A storage trial was carried out to investigate color stability of the iron fortified extruded grains which showed the least color differences compared to natural grains. Grains were stored over 5 months in transparent polyethylene bags at constant room temperature (20°C) under natural lighting conditions.

Texture measurements

The method used for the texture measurements was adapted from previously published methods (Champagne et al., 1998; Okabe, 1979). Fifteen g of extruded rice were soaked for 1 hour in 30 ml tap water at room temperature. The grains were dried with a paper towel and filled in a cylindrical tapered aluminum cup (top diameter 44 mm, bottom diameter 34 mm, height 35.5 mm). 18 ml of tap water were added to the rice grains. The cups were introduced in a household rice cooker (Tatung Corp, Taipei, Taiwan) with the inner pan removed. 30 ml of water were added to the outer pan for moisture equilibration and samples were cooked for 9.5 min. Samples were left in the hot pan for additional 15 min. and then left at room temperature for 90 min. for temperature equilibration. Three intact rice grains were sampled from the center of the cup and texture measured with a TA-XT2 texture analyzer (Godalming, Surrey, UK). Each measurement was repeated 5 times (n=5) with a constant plunger-plate distance of 5 mm with a compression distance of 4.9 mm. The rice grains were compressed within 0.1 mm of the bottom plate. Plunger speed was 1 mm/sec, with a relaxation time of 5 s. Samples were measured using texture profile analysis (Champagne et al., 1998), which simulates a two bite compression. Natural long grain rice with the same
Chapter 5

Amylose content of the rice flour and a commercially available Jasmine rice were used as comparison. Hardness (kg) is defined as the force needed to compress the sample by the plunger-plate distance on the simulated first bite, whereas stickiness (kg) and adhesiveness (kg.s) both describe the force and the work, respectively, needed to bring the plunger back to the original position after compression. Cohesiveness is the ratio between the work for the simulated second bite and the work for the simulated first bite. Springiness is the ratio between the distances traveled by the plunger on the down stroke from sample contact to the end of the down stroke on simulated second bite and simulated first bite.

Iron loss by rinsing

Iron fortified rice grains were soaked in 20 ml of distilled water and agitated in circular shaker (Polytest 30, Fisher Bioblock Scientific, Fisher Scientific International Inc. Hampton, NH) for 30 min. at 30°C at maximum speed of rpm=200. An aliquot of 2 ml was taken from the rinsing water, mineralized using an HNO₃/H₂O₂ mixture and microwave digestion (MLS Ethos Plus, MLS Laborsysteme, Leutkirch im Allgäu, Germany) and analyzed by flame atomic absorption spectroscopy (SpectrAA 400, Varian, Mulgrave, Australia) using a standard addition technique to minimize matrix effects.

Sensory studies

A preliminary visual comparison was made between all mixtures (extruded rice grains and natural rice) with varying dilution ratios (1:100 and 1:200) and iron concentrations (0.5 gFe/100g and 1gFe/100g), as well as different types of natural rice grains (Jasmine, Long Grain, Basmati). The four mixtures which showed the closest similarity to the natural grains were selected to be tested in the sensory study. Two series of triangle tests were performed. First, uncooked rice was evaluated visually. In a second step rice was evaluated after cooking.

Prior to the proper sensory study, 18 subjects (age range 20-45 years) from the student and staff population of the Institute of Food Science and Nutrition in Zurich were trained in visual and taste triangle tests, using two similar rice varieties (Long Grain and Jasmine) in the raw and cooked form.
For the visual evaluation four types of fortified rice mixed 1:100 or 1:200 with natural rice were presented in a balanced design to be compared with natural unfortified rice. Two of the four samples were tested in duplicate.

Twenty-five grams of natural/extruded rice mixture with an iron content of 5 mg Fe/100 g were presented in tapered aluminum plates (111 mm diameter, 45 mm height). Samples were mixed thoroughly before presentation and the panel performed six visual triangle tests in one session.

In the cooked form, two combinations of fortified extruded grains and natural grains were compared to natural unfortified rice in different sessions, not exceeding two taste evaluation-triangle tests per session. The formulations selected were chosen according to the outcome of the first sensory study and were tested in duplicate. Rice was cooked in a household rice cooker (Severin GmbH, Sunden, Germany) with water to rice ratio of 2:1 (wt. /wt.). Preheated porcelain cups were used for tasting, and a dose of ten grams of cooked rice was served. To ensure that every 10 g dose contained at least one extruded rice grain a higher premix concentration was chosen (1.5:100). Rice was kept at 50 C in a rice cooker for max. 30 min. All tests were performed in partitioned booths under uniform lighting conditions and the subjects were not informed about the background of the study. The extruded rice grains were manufactured two months before the triangle tests.

Statistical analysis

Colorimetric data were expressed as mean (SD) for each type of extruded rice grain. In the texture testing and in the iron loss by rinsing measurements differences between rices were tested by one way-ANOVA, with LSD as the post hoc test (SPSS Inc. Version 11.5, Chicago, IL). Results of the triangle tests were evaluated with the Chi square statistic. For replicated tests following expected probabilities were used: 1 correct answer: p=4/9; 2 correct answers p=1/9; 0 correct answers: p=4/9. For single tests expected probabilities were p=1/3 for correct guess and p=2/3 for wrong guess. Significance was set at p=0.05.
Results and Discussion

Color measurements

Table 1 shows the color profile of the iron fortified extruded rice grains two weeks after production. Unfortified extruded rice grains had a difference in color of $\Delta E=3.1$ and $\Delta E=3.4$ compared to Basmati rice and Jasmine rice, respectively. When extruded grains were fortified with 0.5 g Fe/100g reagent grade ferric pyrophosphate, this difference decreased to $\Delta E=1.4$ and $\Delta E=2.1$, indicating that reagent grade ferric pyrophosphate had color masking properties. Increasing the iron concentration of the extruded grains increased the color deviation from natural and from extruded unfortified rice in all but one fortification compound (reagent grade ferric pyrophosphate). This is probably related to impurities in the food grade iron pyrophosphate, which is not manufactured with the same purification standard as reagent grade ferric pyrophosphate.
Table 1- Color scores of iron fortified simulated rice grains (premix grains) after 2 weeks of storage at room temperature (25°C) in PE zipper bags. L: whiteness; a>0: redness, a<0: greenness; b>0: yellowness, in brackets (SD). ΔE indicates the overall color difference relative to Basmati Rice, Jasmine Rice and extruded unfortified rice.

<table>
<thead>
<tr>
<th></th>
<th>L:</th>
<th>a:</th>
<th>b:</th>
<th>Color appearance</th>
<th>ΔE to Basmati rice</th>
<th>ΔE to Jasmine rice</th>
<th>ΔE to extruded unfort. rice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Natural Grains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jasmine Rice</td>
<td>72.89(0.02)</td>
<td>0.06(0.01)</td>
<td>13.03(0.03)</td>
<td>White-opaque</td>
<td>1.08</td>
<td>0.00</td>
<td>3.43</td>
</tr>
<tr>
<td>Basmati Rice</td>
<td>72.89(0.01)</td>
<td>0.04(0.02)</td>
<td>14.11(0.02)</td>
<td>White-opaque</td>
<td>0.00</td>
<td>1.08</td>
<td>3.11</td>
</tr>
<tr>
<td><strong>Extruded Simulated Rice Grains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extruded Rice unfortified</td>
<td>69.73(0.02)</td>
<td>-0.29(0.01)</td>
<td>12.06(0.02)</td>
<td>White translucent</td>
<td>3.11</td>
<td>3.43</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Ferric Pyrophosphate Compounds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent Grade Ferric pyrophosphate 0.5 g Fe/100g</td>
<td>71.44 (0.03)</td>
<td>-0.94(0.00)</td>
<td>12.59(0.03)</td>
<td>White, opaque</td>
<td>1.44</td>
<td>2.10</td>
<td>1.74</td>
</tr>
<tr>
<td>Reagent Grade Ferric pyrophosphate 1 g Fe/100g</td>
<td>70.43(0.02)</td>
<td>0.27(0.02)</td>
<td>12.33(0.02)</td>
<td>White, opaque</td>
<td>2.49</td>
<td>3.05</td>
<td>1.03</td>
</tr>
<tr>
<td>Micronized dispersible ferric pyrophosphate 0.5 g Fe/100g</td>
<td>67.11(0.01)</td>
<td>0.23(0.01)</td>
<td>14.18(0.02)</td>
<td>White opaque, yellow tone</td>
<td>5.82</td>
<td>5.78</td>
<td>2.99</td>
</tr>
<tr>
<td>Micronized dispersible ferric pyrophosphate 1 g Fe/100g</td>
<td>66.28(0.01)</td>
<td>0.82(0.02)</td>
<td>13.63(0.01)</td>
<td>White opaque, yellow tone</td>
<td>6.6</td>
<td>6.67</td>
<td>3.71</td>
</tr>
<tr>
<td>Ferric pyrophosphate (20 μm) 0.5 g Fe/100g</td>
<td>69.14(0.02)</td>
<td>-0.18(0.02)</td>
<td>17.24(0.04)</td>
<td>White opaque, yellow tone</td>
<td>5.59</td>
<td>4.89</td>
<td>4.45</td>
</tr>
<tr>
<td>Ferric pyrophosphate (20 μm) 1 g Fe/100g</td>
<td>67.55(0.03)</td>
<td>0.3(0.01)</td>
<td>18.06(0.02)</td>
<td>White opaque, yellow tone</td>
<td>7.28</td>
<td>6.65</td>
<td>5.70</td>
</tr>
<tr>
<td>Ferric pyrophosphate (=2.5 μm) 0.5 g Fe/100g</td>
<td>70.52(0.03)</td>
<td>-0.26(0.01)</td>
<td>17.57(0.01)</td>
<td>White opaque, yellow tone</td>
<td>5.09</td>
<td>4.20</td>
<td>4.81</td>
</tr>
<tr>
<td>Ferric pyrophosphate (=2.5 μm) 1 g Fe/100g</td>
<td>69.46(0.02)</td>
<td>0.11(0.01)</td>
<td>18.97(0.02)</td>
<td>White opaque, yellow tone</td>
<td>6.05</td>
<td>5.24</td>
<td>5.26</td>
</tr>
<tr>
<td><strong>Encapsulated Compounds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposome composition (encaps. FeSO4) 0.5 g Fe/100g</td>
<td>51.34(0.02)</td>
<td>1.7(0.01)</td>
<td>8.27(0.02)</td>
<td>Black</td>
<td>22.05</td>
<td>22.39</td>
<td>19.05</td>
</tr>
<tr>
<td>Encapsulated FeSO4 (hydr.fat) 0.5 g Fe/100g</td>
<td>57.85(0.04)</td>
<td>2.34(0.02)</td>
<td>12.85(0.03)</td>
<td>Brown</td>
<td>15.13</td>
<td>15.27</td>
<td>12.17</td>
</tr>
<tr>
<td><strong>Ferrous Sulfate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeSO4 1g Fe/100g</td>
<td>46.46(0.02)</td>
<td>4.96(0.03)</td>
<td>13.25(0.01)</td>
<td>Dark Brown</td>
<td>26.80</td>
<td>26.90</td>
<td>23.86</td>
</tr>
<tr>
<td>FeSO4 0.5g Fe/100g</td>
<td>53.68(0.01)</td>
<td>2.94(0.02)</td>
<td>11.61(0.02)</td>
<td>Dark Brown</td>
<td>19.40</td>
<td>19.59</td>
<td>16.42</td>
</tr>
<tr>
<td><strong>Elemental Fe and NaFeEDTA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaFeEDTA 0.5g Fe /100 g</td>
<td>46.65(0.01)</td>
<td>7.34(0.01)</td>
<td>8.92(0.01)</td>
<td>Brown-Reddish</td>
<td>27.46</td>
<td>27.73</td>
<td>24.62</td>
</tr>
<tr>
<td>Elemental Fe electrolytic 0.5 g Fe/100 g</td>
<td>61.02(0.02)</td>
<td>-0.63(0.02)</td>
<td>6.37(0.02)</td>
<td>White-Grey</td>
<td>13.56</td>
<td>14.19</td>
<td>10.85</td>
</tr>
<tr>
<td>Elemental Fe electrolytic 1g Fe/100 g</td>
<td>52.07(0.02)</td>
<td>-0.24(0.01)</td>
<td>4 (0.02)</td>
<td>Dark Grey</td>
<td>22.62</td>
<td>23.15</td>
<td>19.74</td>
</tr>
</tbody>
</table>
Other ferric pyrophosphate compounds resulted in slightly elevated $\Delta E$-values but much lower than fortification with ferrous sulfate, encapsulated ferrous sulfate, elemental iron, or NaFeEDTA. Micronized dispersible ferric pyrophosphate caused a decrease in whiteness (L-values) and an increase in yellowness (b-values). A larger increase in yellowness could be seen with the addition of regular food grade ferric pyrophosphate of both particle sizes, whereas whiteness values seemed to be less affected than with micronized dispersible ferric pyrophosphate. Fortification with ferrous sulfate to the premix resulted in highly discolored (dark brown) rice grains, with decreased whiteness values and increased yellowness and redness (a-values). Addition of encapsulated ferrous sulfate did not prevent color development and resulted in similar redness and yellowness values, but in a less pronounced decrease in whiteness (brown color). Fortification with elemental iron resulted in grey rice grains, with lower yellowness than natural rice grains and lower whiteness. Rice grains fortified with NaFeEDTA had a brown-reddish color, and whiteness values similar to those of ferrous sulfate. Overall, ferric pyrophosphate compounds gave the best color match to natural rice, with a concentration dependent increase in color for all food grade compounds. The purified reagent grade ferric pyrophosphate gave the best color match of all compounds tested.

**Figure 1** represents the overall color difference ($\Delta E$) of selected iron fortified extruded rices during 5 months storage. The overall color difference relative to natural rice (Jasmine) was stable over 5 months for grains fortified with ferric pyrophosphate of either particle size (20 and 2.5 $\mu$m), and for reagent grade ferric pyrophosphate. Extruded grains fortified with micronized dispersible ferric pyrophosphate (0.5 $\mu$m) experienced a weak trend to an increase in color compared to natural rice. Such range of discoloration however, was far from the discolorations registered with unsuitable fortificants. Overall color difference between extruded rice grains fortified with all forms of ferric pyrophosphate and natural Jasmine rice changes very little over 5 months storage.
Figure 1- Overall color difference (expressed as ΔE) between differently fortified extruded rice grains and unfortified Jasmine rice over a storage period of 5 months at room temperature in polyethylene transparent zipper bags.

All Texture parameters of differently fortified rices were significantly different from each other when tested with a one-way ANOVA model (p<0.01). To identify specific differences between the different formulations LSD tests were performed as post hoc tests. All cooked extruded rice grains showed higher cohesiveness and springiness values than natural rice grains (p<0.05, Table 2). This effect is more strongly pronounced with unfortified extruded rice grains. The addition of iron decreased cohesiveness and springiness values in all iron fortified grains tested. However, increasing the iron concentration from 0.5 to 1 g Fe/100g did not result in a consistent further decrease in cohesiveness or springiness. The increased cohesiveness with extrusion is consistent with findings from other authors(Lee and Schwarz, 2004) and the values might be explained by the action of structuring forces in the extrusion process, where starch is plasticized and proteins are reversibly or irreversibly denatured.
Table 2 – Texture profile analysis of cooked rice grains fortified with micronized dispersible ferric pyrophosphate (0.5μm) and small particle sized ferric pyrophosphate (2.5μm) at 0.5g Fe/100g rice and 1 g Fe/100g. Results are expressed in Mean and SD (n=5). In columns, values without a common letter differ (P<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Cohesiveness (-)</th>
<th>Hardness (kg)</th>
<th>Springiness (-)</th>
<th>Adhesiveness (kg.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Natural Rice (Jasmine rice)</td>
<td>Mean 0.42e</td>
<td>11.1cd</td>
<td>0.16d</td>
<td>-250e</td>
</tr>
<tr>
<td></td>
<td>sd 0.01</td>
<td>0.9</td>
<td>0.01</td>
<td>43.7</td>
</tr>
<tr>
<td>2 Natural Rice (Long grain rice)</td>
<td>Mean 0.42e</td>
<td>8.95e</td>
<td>0.18de</td>
<td>-329e</td>
</tr>
<tr>
<td></td>
<td>sd 0.01</td>
<td>0.8</td>
<td>0.04</td>
<td>51.3</td>
</tr>
<tr>
<td>3 Unfortified, extruded rice</td>
<td>Mean 0.62a</td>
<td>13.0a</td>
<td>0.29a</td>
<td>-870a</td>
</tr>
<tr>
<td></td>
<td>sd 0.03</td>
<td>1.0</td>
<td>0.04</td>
<td>108</td>
</tr>
<tr>
<td>4 Micronized dispersible ferric pyrophosphate 0.5g Fe/100g</td>
<td>Mean 0.54bc</td>
<td>12.4ab</td>
<td>0.20bd</td>
<td>-721cb</td>
</tr>
<tr>
<td></td>
<td>sd 0.01</td>
<td>0.7</td>
<td>0.04</td>
<td>59.1</td>
</tr>
<tr>
<td>5 Micronized dispersible ferric pyrophosphate 1g Fe/100g</td>
<td>Mean 0.50d</td>
<td>11.8abc</td>
<td>0.19ed</td>
<td>-648cd</td>
</tr>
<tr>
<td></td>
<td>sd 0.02</td>
<td>1.3</td>
<td>0.02</td>
<td>79.8</td>
</tr>
<tr>
<td>6 Ferric pyrophosphate 0.5g Fe/100g</td>
<td>Mean 0.52bd</td>
<td>10.6d</td>
<td>0.22plce</td>
<td>-617d</td>
</tr>
<tr>
<td></td>
<td>sd 0.03</td>
<td>1.4</td>
<td>0.04</td>
<td>90.1</td>
</tr>
<tr>
<td>7 Ferric pyrophosphate 1g Fe/100g</td>
<td>Mean 0.55b</td>
<td>11.3bd</td>
<td>0.23ab</td>
<td>-749b</td>
</tr>
<tr>
<td></td>
<td>sd 0.03</td>
<td>0.7</td>
<td>0.04</td>
<td>25.1</td>
</tr>
</tbody>
</table>

Adhesiveness, a parameter that often reflects stickiness was also increased in all extruded grains (p<0.05) relative to natural rice grains. This increased value is most probably due to the higher amount of extra cellular starch in the extruded grain compared to the natural grain. Although hardness was significantly increased in extruded unfortified rice grains when compared to natural rice grains (p<0.05), this difference was no longer significant between natural rice and 3 out of four iron fortified extruded grains. These tests indicate that iron fortified extruded rice grains had a consistent and measurable texture after cooking. However, differences in cohesiveness and adhesiveness between extruded rice and natural rice are detectable instrumentally.
Iron loss by rinsing

In developing countries rice is often washed before cooking due to the presence of natural impurities and stones. Grains fortified with electrolytic Fe showed the lowest iron loss (0.6% and 0.2%, for 0.5 and 1g Fe/100g, respectively) which was significantly different from the iron loss measured for other Fe fortified rices (p<0.05, Table 3). Extruded rice fortified with micronized dispersible ferric pyrophosphate at 0.5g Fe/100g had the highest Fe loss (2.6%). The losses found are minimal and are of little effect on the amount of iron ingested by the consumer. Iron retention after cooking was reported to be 100% from grains fortified with electrolytic iron and coated with hydroxypropylmethylcellulose and methylcellulose (Peil et al., 1982).

Table 3- Iron loss (%) of fortified extruded rice grains with 5 mg Fe/100g shaken in water for 30 min at 30°C. Samples without a common letter differ (p<0.05).

**Sensory study**

After a preliminary color evaluation, four formulations were chosen to be evaluated with triangle tests (Table 4). In a first step iron fortified rice was visually compared to raw unfortified (natural) rice. Basmati rice fortified with ferric pyrophosphate of both 0.5μm and 2.5μm particle sizes and 0.5 g
Fe/100g in the premix could not be detected as significantly different (p=1.00 and p=0.32, respectively); similarly, Jasmine rice fortified with ferric pyrophosphate (0.5 μm 1 g Fe/100g premix, p=0.57), could not be identified as significantly different from unfortified natural rice (Table 4). Basmati rice fortified with ferric pyrophosphate (0.5μm, 1 g Fe/100g) was identified to be significantly different from unfortified natural rice (p=0.04).

Two formulations out of the four previously analyzed were selected to be compared in the cooked form relative to unfortified natural rice. Both samples, namely Jasmine rice fortified with ferric pyrophosphate (0.5 μm) and Basmati rice fortified with ferric pyrophosphate (2.5μm) could not be identified as significantly different from unfortified natural rice (Table 5) even at higher premix concentration of 1.5:100, with p=0.41 for Jasmine rice fortified with ferric pyrophosphate (0.5 μm) and p=0.19 for Basmati rice fortified with ferric pyrophosphate (2.5μm).

Because low power of the test, it is not possible to conclude that the fortified and unfortified rice are perceived as identical. However, the low proportion of correct guesses (less than half the panel in all the tests) suggests that any differences between fortified and unfortified rice are minimal. Further sensory and acceptability studies will need to be done in rice eating populations.
### Table 4 - Results of triangle test limited to visual evaluation comparing uncooked fortified rice to uncooked natural rice in four different formulations providing 5 mg Fe/100g Rice.

<table>
<thead>
<tr>
<th>Iron compound</th>
<th>Natural rice grains</th>
<th>Iron conc. in premix (mg Fe/100g Rice)</th>
<th>Premix concentration</th>
<th>Correct answers</th>
<th>p^1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferric pyrophosphate (2.5 μm)</td>
<td>Basmati rice</td>
<td>500</td>
<td>1:100</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>Micronized dispersible ferric pyrophosphate (0.5 μm)</td>
<td>Basmati rice</td>
<td>500</td>
<td>1:100</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>Micronized dispersible ferric pyrophosphate (0.5 μm)</td>
<td>Basmati rice</td>
<td>1000</td>
<td>1:200</td>
<td>36</td>
<td>17</td>
</tr>
<tr>
<td>Micronized dispersible ferric pyrophosphate (0.5 μm)</td>
<td>Jasmine rice</td>
<td>1000</td>
<td>1:200</td>
<td>36</td>
<td>15</td>
</tr>
</tbody>
</table>

^1P values were calculated with the chi square method.

### Table 5 - Results of evaluation of cooked rice in triangle test comparing fortified rice to natural rice in two different formulations of iron fortified rice.

<table>
<thead>
<tr>
<th>Iron compound</th>
<th>Natural rice grains</th>
<th>Iron conc. in premix (mg Fe/100g Rice)</th>
<th>Premix concentration</th>
<th>Correct answers</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micronized dispersible ferric pyrophosphate (0.5 μm)</td>
<td>Jasmine rice</td>
<td>1000</td>
<td>1.5:100</td>
<td>34</td>
<td>15</td>
</tr>
<tr>
<td>Ferric pyrophosphate (2.5 μm)</td>
<td>Basmati rice</td>
<td>500</td>
<td>1.5:100</td>
<td>34</td>
<td>16</td>
</tr>
</tbody>
</table>

^1P values were calculated with the chi square method.

### Conclusion

Our data shows that production of iron fortified rice closely resembling natural unfortified rice is possible through an extrusion premix approach using ferric pyrophosphate. Encapsulated ferrous sulfate, even if extruded at low temperatures, produced strong discoloration. Elemental iron, NaFeEDTA, and ferrous sulfate were also unsuitable because of color changes.
Chapter 5

Reducing the particle size of poorly-soluble elemental iron powders has been shown to improve their bioavailability in animal models (Motzok et al., 1975; Swain et al., 2003). In Hb repletion studies in rats, ferric pyrophosphate with a mean particle size of ≈2.5μm and 0.5μm dispersible ferric pyrophosphate have relative bioavailability of ≈70% and 95%, respectively, compared to ferrous sulfate (Wegmiller and others 2004). In contrast to that, regular particle sized ferric pyrophosphate has relative bioavailability ranging from 45 to 58% (Hurrell, 2002). Sakaguchi and coworkers (Sakaguchi et al., 2004) also showed micronized dispersible ferric pyrophosphate to be of similar bioavailability as ferrous sulfate in rats. The same compound had similar bioavailability to ferrous sulfate in human subjects (Fidler et al., 2004). Micronized ferric pyrophosphate or higher concentrations of 2.5 m or regular sized ferric pyrophosphate could therefore be a potentially efficacious fortificant in rice.

Extruded rice grains containing ferric pyrophosphate, when mixed with natural rice to provide an iron concentration of 5 mg Fe/100g, closely resemble unfortified rice in triangle tests. Iron losses during rinsing were low (<3%), and a consistent texture can be created in the cooked extruded rice grains. Bulk density of extruded rice flour does not change with extrusion (Kadan et al., 2003; Zilberboim, 1994), so that segregation of similarly-shaped extruded rice grains from the blend is unlikely. However, if this fortification approach is to be sustainable, several issues will need to be resolved. Extrusion can be done at low cost and high output (Harper and Jansen, 1985), and the use of rice flour derived from broken rice might further decrease costs. Central, large-scale processing would be particularly effective in reaching the growing urban poor. Effective distribution of the fortified rice would be crucial for the success of the program in rural areas. Small scale, local approaches, where schools or large employers included fortified rice in feeding and lunch programs have been explored with Vitamin A fortified rice (PATH, 2000). Finally, further studies on iron bioavailability and efficacy from extruded rice grains are needed, as well as consumer acceptability tests in rice-eating populations.
Acknowledgements

This study was partly supported by Tayio Kagaku Ldt., Japan, the NJAES/Cook College, Rutgers University, and the Swiss Federal Institute of Technology. We express our gratitude Dr.Paul Lohmann GmbH, Emmerthal Germany; Lipotech Ldt, Buenos Aires, Argentina, for providing iron fortification compounds. We are also grateful to Sabine Renggli for the help in cooking and preparing the samples and to the subjects who participated to the sensory study.

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PATH (2000): Ultra Rice Brochure, PATH.


CHAPTER 6

IRON STATUS AND FOOD MATRIX STRONGLY AFFECT THE RELATIVE BIOAVAILABILITY OF FERRIC PYROPHOSPHATE IN HUMANS

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Abstract

Background: Although ferric pyrophosphate is a promising compound for iron fortification of foods, there are few data on the effect of food matrices, processing, and ascorbic acid on its bioavailability.

Objective: We compared the relative bioavailability (RBV) with respect to ferrous sulfate of an experimental form of micronized dispersible ferric pyrophosphate (MDFP) in a wheat-milk infant cereal given with and without ascorbic acid to the RBV of MDFP from a processed and unprocessed rice meal.

Design: Using a crossover design, iron absorption was measured in young women (n=26) from test meals fortified with isotopically labeled $^{57}$Fe-MDFP and $^{58}$Fe-ferrous sulfate, based on erythrocyte incorporation of stable isotope labels 14 days later.

Results: Geometric mean iron absorption from the wheat-based meal fortified with MDFP was 2.0% compared to 3.2% for ferrous sulfate (RBV=62). Addition of ascorbic acid at a 4:1 molar ratio to iron increased iron absorption from MDFP to 5.8% and from ferrous sulfate to 14.8% (RBV=39). In the rice meals, mean iron absorption from MDFP added to the rice at time of feeding was 1.7% compared to 11.6% for ferrous sulfate (RBV=15). The mean iron absorption from MDFP extruded into artificial rice grains was 3.0% compared to 12.6% from ferrous sulfate in unprocessed rice (RBV=24). Sixteen subjects of 26 were iron deficient. Iron status was a highly significant predictor of the RBV of MDFP (P<0.001).

Conclusion: RBV of the experimental MDFP varied markedly with food matrix and iron status. Assigning a single RBV to poorly soluble compounds may be of limited value in evaluating their suitability for food fortification.
Introduction

Iron fortification of foods is challenging because poorly water-soluble iron compounds cause fewer sensory problems when added to foods, but have lower bioavailability than soluble iron (Hurrell, 2002). A potential strategy to overcome this problem is to reduce the particle size of poorly water-soluble iron compounds to increase their dissolution rate, and thereby, improve their bioavailability. Reducing the particle size of elemental iron powders increases their relative bioavailability with respect to ferrous sulfate (RBV) in humans (Bjorn-Rasmussen et al., 1977; Verma et al., 1977) and animals (Swain et al., 2003). A micronized ferric pyrophosphate with a mean particle size (MPS) of 0.5 μm, coated in mono- and diglycerides to minimize aggregation, has been developed (SunActive Fe, Taiyo Kagaku, Ldt., Yokkaichi, Japan; Nambu et al., 1998) and reported to have a RBV in humans of 82% and 92% from a wheat-milk infant cereal and a yoghurt drink, respectively (Fidler et al., 2004).

MDFP was developed for addition to liquid products, but its high bioavailability makes it potentially useful in other food vehicles that readily undergo adverse sensory changes when fortified with soluble iron, such as rice, infant cereals, and salt. MDFP has excellent sensory qualities when extruded into artificial rice grains (Moretti et al., 2005). A ground form of micronized ferric pyrophosphate, with a MPS=2.5 μm, has recently been shown to be efficacious in fortified salt in North Africa (Zimmermann et al., 2003).

Although MDFP is a promising iron fortificant, the potential influence of food matrix, food processing, and absorption enhancers on its bioavailability is uncertain. Hallberg et al. reported that food matrix influenced the bioavailability of poorly soluble carbonyl iron, presumably through effects on gastric pH and gastric emptying (Hallberg et al., 1986). Food processing may also influence the RBV of poorly soluble iron compounds, although the effects have not been consistent. In a human study, the RBV of ferric pyrophosphate fell from 75 to 21% when processed into a vacuum-dried chocolate drink (Hurrell et al., 1991), whereas in a rat study, its RBV increased when added to canned liquid infant formula (Theuer et al., 1971). One concern of feeding
MDFP to humans is that its very small MPS (0.3 μm) could result in it not entering the common iron pool and possibly by-passing normal absorptive control mechanisms for dietary iron. Therefore, our study aims were to: 1) determine the influence of the food matrix on the RBV of MDFP by measuring iron absorption of MDFP and ferrous sulfate from wheat- and rice-based meals; 2) investigate if MDFP enters the common iron pool by measuring iron absorption from the same meals with added ascorbic acid; and 3) measure the effect of rice extrusion processing on the bioavailability of MDFP.

Subjects and Methods

Subjects
Twenty six apparently healthy young women (age 20-40 y; body weight <60 kg) were recruited from the student and staff population at the Swiss Federal Institute of Technology and University of Zurich. Exclusion criteria were pregnancy or lactation and known gastrointestinal or metabolic disorders. No medication (except oral contraceptives) or vitamin/mineral supplements were allowed during the study. Subjects who regularly consumed vitamin/mineral supplements were asked to discontinue 2 weeks before the start of the study. None of the subjects had donated blood within 4 months from the start of the study. The study protocol was approved by the ethical committee at the Swiss Federal Institute of Technology, Zurich, Switzerland. Written, informed consent was obtained from all subjects.

Preparation of stable isotope labels
The $^{57}$Fe-MDFP used for the absorption study was an experimental form of MDFP prepared in the laboratory according to the method of Nambu et al. (Nambu et al., 1998). The experimental MDFP differed from the commercial form of Sunactive Fe (Taiyo Kagaku; Yokkaichi, Japan) which is manufactured by the same procedure but on an industrial scale. Firstly, the particle size was more than 2-fold the specified particle size of the commercial compound. Mean particle size of the $^{57}$Fe-MDFP, expressed as mean Sauter diameter, was 0.77 μm, compared to 0.3 μm specified by the manufacturer.
Relative bioavailability of ferric pyrophosphate

(Nambu et al., 1998). Secondly, the iron content of the \(^{57}\text{Fe}\)-MDFP was 14.6 mg Fe/g compared to the 12.0 mg Fe/g specified for the commercial product.

Isotopically labeled \(^{58}\text{Fe}\)-FeSO\(_4\) was prepared from isotopically enriched elemental iron by dissolution in diluted sulfuric acid. The solution was stored in Teflon containers and flushed with argon to keep the Fe in the +II oxidation state.

\(^{57}\text{Fe}\) labeled MDFP was prepared from isotopically enriched elemental iron (Chemgas, Boulogne, France) by firstly dissolving the elemental iron in concentrated hydrochloric acid followed by oxidation of ferrous to ferric iron by H\(_2\)O\(_2\). For purification \(^{57}\text{FeCl}_6\)\(^3-\) was extracted into diethyl ether, followed by re-extraction into water. The aqueous \(^{57}\text{Fe}\)-FeCl\(_3\) solution was evaporated under vacuum at 80°C using rotary evaporation (Rotavapor, Buechi, Flawil, Switzerland). The obtained dark red paste was crystallized to bright yellow \(^{57}\text{Fe}\)-FeCl\(_3\)\(\times\)6H\(_2\)O crystals by scratching the glass surface. From this base compound, micronised, dispersible \(^{57}\text{Fe}\)-ferric pyrophosphate was produced in collaboration with Taiyo Kagaku by mixing \(^{57}\text{Fe}\)-FeCl\(_3\)\(\times\)6H\(_2\)O, emulsifiers (enzymatically hydrolyzed lecithin and polyglycerol fatty acid ester) and sodium pyrophosphate (Nambu et al., 1998). Particle size distribution of \(^{57}\text{Fe}\)-MDFP was measured with a laser diffraction particle seizer (Mastersizer 2000, Malvern Instruments Ltd, Worcestershire, UK).

Study Design

Two iron absorption studies were done. In study 1, 10 subjects were fed a wheat-milk infant cereal fortified with MDFP or ferrous sulfate, with or without added ascorbic acid. In study 2, 16 subjects were fed a rice meal with MDFP added either at the time of feeding or extruded into artificial rice grains (Moretti et al., 2005), and ferrous sulfate added at the time of feeding. In both studies 1 and 2, each subject consumed 4 test meals in a crossover design. In study 1, the wheat-milk infant cereal fortified with \(^{56}\text{Fe}\)-ferrous sulfate and the same test meal fortified with \(^{57}\text{Fe}\)-MDFP were fed on two consecutive days (days 1 and 2). The isotopically labeled iron compounds were added
directly to the prepared test meal at the time of feeding. After 14 days (days 16 and 17), the same test meals were served with the addition of 63 mg of ascorbic acid. In study 2, the rice and vegetable meal fortified with $[^{58}\text{Fe}]$-ferrous sulfate or $[^{57}\text{Fe}]$-MDFP, both added directly to the prepared test meal at the time of serving, were given on days 1 and 2. On days 30 and 31, rice and vegetable meals with $[^{58}\text{Fe}]$-ferrous sulfate were again compared to those with $[^{57}\text{Fe}]$-MDFP, but the labeled MDFP was first extruded into artificial fortified rice grains (Moretti et al., 2005). All test meals were served between 7.00 and 9.00 AM after an overnight fast, under standardized conditions and close supervision. No foods or drinks were allowed for 3 hours after consuming the meals. Fasting venous blood was drawn into EDTA-treated tubes at baseline (day 0), at day 16 and at day 32 for study 1, days 14, 29, 45 for study 2. Calculation of iron absorption was based on the shift in the $^{58}\text{Fe}/^{57}\text{Fe}$ isotopic ratio after a 14-d incorporation period (Davidsson et al., 1994).

Production of extruded rice fortified with $[^{57}\text{Fe}]$-MDFP

Three hundred fifty grams of long grain rice flour were mixed in a Hobart mixer (Hobart Corporation, Troy, OH) and 194.1 mg of $^{57}\text{Fe}$ as $[^{57}\text{Fe}]$-MDFP were added. To reach a water content of 25%, distilled water was added to the mixture and mixed for 30 minutes to ensure water absorption on the flour particles and to obtain a free flowing powder. To obtain maximum homogeneity, the liquid $[^{57}\text{Fe}]$-MDFP formulation was slowly added to the rice flour during mixing using a manual spray vaporizer. Extrusion was performed with a Brabender single screw extruder (Model 2003, C.W. Brabender Instruments Inc, S.Hackensack, NJ) with a 20:1 length/diameter ratio using a screw with a 3:1 compression ratio, as described previously (Moretti et al., 2005). Extruded rice grains underwent an extrusion processing step at 90-95 C for $\approx$30 seconds.

Homogeneity of iron distribution in the rice flour

The homogeneity of the rice flour-MDFP mixture after mixing was tested before extrusion. 350 g of rice flour and 194.1 mg non labeled MDFP were
mixed in a food mixer for 30 min, with addition of distilled water to reach a moisture content of 25%. Eleven aliquots (n=11) of ≈0.5 g were taken from the mixed rice flour and the iron content analyzed in triplicate (n=3). The fortified rice flour was mineralized using an HNO₃/H₂O₂ mixture and microwave digestion. Iron content of the solution was determined by atomic absorption spectrometry (AAS) using a commercial iron standard (Titrisol, Merck, Darmstadt, Germany) and standard addition technique to minimize matrix effects.

**Concentration of iron isotopic labels in solution and in the labeled rice grains**
Isotope dilution mass spectrometry was used to determine the concentration of the isotopic labels in solution and in the rice. The iron standard used was prepared gravimetrically from an isotopic reference material (IRM-014, EU Institute of Reference Materials, Geel, Belgium). Isotopic analysis was done with negative thermal ionization mass spectrometry using a magnetic sector field mass spectrometer (MAT 262) equipped with a multi-collector system for simultaneous ion beam detection (Walczyk, 1997). Iron absorption was calculated based on the shift in the iron isotope ratios, the determined isotope ratios of the pure isotopic labels, and the iron isotopic composition of the used iron isotopic standard.

**Measurements of iron status and isotopic composition in blood**
Hemoglobin (Hb) was measured by the cyanmethemoglobin method, and serum ferritin (SF) was measured by immunoassay (Ramco Laboratories, Houston, Texas), using quality control materials for Hb (Digitana, Horgen, Switzerland) and SF (Ramco). Anemia was defined as Hb <120 g/L, and iron deficiency was defined by a SF <12µg/L. Each isotopically-enriched blood sample was analyzed in duplicate for its iron isotopic composition under chemical blank monitoring. Whole blood samples were mineralized using a HNO₃/H₂O₂ mixture and microwave digestion followed by separation of the sample iron from the matrix by anion-exchange chromatography and a solvent/solvent extraction step into diethyl ether(Walczyk et al., 1997). All
isotopic analysis were performed by negative thermal ionization mass spectrometry (NTI-MS).

Calculation of Fe absorption
Circulating Fe was calculated based on the blood volume, which was estimated with height and weight according to Brown (Brown et al., 1962). For calculation of fractional absorption, 80% incorporation of the absorbed Fe into red blood cells was assumed (Walczyk et al., 1997). Corrections for enriched baseline values were made when calculating Fe absorption from the third and fourth test meal.

Test Meals
The test meal for study 1 was a roller-dried wheat-milk infant cereal (Nestl< PTC, Orbe, Switzerland) prepared with reconstituted milk (50 g cereal plus 8 g milk powder and 75 mL water). Each test meal contained 5 mg of added Fe, either as 4 mg $[^{56}\text{Fe}]$-FeSO$_4$ plus 1 mg FeSO$_4$ of natural iron isotopic composition (test meal 1) or as 5 mg $[^{57}\text{Fe}]$-MDFP (test meal 2). To the test meals 3 and 4, ascorbic acid (Haenseler AG, Herisau, Switzerland) was added in a molar ratio of ascorbic acid to Fe of 4:1 (63 mg ascorbic acid).

In study 2, test meals were composed of 41 g of natural cooked rice and 9 g of extruded rice. Each test meal contained 5 mg of added Fe: as 4mg $[^{56}\text{Fe}]$-FeSO$_4$ plus 1mg FeSO$_4$ of natural isotopic composition (test meal 1 and 3); as 5 mg $[^{57}\text{Fe}]$-MDFP added in solution immediately prior consumption (test meal 2); or in form of extruded rice grains fortified with $[^{57}\text{Fe}]$-MDFP (test meal 4). Eight g of mixed vegetable sauce (44% Chinese cabbage, 21% carrots, 21% Zucchini, 12% Onions, 2% by oil and salt) were added to the rice meal. The sauce was prepared in bulk and boiled for 2h in excess water, so to have a negligible amount of ascorbic acid. After boiling, the vegetables were blended in a kitchen blender, and stored in portions at -18 C. Rice was steamed in food grade, heat resistant, plastic containers using nanopure water at a water to rice ratio of 2/1(w/w). The extruded grains were boiled at 100 C for 15 minutes.
Individual rice portions were prepared the evening before and stored at 4 C. Just before meal administration the sauce was added, and the test meals were heated in a microwave oven. Nanopure water (200 g) was served as a drink with the test meals. To minimize aggregation, $[^{57}\text{Fe}]-\text{MDFP}$ was treated in an ultrasound bath for 10 minutes prior to rice extrusion, and before direct administration.

**Statistical analysis**
Statistical Analyses were done using SPSS 13.0 (SPSS Inc., Chicago, IL) and SYSTAT 10 (Systat Software Inc, Richmond, CA). Non-normally distributed data were log transformed for statistical analysis. Iron absorption was presented as geometric means, and compared with paired or unpaired t-tests. P values <0.05 were considered significant. Stepwise linear regression was used with iron absorption from ferrous sulfate, iron absorption from MDFP, and RBV as dependent variables, and SF, food matrix, MDFP processing, and presence of ascorbic acid as independent variables. Data were standardized before analysis to avoid co-linearity according to the formula $Z_x=\frac{x-\text{mean}(x)}{\text{SD}(x)}$, where $x$ is the log transformed nonstandardized variable and $Z_x$ is the standardized variable. A parameter was included in the model at the significance level of 0.05.

**Results**

*Iron status of the test subjects*
In study 1, 2 of the 10 test subjects were iron deficient anemic (hemoglobin <120 g/L, SF <12μg/L), and five were iron deficient (SF<12μg/L). Within the 16 subjects enrolled in study 2, three subjects had iron deficiency anemia and 11 had iron deficiency.

*Composition of the test meals*
Mean iron concentration (range) of iron fortified rice flour for extrusion was 0.49 (0.48-0.52) mg /g, as compared to an expected iron concentration, considering water content, of 0.484 mg Fe/g; inter- and intra-measurement
CVs were 4.0% (n=11) and 3.6% (n=3), respectively. The wheat based infant cereal contained 0.6 0.05 mg native iron, 158 15 mg calcium (148 mg calcium/100g cereal, 1055 mg calcium/100 g milk powder) and 84 8 mg phytic acid (168 mg phytic acid/100g cereal). The ascorbic acid concentration of the cereal and milk powder was not measured as it was assumed to be negligible. The rice meal with vegetable sauce had a mean (SD) native iron content of 0.4 0.05 mg, a phytate content of 52 5 mg, and an ascorbic acid concentration of 1.5 0.1 mg. The phytate to iron ratio of the rice meal was ≈0.8:1, whereas the phytate to iron ratio for the wheat based infant cereal was ≈1.3:1.

Iron absorption from wheat based infant cereal
Mean $[^{57}\text{Fe}]$-MDFP absorption (range) from wheat based infant cereal was 5.8%(3.2-10.5) and 2.0%(1.3-3.0), with and without ascorbic acid, respectively. Mean $[^{58}\text{Fe}]$-ferrous sulfate absorption increased from 3.2%(1.4-7.2) to 14.8%(7.1-30.9) when given with ascorbic acid (Table 1). Mean iron absorption from $[^{57}\text{Fe}]$-MDFP and $[^{58}\text{Fe}]$-ferrous sulfate were significantly different from each other when administered with (P<0.001) and without ascorbic acid (P<0.05). The RBV of MDFP to ferrous sulfate when given with and without ascorbic acid was 39% and 62%, respectively (P<0.01).
Table 1- Iron absorption from a wheat based infant cereal (Study 1) fortified with 5 mg ferrous sulfate or 5 mg MDFP.

<table>
<thead>
<tr>
<th>Test meal</th>
<th>Subjects (n=10)</th>
<th>Geometrical mean iron absorption (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hb(^1) (g/L)</td>
<td>Serum ferritin(^2) (µg/L)</td>
</tr>
<tr>
<td>Wheat-milk infant cereal without ascorbic acid</td>
<td>128 9 (3.3, 35.6)</td>
<td>13.1 (1.3, 3.0)</td>
</tr>
<tr>
<td>Wheat-milk infant cereal with ascorbic acid</td>
<td></td>
<td>5.8(^6) (3.2, 10.5)</td>
</tr>
</tbody>
</table>

\(^1\) As means±SD.  
\(^2\) As geometric means (-1 SD, +1 SD, all such values).  
\(^3\) Compared using paired t-tests.  
\(^4\) Significant interaction between ascorbic acid and iron compound: \(^4\)P<0.05  
\(^5,7,9,11\) Significantly different between compounds: \(^5\)P<0.05, \(^7,9,11\) P<0.0001  
\(^6,8\) Significantly different with the addition of ascorbic acid \(^6\)P<0.0001, \(^8\)P<0.01
Iron absorption from processed and unprocessed rice test meals

Mean iron absorption from the rice meal fortified with $[^{57}\text{Fe}]$-MDFP added at the time of feeding was 1.7% (1.0-2.9), whereas mean iron absorption from the same meal with added $[^{58}\text{Fe}]$-ferrous sulfate was 11.6% (5.5-24.7; Table 2). When the $[^{57}\text{Fe}]$-MDFP compound was extruded into iron fortified rice, iron absorption was 3% (1.3-6.6), significantly higher than that of unprocessed $[^{57}\text{Fe}]$-MDFP (P<0.05). RBV of the MDFP compared to ferrous sulfate was 15 for the unprocessed rice meal and 24 for the processed rice meal (P<0.05). Iron absorption comparing the ferrous sulfate-fortified meals (meals 1 and 3) that were given twice to calculate RBV was not significantly different.

**Table 2**- Iron absorption from a rice meal with vegetable sauce (study 2) fortified with 5 mg MDFP given either at time of feeding or fortified into artificial rice grains. As a comparison, the same meal was fortified with 5 mg ferrous sulfate given at time of feeding.

<table>
<thead>
<tr>
<th>Composition of test meals</th>
<th>Subjects (n=16)</th>
<th>Iron bioavailability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hb(^1) (g/L)</td>
<td>Serum ferritin(^2) (µg/L)</td>
</tr>
<tr>
<td>Rice meal fortified with MDFP at time of feeding(^3)</td>
<td>126 9</td>
<td>7.7 (2.7, 20.4)</td>
</tr>
<tr>
<td>Rice meal fortified with FeSO(_4) at time of feeding(^3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rice meal fortified with MDFP extruded into rice grains(^3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rice meal fortified with FeSO(_4) at time of feeding(^3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)As means?SD.

\(^2\)As geometric means (-1 SD, +1 SD).

\(^3\)Compared using paired t-tests

\(^4\)Significantly different between compounds: \(^4\)P<0.0001

\(^5\)Significantly different with processing of MDFP: \(^5\)P<0.05
Factors influencing RBV and absorption from ferrous sulfate and MDFP

Presence of ascorbic acid (P<0.001), SF (P<0.01) and meal type (P<0.01) were significant predictors of iron absorption from ferrous sulfate. In contrast, presence of ascorbic acid (P<0.001) was the only significant predictor of iron absorption from MDFP; processing (P=0.073), type of meal (P=0.133) and SF (P=0.225) were not significant determinants. In the regression with RBV of MDFP as the dependent variable, meal type (P<0.001), SF (P<0.001) and processing of the MDFP compound (P<0.01) were all significant predictors, whereas presence of ascorbic acid (P=0.22), hemoglobin concentration (P=0.77) and subject (P=0.52) were not significant determinants. The regression formula was:

\[
Z(\text{RBV}) = Z(\text{SF}) \times 0.390 + Z(\text{Food Matrix}) \times (-0.666) + Z(\text{Processing}) \times 0.556 + 0.343.
\]

The inverse relationship between iron stores (represented by SF) and iron absorption from both ferrous sulfate and MDFP is shown in Figure 1. In study 1, there was a negative correlation between SF and absorption from ferrous sulfate (r=-0.83; P=0.003 with ascorbic acid; r=-0.79; P=0.006, without ascorbic acid). Absorption of iron from MDFP was also negatively correlated with SF, both with and without ascorbic acid (r=-0.77; P=0.010 and r=-0.64; P=0.046, respectively). In study 2, iron absorption from ferrous sulfate and SF were negatively correlated (r=-0.71; P=0.002), whereas for MDFP the inverse relationship was significant only for the processed form (r=-0.49; P=0.053). The greater absorption of ferrous sulfate at low SF concentrations results in a positive correlation between SF and RBV (r=-0.56, P<0.001; Figure 2).
Figure 1 - Iron absorption plotted against serum ferritin concentration from a wheat-based infant cereal with (n=10) and without (n=10) the addition of ascorbic acid (4:1 ascorbic acid/iron molar ratio) fortified with micronised dispersible ferric pyrophosphate (MDFP, o) or ferrous sulfate (x). Linear regression on MDFP is represented by full lines; linear regression on ferrous sulfate is represented by broken lines. Statistical analysis on log10 transformed data, \(^1P<0.005\), \(^2P<0.05\). Significant interaction between iron compound x ascorbic acid (P<0.001) and iron compound x serum ferritin (P<0.05) with univariate general linear model. Slopes were significantly different between each other using paired t-tests (P<0.05).
Relative bioavailability of ferric pyrophosphate

Figure 2: Relationship between serum ferritin and the average relative bioavailability obtained in the study subjects (n=26). *Pearson correlation coefficient, P<0.001.

Discussion

One of the major findings from this study is that the RBV of MDFP (particle size 0.77 μm) varied depending on the food vehicle, being 62% in a wheat-milk infant cereal and only 15-25% in a rice meal. This compares with previously reported RBV values for an MDFP (particle size 0.3 μm) of 95% from a yoghurt drink and 83% in a wheat-milk cereal (Fidler et al., 2004). In the present study, while the RBV of MDFP was 3-4-fold lower in the two rice meals compared to the wheat-based meal without ascorbic acid, the mean absorption of the MDFP was similar from both meals. Thus, the effect of the food matrix on RBV was primarily due to the much higher iron absorption from ferrous sulfate in the rice meal (11.6% and 12.6%) compared to the wheat-based meal (3.2%). Although these results were obtained with ferric pyrophosphate, two previous studies also suggested that food matrix
influences iron absorption from ferrous sulfate to a greater degree than iron absorption from poorly water-soluble iron compounds.

In a study by Hallberg et al. (Hallberg et al., 1986) absolute absorption from carbonyl iron in presence of meat increased from 1% to 1.7%, whereas common pool iron absorption increased from 5.6% to 12.7%, resulting in a reduction in RBV of the carbonyl iron from 20% to 11%. In a similar study with a complex ferric orthophosphate, RBV was 64% in an infant cereal; 37% in fortified bread rolls served with margarine, cornflakes, sour milk, cheese and coffee; and 30% in bread rolls served with a meat broth (Hallberg et al., 1989). However, in both studies, addition of ascorbic acid at different dose levels did not affect RBV (9, 17).

In previous studies using radioisotope labels, we have reported the RBV of nonmicronized ferric pyrophosphate to be 15% from a purely wheat infant cereal (Hurrell et al., 2000), 39% from a wheat-milk infant cereal (Hurrell et al., 1989), and 75% from a chocolate drink (Hurrell et al., 1991), all added to the test meal just prior to consumption. Therefore, at least for poorly water-soluble iron compounds, using a single RBV value to set a fortification level and predict potential efficacy in all food vehicles may be of limited value.

The reason for the higher iron absorption from ferrous sulfate in the rice meal compared to the wheat-based meal in the present study may be due to both differences in meal composition and iron status of the test subjects. The calcium in the milk given with the wheat-based cereal, as well as the higher phytic acid to iron ratio in the wheat meal (1.3:1 compared to 0.8:1 in the rice meal) likely reduced the iron absorption from ferrous sulfate (Hallberg et al., 1992; Hurrell et al., 2002). It is possible that these compounds are less inhibitory for poorly water-soluble iron compounds, like MDFP, as the iron is dissolved in the common pool at a slower rate. Also, meal composition may affect gastric motility, stomach emptying, and gut pH. These data suggest that, depending on the iron compound, digestion and release of non-heme iron into the common pool and/or its subsequent absorption can be strongly influenced by the effects of food matrix (Hallberg et al., 1986).

There was a significant inverse relationship between iron status (as defined by SF concentration) and iron absorption from both ferrous sulfate and MDFP.
Relative bioavailability of ferric pyrophosphate

(Figure 1). Increased iron absorption with decreasing iron stores is a central mechanism of iron homeostasis in humans (Finch, 1994; Gavin et al., 1994). Our data suggest that this adaptive up-regulation of iron absorption is more effective for ferrous sulfate. The greater absorption from ferrous sulfate than from MDFP at low SF concentrations produced RBV values for MDFP that varied inversely with SF. These data suggest the RBV of a poorly water-soluble iron compound may vary depending on the iron status of the individual (Figure 2).

We previously reported the RBV of MDFP in the same wheat-milk infant cereal used in the present study was 82% compared to ferrous sulfate. The lower RBV in this study (62%) was probably due to the higher mean particle size of the labeled MDFP batch used in this study (0.77 m) compared to that used in the previous study, which more closely matched the commercial specification (0.3 m). Our values for iron absorption of ferrous sulfate from a rice-based meal (=12%) are similar to the range of values (6-13%) reported in previous human studies (Fidler et al., 2003; Tuntawiroon et al., 1990; Walczyk et al., 2005).

From the inverse relationship between SF concentration of the subjects and iron absorption (Figure 1), as well as the ≈3-fold increase in iron absorption on addition of ascorbic acid to the wheat-based meal, it would appear that MDFP enters the common iron pool, and its absorption is regulated by normal mechanisms. Ascorbic acid addition has been previously shown to enhance iron bioavailability from other poorly-soluble iron compounds (Fidler et al., 2004; Forbes et al., 1989; Hallberg et al., 1986; Hallberg et al., 1986). However, with addition of ascorbic acid, the RBV of MDFP decreased from 62% to 39%. A similar decrease in RBV with the addition of ascorbic acid was previously reported for ferric pyrophosphate with larger mean particles sizes (Fidler et al., 2004). Similarly, EDTA increases absorption of iron from ferrous sulfate but not from ferrous fumarate, ferric pyrophosphate and elemental iron (Hurrell et al., 2000). In contrast, studies with ferric orthophosphate and elemental iron found an identical RBV before and after the addition of ascorbic acid (Forbes et al., 1989; Hallberg et al., 1989). Differences in
chemical and crystalline properties of ferric pyrophosphate and orthophosphates may influence their rates of solubility and bioavailability (Hallberg et al., 1989).

In this study, heated extrusion of MDFP to produce artificial rice grains followed by boiling was associated with a small but significant increase in the RBV, mainly due to an increased absorption of iron from the MDFP. It is possible that extrusion into the rice grain fixes the micronized ferric pyrophosphate particles and minimizes their potential aggregation. In a previous study in rats, heat sterilization (121 C, 20 min) of an infant formula containing ferric pyrophosphate increased its RBV from 75% to 125% (Theuer et al., 1973), likely due to solubilization of the ferric pyrophosphate in the liquid formula. In contrast, in a human study using chocolate milk powder, vacuum drying (90 C for 3 hours) decreased iron absorption from ferric pyrophosphate (2.1% and 0.6%, before and after processing, respectively), but did not affect ferrous sulfate absorption (2.8% and 2.6%, before and after), resulting in a decrease in RBV of ferric pyrophosphate from 75% to 21% (Hurrell et al., 1991). The effects of processing on RBV are therefore variable and difficult to predict.

RBV values from animal and human studies have proven valuable for choosing iron compounds to fortify foods. They have been used to divide iron compounds into 3 groups: water-soluble compounds with an RBV close to ferrous sulfate; compounds which dissolve more or less completely in the gastric juice and have an RBV similar to ferrous sulfate in adults (e.g. ferrous fumarate); and compounds only partly soluble in the gastric juice. The latter group includes ferric pyrophosphate, whose bioavailability has been reported to vary from 15% to 75% compared to ferrous sulfate (Hurrell et al., 1989; Hurrell et al., 2000; Hurrell et al., 1991), other phosphate compounds and the different forms of elemental iron. Our findings suggest some explanation for this wide variability. In the case of poorly water-soluble iron compounds, RBV alone should not be used to judge the potential of an iron compound for fortification. The absolute iron absorption in the fortified food is likely to be a better predictor of efficacy.
Acknowledgements

All authors contributed to the study design. DM, RW, and CZ prepared and fed the test meals. All authors contributed to the data and statistical analysis. DM wrote the first draft of the paper, and all authors contributed to its editing. We thank Tung-Ching Lee (Rutgers University, New Jersey) for providing the extrusion facility. The experimental MDFP was produced by Taiyo Kagaku Ldt. (Yokkaichi, Japan). We thank the subjects who participated in these studies and S Renggli (Swiss Federal Institute of Technology Zürich, Switzerland) for excellent technical assistance. None of the authors had a conflict of interest related to this study.
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CHAPTER 7

EXTRUDED RICE FORTIFIED WITH MICRONIZED GROUND FERRIC PYROPHOSPHATE REDUCES IRON DEFICIENCY IN INDIAN SCHOOLCHILDREN: A DOUBLE BLIND, RANDOMISED, CONTROLLED TRIAL

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Abstract
**Background** Iron fortification of rice, could be an effective strategy to reduce IDA in South Asia.

**Objective** To determine if extruded rice grains fortified with micronized ground ferric pyrophosphate (MGFP) would increase body iron stores in children.

**Study Design** In a double-blind, 7-month, school-based feeding trial in Bangalore, India, iron-depleted, 6-13 y-old children (n=184) were randomized to receive either a rice-based lunch meal fortified with 20 mg Fe as MGFP, or an identical but unfortified control meal. The meals were consumed under direct supervision, and daily leftovers were weighed. All children were dewormed at baseline and 3.5 mo. Iron status and hemoglobin (Hb) were measured at baseline, 3.5 and 7 months.

**Results:** At baseline, prevalence of iron deficiency (ID) and IDA were 78% and 25%, respectively. After 7 mo of feeding, there was a significant increase in body iron stores in both study groups (p<0.001), with a greater increase in the iron group compared to control (p<0.05). There was a significant time x treatment interaction for ID, which fell from 78% to 25% in the iron group and from 79% to 49% in the dewormed control. IDA decreased from 30% to 15% (N.S) in the iron group but remained virtually unchanged in the controls (28% and 27%). In sensory tests, the MGFP-fortified rice (fortified at 3 and 5 mg Fe/100g) was indistinguishable from natural rice, in both cooked and uncooked form.

**Conclusion:** Extruded rice fortified with MGFP has excellent sensory characteristics. Fed in a school lunchmeal, it increases iron stores and reduces the prevalence of iron deficiency in Indian children.
Introduction

Iron deficiency (ID) and iron deficiency anemia (IDA) are highly prevalent among young women and children in South and Southeast Asia (1). In low socioeconomic populations in India, the prevalence of IDA may be as high as 64-68% in school aged children and infants (2-3). IDA impairs cognitive performance, infant and child growth, immune status and work capacity (1). Even mild-to-moderate ID without anemia, may lower work capacity and resistance to fatigue (4-5) and impair cognition (6-7).

Rice is a leading staple food in South Asia, and it is typically milled before consumption. Milled rice has an iron content of only ≈4-8 mg Fe/kg (8). A rice-based diet consumed with few other foods might not supply sufficient dietary iron. Although iron fortification of rice could be an effective strategy to reduce ID and IDA (1,9), it is technically challenging. Rice is usually consumed as intact grains, and addition of highly-bioavailable, water-soluble iron compounds to artificial rice grains causes adverse sensory changes, while less reactive, poorly-soluble iron compounds are not well absorbed (10). However, increasing the amount of poorly soluble iron compounds, such as ferric pyrophosphate, can overcome their low bioavailability and may make them more useful food fortificants. Additionally, reducing the particle size might positively influence iron absorption from ferric pyrophosphate (11-14). In a recent intervention trial in Morocco, salt fortified with micronized ground ferric pyrophosphate (MGFP) was highly effective in reducing IDA in children (15).

Using an extrusion method (16, 17), we have developed artificial rice grains fortified with MGFP (mean particle size ̃2.5 μm) (16). In both cooked and uncooked form, the texture, taste, appearance and storage stability of the MGFP-fortified rice closely resembles natural unfortified rice, and losses of iron from the extruded grains during rinsing are <3% (16). The primary aim of this study, done in an urban slum in Bangalore, India, was to test the efficacy of the MGFP-fortified rice in iron-deficient children. We also measured rice
intake, iron intake and iron bioavailability from the local diet, and determined if the extruded MGFP-fortified rice could be distinguished from natural rice by the local population in raw form and when cooked into local meals.

Subjects and methods

Study site
Bangalore is situated on the Deccan plateau in South India (~ 900 m above sea level), and has a population of ~ 4.3 million (2001 census, 18). This region is not endemic for malaria, and it is estimated that incidence of the disease is <2% (19). The study site was the Franciscan School, a primary school serving the population of the Rock-Colony neighborhood, a crowded urban slum. The school has ~ 970 students aged 4-14 yr. A subsidized lunch feeding program is in place that provides the students with a 200-300 g meal of cooked rice daily. Informed, written consent was obtained from the parents of the children and oral consent was obtained from the children. The protocol of the study was approved by the ethical committee at St John's National Academy of Health Sciences, Bangalore and by the ethical committee of the Swiss Federal Institute of Technology, Zurich, Switzerland. The study period was August 2004 to April 2005.

Estimation of rice and iron intake and iron bioavailability from the local diet
Food intake was assessed by three day weighed food records in 20 randomly selected families living in the Rock Colony neighborhood adjacent to the school. Records were kept for 3 days during which households were asked to maintain their usual food habits. Edible portions of all foods were weighed during preparation and at consumption using food-scales with a precision of 1g (Soenle-Waagen GmbH, Murrhardt, Germany). Foods consumed outside home were reported and quantities estimated by the study participants. Consumption data was entered on a dietary survey program (Ebispro-Nutrisurvey, 2004, University of Hohenheim/Stuttgart, Germany) into which food composition data from the Indian food composition database (20) was integrated. Dietary intakes of heme and non-heme iron, as well as vitamin C

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were calculated. The phytate content of local staple foods (rice, millet, wheat flour) and locally consumed pulses (black gram dahl, tur dahl, Bengal gram dahl, moong dahl and beans) was directly analyzed (21). The percentage of heme iron in animal foods was estimated to be as follows: chicken, 30%; mutton, 70%; and fish, 25% (22, 23). Dietary Fe bioavailability was then estimated using published algorithms to estimate iron absorption (24, 25, 26, 27) and adjusted for body iron stores (28). Iron intake and bioavailability were calculated on individual basis and averages were calculated for different age groups.

Sensory tests
Triangle tests (29) were performed to determine if local women could distinguish the iron-fortified rice from unfortified rice. Four local recipes were tested along with cooked and uncooked rice. The panel was composed of 24 middle class Indian women. Subjects were blinded and were informed about the procedures of the test only after completion of the entire study. Samples were presented using a randomized block design, and not more than three consecutive tests per session were performed. Tests were done in a private setting under uniform lighting conditions. Both raw rice and cooked rice were served on coded, rectangular polyethylene cups (dimensions: base 60x70mm; height 30mm). Each portion of raw rice contained 30g of fortified or unfortified Sona Masuri rice (Sona Masuri, Bangalore Rice Traders, Bangalore). The cooked rice portions were simultaneously prepared using pressure cookers equipped with a pressure valve. Rice was prepared using a standardized procedure similar to the traditional preparation in South Indian households. Rice was washed in preparation for the cooking. Rice portions were cooked with seasoning ingredients in household pressure cookers for 8 minutes after reaching peak pressure, after which pressure was released. Test servings contained 85g of cooked rice.
School-based intervention study
At the Franciscan school, serum transferrin receptor (TfR), serum ferritin (SF), and hemoglobin (Hb) were determined in all consenting children (n=554). All children who met the inclusion criteria of iron deficiency and/or low iron stores (as defined by a SF<20 µg/L or a TfR >7.2 mg/L) (n=184) were randomized to either receive the iron-fortified daily lunch meal or a nonfortified control (see details below). The study was double-blind. The sample size was estimated at 70 children per group, to be able to detect a difference of 30% in the geometric mean SF from a mean baseline of 15 µg/L, assuming a significance level of 0.05 (2-tailed) and a power of 90%. Anticipating a drop-out rate of 20%, • 90 children were recruited per group.

At baseline, in the participating children, height and weight were measured and 5 ml of blood were collected by venipuncture into EDTA-containing tubes for determination of Hb, SF, TfR, and C-reactive protein (CRP). Measures were repeated after 3.5 months (midpoint) and after 7 months (endpoint). At baseline and midpoint all study participants were dewormed with 400 mg albendazole (Low-Cost pharmaceuticals, Bangalore, India). As part of the current Indian national supplementation campaign, children in the study were treated with vitamin A supplements (200,000 IU) 4 months before the start of the study and near the study midpoint. Subjects who remained anemic after completion of the trial received supervised treatment with oral iron tablets [60 mg Fe (as ferrous sulfate) for 4 d/wk for 12 wk].

Morbidity in both study groups was assessed weekly. Subjects were asked to identify diseases/complaints from a list (see Table 6), and to quantify their severity by estimating the number of days they were affected by it.

Preparation and feeding of the lunch meals
The premix of extruded iron fortified rice was produced as previously reported (13). The extruded rice grains contained 10 mg Fe/g as MGFP. The MGFP was produced by conventional grinding and has a mean particle size (MPS) of • 2.5 µm (Paul Lohmann AG, Emmerthal, Germany). The premix was mixed
with local rice (Sona Masuri, Bangalore Rice Traders, Bangalore) in 50 kg batches at a 1:50 ratio to result in a fortification level of 200 mg Fe / kg rice. Rice was mixed monthly using barrel mixers of 15 kg and 50 kg capacity. First, 1 kg of premix was mixed with 14 kg of natural rice for 15 minutes. Then, the 15 kg blend was mixed for 30 minutes with 35 kg natural rice. The iron-fortified and unfortified rice were packaged in color-coded 10 kg polyethylene bags.

The separate lunch meals containing the two types of rice were prepared daily under supervision in the kitchen of the Division of Nutrition at St John's National Academy of Health Sciences. A dedicated technician was responsible for preparing the ingredients according to the recipes and supervising the cooking. The rice meals were packed into color-coded plastic lunch boxes; the daily portion of rice per lunch was 100g dry rice. This provided 20 mg of iron as MGFP in the iron-fortified meals.

At the school, the group assignment of the participating children was identified using a color-coded personal badge. Lunch was served six days a week (except for school holidays). Three local recipes of rice cooked with different seasoning ingredients, were presented in repeating sequence to maintain interest. The main seasoning ingredients of the 3 recipes were: tomato rice: onions, tomatoes; lemon rice: groundnuts, roasted lentils and lemon juice; and veg. pulao: french beans, beetroot, cauliflower, carrots, onions. The children ate their lunch, confined in large hall, under direct supervision of the study team. After finishing lunch, boxes with visible leftovers were individually weighed and the data recorded.

*Laboratory analyses*

Iron content of the iron-fortified and control meals was analyzed biweekly (fortified meal: n=17, control meal: n=16). The test meals were lyophilized and successively mineralized using an HNO$_3$/H$_2$O$_2$ mixture and microwave digestion. Iron content of the solution was determined by graphite-oven-atomic absorption spectrometry (GAAS) using a commercial iron standard for
Efficacy of iron fortified rice calibration (Titrisol, Merck, Darmstadt, Germany). Vitamin C content of the menus was measured by HPLC with reversed phase column and photometric detection (30) and the phytate content was measured using a modification of the Makower method (21) in which Ce replaced Fe in the precipitation step.

Hb was measured with an AcT8 Counter (Beckman Coulter, Krefeld, Germany) on the day of blood collection. Serum samples were aliquotted and frozen at -80 °C until analysis. SF and TfR were measured using commercial immunoassays and control materials (TfR: RAMCO, Houston, TX; SF IMMULITE 1000 DPC, Los Angeles, CA). CRP was measured using nephelometry (TURBOX, Orion Diagnostica, Espoo, Finland). Analytical sensitivity was 1.5 µg/L for SF, 0.6 mg/L for sTfR and 5 mg/L for CRP. The coefficient of variation (intraassay) for the assays were: SF: CV=7.9-9.7%; sTfR: CV=4.4-5.0%; CRP: CV=5.6-6.7% Reference values were SF, 15-300 g/L; TfR, 2.9-7.6 mg/L (31) and CRP <10 g/L. Fe deficiency was defined as either SF < 15 g/L or TfR > 7.6 mg/L (32-34). Anemia was defined as Hb < 115 g/L in children aged 5–11 y (30). Serum ferritin values from subjects with an elevated CRP (CRP>10 mg/l) were exluded from the analysis.Total body iron was calculated from the ratio of the TfR to SF using the method of Cook et al (35, 36). Iron absorption from the extruded rice was calculated comparing the change in body iron in the treatment and control groups, and by estimating the total iron dose given during the study.

Statistical methods
Data processing and statistical analysis were performed with SPSS (version 13.0, 2004, Chicago) and with Microsoft Excel (2002, Microsoft corporation, Seattle). Epinfo™ was used for anthropometry calculations (Epinfo version 3.3.2, CDC, Boston). Normality of data was checked before analysis with the Kolmogoroff-Smirnoff test and graphically evaluating Q-Q plots. Results were analyzed with a univariate general linear model using the “subject” variable as random factor to correct for repeated measures. Effect of treatment and time were evaluated for all outcome parameters: Hb, SF, TfR, CRP. If a significant time x treatment interaction could be found, data was further analyzed using
Tukey's tests as a post hoc tests. Normality of residual distribution was checked graphically for the univariate model. To analyze the morbidity data, a GLM model was used with the sum of days of sickness as the dependent variable and treatment and type of disease as the fixed factors. Results of the sensory study were evaluated with the binomial distribution. Significance was set at $P=0.05$.

Results

Assessment of iron bioavailability from the diet
Iron intake and bioavailability from the local diet and factors used in the estimation are shown in Table 1. Mean SD daily iron intakes in 6-13 y-old children were 5.0 2.2 mg (boys), and 4.7 2.2 (girls). Only $\approx6\%$ was heme iron. Estimated dietary iron bioavailability ranged between 4.5 2.3% and 6.5 2.9% (boys) and between 4.6 2.0% and 7.1 8.2% (girls) depending on the model used to assess iron bioavailability.
Table 1. Daily nutrient intake and Fe bioavailability form the diet determined using 3-day weighed food records in children aged 6-13 years living in the Rock Colony Slum, Koramangala, Bangalore.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>Rice (g)</td>
<td>165.3 (73)</td>
<td>167.9 (50)</td>
</tr>
<tr>
<td>Fe (mg)</td>
<td>5.0 (2.2)</td>
<td>4.7 (2.2)</td>
</tr>
<tr>
<td>Heme Fe (mg)</td>
<td>0.33(0.45)</td>
<td>0.27(0.27)</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>33(20)</td>
<td>38(35)</td>
</tr>
<tr>
<td>Phytate (mg)</td>
<td>928(423)</td>
<td>962(305)</td>
</tr>
<tr>
<td>Meat, fish and poultry</td>
<td>38.5(16.8)</td>
<td>20.7 (12.9)</td>
</tr>
<tr>
<td>Fe Bioavailability (24)</td>
<td>4.5 (2.3)</td>
<td>4.6 (2.0)</td>
</tr>
<tr>
<td>Fe Bioavailability (25)</td>
<td>5.5 (2.8)</td>
<td>5.3 (2.5)</td>
</tr>
<tr>
<td>Fe Bioavailability (26)</td>
<td>6.5 (2.9)</td>
<td>6.6 (2.6)</td>
</tr>
<tr>
<td>Fe Bioavailability (27)</td>
<td>6.4 (9.6)</td>
<td>7.1 (8.2)</td>
</tr>
</tbody>
</table>


Organoleptic tests

Results of the sensory study are shown in Table 2. At both 3 and 5 mg iron/100g rice, fortified and unfortified uncooked rice were indistinguishable. Similarly, in all of the cooked recipes —plain white rice, lemon rice, vegetable rice, tomato rice and tamarind rice— meals containing rice fortified at 3 mg Fe/100g were indistinguishable from meals containing nonfortified rice.
Table 2. Results of the triangle tests comparing rice or rice-based meals fortified with extruded rice grains containing micronized ground ferric pyrophosphate to nonfortified control meals in a panel of Indian women (n=24).

<table>
<thead>
<tr>
<th>Test meals (3 mg Fe/100 g)</th>
<th>Subjects</th>
<th>Correct answers¹</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw rice</td>
<td>24</td>
<td>8</td>
<td>0.576</td>
</tr>
<tr>
<td>Raw rice (5mg/100g)</td>
<td>24</td>
<td>11</td>
<td>0.140</td>
</tr>
<tr>
<td>Cooked plain rice</td>
<td>24</td>
<td>8</td>
<td>0.576</td>
</tr>
<tr>
<td>Tomato rice</td>
<td>24</td>
<td>11</td>
<td>0.140</td>
</tr>
<tr>
<td>Lemon Rice</td>
<td>24</td>
<td>12</td>
<td>0.068</td>
</tr>
<tr>
<td>Vegetable Rice</td>
<td>23</td>
<td>8</td>
<td>0.519</td>
</tr>
<tr>
<td>Tamarind Rice</td>
<td>23</td>
<td>10</td>
<td>0.206</td>
</tr>
</tbody>
</table>

¹ Calculated with the binomial distribution
² The panel was asked to identify the odd samples within 3 presented samples of unfortified and fortified rice. The number of correct answers provides information on the panel's ability to distinguish iron fortified rice from unfortified rice.

Test meal composition

Study subjects received 100g of raw rice per meal. Table 3 gives an overview of the composition of the test meals. Mean SD iron content of the lunch meals served to the iron-fortified and control groups was 19.2 2.5 mg Fe/meal and 1.2 0.6 mg Fe/meal, respectively. For the specific meals, mean SD iron content per daily serving of the unfortified tomato rice, lemon rice, and vegetable pulao meals was 1.0 0.2 mg, 1.6 0.2 mg and 1.4 0.6 mg, respectively. The mean SD phytate content of the tomato rice, lemon rice, and vegetable pulao meals was 95 20 mg, 120 27 mg and 175 15 mg, respectively. No detectable ascorbic acid was present in any of the cooked recipes.

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Table 3. Composition of the rice-based lunch meals in the feeding trial1.

<table>
<thead>
<tr>
<th></th>
<th>Tomato Rice</th>
<th>Lemon Rice ('Chitrana')</th>
<th>Vegetable Rice ('Veg Pulao')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)2</td>
<td>477</td>
<td>514</td>
<td>511</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>8.3</td>
<td>10.2</td>
<td>8.3</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>11.0</td>
<td>14.2</td>
<td>15.3</td>
</tr>
<tr>
<td>Carbohydrates (g)</td>
<td>86.1</td>
<td>87.0</td>
<td>84.9</td>
</tr>
<tr>
<td>Phytate (mg)</td>
<td>95(20)</td>
<td>120(30)</td>
<td>175(15)</td>
</tr>
<tr>
<td>Fe (mg)</td>
<td>1.0(0.2)</td>
<td>1.6 (0.2)</td>
<td>1.4 (0.6)</td>
</tr>
</tbody>
</table>

1 One hundred g of dry rice were served in each meal, cooked with seasoning ingredients, in three rotating recipes.
2 Values are arithmetic means (SD) (all such values).

Compliance with feeding

There were a total of 137 feeding days over 7 months. Due to school vacations, there were 60 feeding days between baseline and midpoint, and 77 feeding days between midpoint and final point of the study. On average, 158 subjects ate lunch daily (an average of 76 from the iron-fortified group and 81 from the control group). The mean serving size was 414 g of cooked rice, and the mean SD daily consumption was 340 81 g. In the iron-fortified and control groups, mean SD daily consumption was 347 77 g and 333 85, respectively. The children in the iron-fortified group consumed a mean of 16.1 mg iron daily from their meals, whereas children in the control group consumed a mean of 1.07 mg iron form the control meal. However, considering absenteeism, when averaged over all of the feeding days in the study, the daily mean SD iron intake per subject in the iron group was 13 2.4 mg, and in the control group, was 0.99 0.44. The school had a 3-wk vacation in October, which interrupted the feeding program during this period.
Changes in Hb, iron status, ID and IDA

Table 4 shows there were no significant differences in baseline characteristics of the two groups after randomization. Of the 184 subjects enrolled, 170 completed the study. Twelve of the 14 subjects who discontinued the study were in the iron fortification group, 2 were in control group. The main reasons for dropping out were: 1) leaving school (n=9) and 2) loss of interest in the study (n=5). Three subjects who dropped out because of lack of interest were in the iron group, two were in the control group.

Table 4. Baseline characteristics of the children in the groups receiving the iron-fortified rice meals and the control, nonfortified rice meals.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Iron fort. group (n=92)</th>
<th>Control group (n=92)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>8.0(2.1)</td>
<td>8.0(1.9)</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>120(12)</td>
<td>119(13)</td>
</tr>
<tr>
<td>Serum Ferritin (g/L)</td>
<td>17 17</td>
<td>15 13</td>
</tr>
<tr>
<td>Transferrin Receptor (mg/l)</td>
<td>9.2(3.9)</td>
<td>8.7(3.2)</td>
</tr>
<tr>
<td>C-reactive protein (mg/l)</td>
<td>4.4(6.5)</td>
<td>5.3(8.2)</td>
</tr>
<tr>
<td>Body iron stores (mg/kg)</td>
<td>1.2(4.0)</td>
<td>0.9(3.7)</td>
</tr>
<tr>
<td>Prevalence of ID (%)</td>
<td>78</td>
<td>79</td>
</tr>
<tr>
<td>Prevalence of IDA (%)</td>
<td>30</td>
<td>28</td>
</tr>
<tr>
<td>Height for age&lt;sup&gt;3&lt;/sup&gt;</td>
<td>-1.39(1.18)</td>
<td>-1.33(1.15)</td>
</tr>
<tr>
<td>Weight for age</td>
<td>-2.11(1.17)</td>
<td>-2.08(1.05)</td>
</tr>
<tr>
<td>Weight for height</td>
<td>-1.70(1.06)</td>
<td>-1.83(1.18)</td>
</tr>
</tbody>
</table>

<sup>1</sup> Data expressed as means (SD) (all such values).  <sup>2</sup> Data expressed as geometric mean SD. There were no significant differences between groups.  <sup>3</sup> Data expressed as Z-scores (all such values).

The results of the fortification trial are shown in Table 5. Time x treatment interactions were significant for Hb (P<0.05), SF (P<0.05), TfR (P<0.05) and body iron stores (P<0.001). CRP did not show a significant time x treatment
interaction. Body iron stores were greater in the iron-fortified group after 7 mo (P<0.01); the increase in iron stores from baseline to 7 mo was 2.7 mg/kg body weight in the iron group and 1.2 mg/kg body weight in the dewormed control group (P<0.001). Hb concentrations were not significantly different between groups after 7 mo (P=0.096). There was no significant change in Hb in the iron group, whereas in the control group, Hb was significantly decreased (P<0.001).
Table 5. Iron status parameters and C-reactive protein concentrations in children fed the iron-fortified (iron group) and the nonfortified rice-based lunch meals (control group) during the fortification trial.

<table>
<thead>
<tr>
<th></th>
<th>Study Group</th>
<th>Baseline</th>
<th>3.5 months</th>
<th>7 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin 1,2 (g/l)</td>
<td>Iron</td>
<td>121(12)a</td>
<td>123(10)a</td>
<td>119(9)a</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>121(13)a</td>
<td>122(12)a</td>
<td>116(11)b</td>
</tr>
<tr>
<td>Serum Ferritin 1,3 (µg/l)</td>
<td>Iron</td>
<td>16.8 17a</td>
<td>17.5 14a</td>
<td>26.3 19b,4</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>15.4 13a</td>
<td>12.2 18a</td>
<td>17.7 17b</td>
</tr>
<tr>
<td>Transferrin receptor (mg/l)</td>
<td>Iron</td>
<td>9.2(3.9)a</td>
<td>8.6(3.3)a</td>
<td>6.1(2.3)b,4</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>8.7(3.2)a</td>
<td>9.1(3.0)a</td>
<td>7.2(3.2)b</td>
</tr>
<tr>
<td>Body iron stores (mg/kg body weight)</td>
<td>Iron</td>
<td>1.2(4.0)a</td>
<td>1.4(3.7)a</td>
<td>4.1(3.4)b,b</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.9(3.7)a</td>
<td>0.1(4.0)a</td>
<td>2.0(4.2)b</td>
</tr>
<tr>
<td>C-reactive protein (mg/l)</td>
<td>Iron</td>
<td>4.4 6.5</td>
<td>9.0 15</td>
<td>4.4 5.9</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>(7.6%)a</td>
<td>(20%)b</td>
<td>(10%)a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.3 8.2</td>
<td>9.2 20</td>
<td>3.7 4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(9.0%)a</td>
<td>(17%)b</td>
<td>(8.6%)a</td>
</tr>
</tbody>
</table>

1,5 Significant time x treatment interaction 1P<0.05; 3P<0.01 (univariate analysis of variance). All data expressed as arithmetic mean (SD) except for 2 expressed as median (SD) and 3 expressed as geometric mean SD. Means in a row not sharing a common letter are significantly different (P<0.001) based on Turkey's tests.

4,6 Significantly different from control at the same time point 4P<0.05, 6P<0.01 (independent sample t-tests).
Figure 1 shows the changes in the prevalences of ID and IDA in the treatment and in the control groups. Over the 7 mo study, the prevalence of ID decreased from 78% to 25% in the iron group and from 79% to 49% in the control group. By logistic regression, there was a significant time x treatment interaction for ID, whereas IDA was not significantly affected by treatment (P=0.161) or time (P=0.453). However, prevalence of IDA decreased from 30% to 15% in the iron group and remained virtually unchanged in the controls (28% and 27%).

![Graph showing changes in ID and IDA prevalences](image)

**Figure 1.** Iron deficiency (ID) and iron deficiency anemia (IDA) prevalence (%) in children receiving a lunch meal containing iron-fortified rice (n=92) and a control group receiving a nonfortified rice meal (n=92). Both groups were dewormed at baseline and 3.5 months. By logistic regression, there was a significant time x treatment interaction for ID (P=0.004), but not for IDA.

There was no significant difference in mean CRP or the prevalence of elevated CRP values between the two groups at any point in the study. However, there was an increase in the prevalence of elevated CRP values in
both groups from baseline to the midpoint of the study (P<0.001), with the prevalence increasing from 7.6% to 20% in the iron group and from 9% to 17% in the control group.

Morbidity and anthropometry
There was no significant evidence of an effect of treatment on the frequency and/or severity of infectious disease, as measured by the questionnaire (P=0.380, Table 6).

At baseline, mean Z scores in the entire sample were: height for age, Z=\(-1.36; SD=1.17\); weight for age, Z=\(-2.09, SD=1.11\); and weight for height, Z=\(-1.77; SD=1.12\). There was no significant difference between groups in these anthropometric measures at any point during the study. There was an increase over the 7 mo study in both groups in height for age and weight for age (P<0.001), but not weight for height (P=0.07).
Table 6. Self reported morbidity in children fed the iron-fortified (iron group) and the nonfortified rice-based lunch meals (control group) during the seven-month trial. The numbers represent the sum of days affected by the specific disease/sickness. Morbidity was assessed weekly by questionnaire.

<table>
<thead>
<tr>
<th>Group</th>
<th>Fatigue</th>
<th>Cold</th>
<th>Fever</th>
<th>Diarrhea</th>
<th>Vomiting</th>
<th>Stomach pain</th>
<th>Ear pain</th>
<th>Skin problem</th>
<th>Eye infection</th>
<th>Measles</th>
<th>Throat pain</th>
<th>Others</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron¹</td>
<td>263</td>
<td>1167</td>
<td>680</td>
<td>94</td>
<td>147</td>
<td>601</td>
<td>165</td>
<td>19</td>
<td>79</td>
<td>24</td>
<td>171</td>
<td>214</td>
<td>3624</td>
</tr>
<tr>
<td>Control</td>
<td>210</td>
<td>1175</td>
<td>734</td>
<td>184</td>
<td>194</td>
<td>692</td>
<td>207</td>
<td>47</td>
<td>178</td>
<td>9</td>
<td>107</td>
<td>254</td>
<td>3991</td>
</tr>
</tbody>
</table>

¹With a univariate general linear model model, there was no significant evidence that iron had an effect on the prevalence of infectious diseases (P=0.380).
Discussion

This study demonstrates that extruded rice fortified with MGFP, fed in a school lunch, increases body iron stores and decreases the prevalence of iron deficiency. However, despite a decrease in IDA prevalence from 30% to 15% in the treatment group—with virtually no effect in the control group—we found only a nonsignificant trend for an effect of iron fortification on IDA (P=0.161). The lack of significance was likely due to the small number of participating children with IDA. ID, rather than IDA, was the inclusion criteria, and the study was designed to detect an increase in body iron stores in iron-deficient children. What was surprising was the lack of change in mean Hb concentration in the iron group despite a clear improvement in iron status. A similar pattern of response was found in a recent iron supplementation trial in children in East Africa, where iron had no effect on Hb concentration or mild or moderate anemia but improved SF and erythrocyte protoporphyrin (Stoltzfus et al., 2004). The authors suggested the lack of effect on Hb was due to endemic infections and concurrent nutrient deficiencies. In our study, the lack of effect on Hb in the iron group may also have been due to widespread infection and inflammation in the children (as reflected in the high prevalence of elevated CRP concentrations, up to 20% of children), impairing iron absorption and utilization (34, 35).

In the control group, there was a significant improvement in iron status during the study. Because feeding was strictly supervised, we do not feel this was due to exchange of rice meals between children in the two groups. Rather, it was likely due to deworming of all children in the study at both baseline and at the midpoint of the study. Reducing the load of intestinal parasites, and particularly hookworm, in endemic areas has clear benefits on iron status (36, 37). Several studies have shown a strong association between intensity of hookworm infection and anemia (Stoltzfus et al., 2000; Stoltzfus et al., 1997). Intestinal parasitoses contribute to negative iron balance through occult gastrointestinal blood loss (40) and may interfere with iron absorption (38).
Although we did not measure parasite load in the children in this study, Indian children residing in urban slums typically have a high prevalence of hookworm infections (41).

Compared to most other iron compounds, ferric pyrophosphate has superior sensory qualities: its white color and lack of reactivity with the food matrix, even at high concentrations, make it attractive for vehicles such as salt and rice (15, 16, 42). We have previously demonstrated that artificial rice grains containing MGFP, produced by heat extrusion, have a texture, taste and appearance in both cooked and uncooked form very similar to natural unfortified rice (16). In the present study, using triangle tests in a panel of Indian women, the iron-fortified rice was indistinguishable from natural rice in raw form (at 3 and 5 mg Fe/100g) and in five local recipes fortified at 3 mg Fe/100g. In addition, the iron-fortified rice lunch meals were well accepted by the children in the study: compliance and rice consumption in the fortified rice group was not significantly different to that of the control rice meals.

Considering the moderate bioavailability of MGFP (10, 13, 14), we chose to fortify the extruded rice grains with 10 mg iron/g as MGFP, to provide • 20 mg of iron/day. MGFP has also been shown to be efficacious in iron-fortified salt in northern Morocco (15).

In the iron group, the mean ± SD cumulative iron dose was 2.06 ± 0.4 g over the 7 mo study. Based on the mean difference in body iron stores between the iron and control groups after 7 mo (1.5 mg Fe/kg body weight), the calculated mean iron absorption from the fortified rice grains was • 2.1%. This absorption value is similar to that from the recent study of iron-fortified salt, where iron absorption from MGFP over a 10-mo feeding trial was • 2% (15). However, despite the low rate of absorption, the MGFP was efficacious: it significantly increased iron stores and reduced the prevalence of ID (Figure 1).

In the present study, the MGFP was given at a high daily dose to show proof-of-concept. In a long-term fortification program, the iron fortification level could be set lower. Based on our measures of local rice consumption (Table 1), a
hypothetical fortification program in Bangalore using MGFP-fortified extruded rice at a concentration of 5 mg iron/100 mg rice (≈ 40% the level used in the present study) would provide 10 mg of iron to women and 6.4 mg of iron to 3-8 y-old children. Our local food record data indicate iron intakes in these two target groups are only about 40% of RDA, and provision of an extra 6-10 mg of iron/day would allow many to achieve adequate iron intakes. If absorption of the iron was 2-3 %, based on the results of this trial and on our estimation of iron bioavailability from the local diet, such a program would supply an additional 0.2-0.3 mg of absorbed iron daily to women and 0.1-0.2 mg to young children.

Several other techniques have been proposed for iron fortification of rice. In industrialized countries, rice is often enriched with iron to restore the iron content found in the unmilled grains (43). Several methods, including coating or extrusion of a grain premix, have been reported (44), but none of these techniques has been used extensively in developing countries due to technical problems. Cold extrusion with has been reported for the production of vitamin A fortified rice (45,46), and PATH (Program for Appropriate Technology in Health, Seattle, WA) has recently promoted extruded fortified rice grains fortified with iron (47). In the Philippines, a large scale rice fortification program has been reported using ferrous sulfate in coated rice kernels (48). Efforts are also underway to biofortify rice with iron by selective breeding. A high-iron rice has been reported to improve iron status in iron-deplete nonanemic women in the Philippines (49). Finally, a genetically-modified rice containing a ferritin gene has been developed (50). Although biofortification and/or genetic engineering are promising approaches, extruded iron-fortified rice grains would offer greater flexibility in fixing the level of iron fortification and avoid concerns about the genetic modification of rice.

Iron supplementation trials in anemic children have found positive effects on growth and conflicting results on the effects on morbidity (52, 53, 54). Our study, there was no significant effect of iron fortification on growth and morbidity. Z scores for weight for age and height for age increased
Efficacy of iron fortified rice

significantly in both groups, but no additional effect of iron could be detected. Our inability to detect a growth effect may have been due to the improvement in iron status in the control group due to deworming, the short duration of the study, and/or the fact that the participating children were mostly iron deficient, but not anemic (55).

Our findings indicate providing iron-fortified extruded rice grains in a school feeding program is an effective iron fortification strategy. Whether applied more generally or targeted to school-feeding programs, extruded iron-fortified rice could help reduce the large burden of ID and IDA in the rapidly growing urban populations of South and Southeast Asia.

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Chapter 7


Conclusions and perspectives

CONCLUSIONS AND PERSPECTIVES

Due to its low reactivity and white color, ferric pyrophosphate can be used to fortify rice using an extrusion premix approach. We found that extruded fortified rice fortified with ferric pyrophosphate of different particle sizes and blended with natural rice at a 1:100 or 1:200 ratio closely resembles natural rice in both cooked and uncooked form. Texture of cooked extruded grains was in the same order of magnitude that natural rice and losses after rinsing were <3%. Therefore extruded iron fortified rice could be a suitable vehicle for iron fortification in rice eating populations.

Relative bioavailability (RBV) of poorly water soluble iron fortification compounds is often used as a proxy of their bioavailability and is used to classify iron fortification compounds and estimate their potential efficacy. It has however been suggested that RBV can be influenced by food matrix (Hallberg et al., 1986; Hallberg et al., 1989). Iron stores indirectly regulate iron absorption, and iron replete subjects absorb iron merely to cover physiological losses (Hallberg et al., 1997). Therefore, in iron replete subjects, a smaller relative difference in iron absorption from ferrous sulfate and from a poorly soluble iron sources can be expected. Inversely, the relative difference would be higher in iron deficient subjects. In the studies performed as part of this thesis, the RBV of an experimental form of micronised ferric pyrophosphate was significantly affected by iron status and food matrix. Thus, RBV should be interpreted with caution as it is a widely variable parameter for poorly water soluble iron compounds. The absolute amount of iron incorporated into erythrocytes might be more informative to evaluate the potential impact of an iron compound. Ultimately, the efficacy of an iron compound and a food vehicle should be tested in controlled field trials.

The results from our 7 month efficacy trial demonstrate a significant improvement in body iron and a significant decrease in iron deficiency prevalence in dewormed school aged children given an average of 13 mg Fe as micronised ground ferric pyrophosphate (MPS=2.5 μm). Extruded rice
Conclusions and perspectives

Fortified with micronised ground ferric pyrophosphate may therefore be an efficacious vehicle for iron fortification in rice eating populations. In an urban slum area of Bangalore, among women and young children (4-8 y-old), average raw rice consumption per day is 199 g and 141 g, respectively. With a fortification level of 5 mg Fe/100 g rice, women and children would consume additional \( \approx 10 \) mg and \( \approx 7 \) mg Fe per day. With estimated absorption from the whole diet of 3-4%, these fortification levels would provide additional 0.3-0.4 and 0.2-0.3 mg absorbed Fe to women and children, respectively. These amounts would likely improve the iron status and contribute to decrease the large burden of iron deficiency in rice eating populations.

There are strong indications that decreasing the particle size of ferric pyrophosphate would improve its bioavailability. Therefore we decided to use a micronised ground ferric pyrophosphate with MPS of \( \approx 2.5 \) \( \mu \)m. Whether the use of ferric pyrophosphate with higher particle size (\( \approx 21 \) \( \mu \)m) would have affected the efficacy of extruded iron fortified rice remains to be determined.

Iron fortification - as part of a set of measures to improve sanitary conditions, encourage healthier weaning practices, and to promote targeted supplementation - could greatly contribute to the eradication of iron deficiency.

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