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Robin Sue Malone-Dickmann

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Gale Strasburg
Major Professor's Signature

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# PHYSICOCHEMICAL PROPERTIES AND RELATIVE BIOAVAILABILITY OF FIVE SOURCES OF FERRIC ORTHOPHOSPHATE IN READY-TO-EAT CEREAL

By

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## ABSTRACT

## PHYSICOCHEMICAL PROPERTIES AND RELATIVE BIOAVAILABILITY OF FIVE SOURCES OF FERRIC ORTHOPHOSPHATE IN READY-TO-EAT CEREAL

**By**

**Robin Sue Malone-Dickmann**

Ferric orthophosphate ($FePO_4$) is a promising iron source for food fortification because of its light color and oxidative stability. However, $FePO_4$ has had limited use in cereal due its variable and often reported low bioavailability. An understanding of the mechanisms underlying this variability may facilitate production of a $FePO_4$ source with consistent bioavailability. The bioavailability of iron sources used for fortification is thought to be largely dependent on their solubility in the dilute HCl in the stomach. Solubility is dependent on a combination of physicochemical properties, such as chemical composition, particle size, surface area and crystal structure. This research determined the relative bioavailability values (RBV) of two (control food) hydrogen-reduced iron sources (Sources 2 and 3) and six commercially available $FePO_4$ sources (Sources 4-9) in ready-to-eat cereal (45% RDA, 0.27-0.30 mg iron/g cereal) using the AOAC Rat Hemoglobin Repletion Bioassay (ferrous sulfate standard, RBV 100%). The solubility of the iron sources in dilute HCl was measured *in vitro* over the range of normal physiological HCl concentrations (0.02, 0.05 and 0.1N) using the solubility method of Shah and others (1977) that was modified to decrease assay variability (CV <1%). Regression analysis found it to be a good predictor of RBV, $R^2$ 90%; $P = 0.008$). Particle

size distributions (PSD) and surface areas (SA) were measured by laser light diffraction and nitrogen absorption, respectively.

The RBVs for the $FePO_4$ sources ranged from 51 – 99 ($P<0.05$) and was higher than that reported in the literature. Most of the PS distributions (PSD) were multimodal and the sources could be grouped into three statistically different distributions ($P<0.05$). The first group had a uniform PSD (Median PS 16.5 μm) and the lowest RBVs (51 & 60%); The second group was composed of a bimodal PSD of fine particles (Median PS 2 μm) and intermediate RBVs (78 & 83%), and the remaining Source 4 (RBV 99%) had a broad bimodal PSD (Median PS 9 μm) with one mode composed of larger 50 μm particles and the second mode composed of fine 6 μm particles. Regression analysis of PS and SA were found to partially explain the variable RBV of $FePO_4$.

In an effort to further explain the variability of RBV, the presence of amorphous microstructure was investigated. Amorphous content is known to have important mechanistic properties that affect the moisture absorption, reactivity and stability of many materials. Very little data on the amorphous content of ferric orthophosphate exists in the literature. Dynamic gravimetric vapor sorption was used to estimate the amount of amorphous content (by moisture uptake). A regression model of median particle size and moisture uptake versus solubility in 0.10 N HCl was found to be an excellent predictor of solubility ($R^2$ 91%; $P = 0.001$).

In conclusion, two factors, mean particle size and moisture uptake (as determined by amorphous content) were found to influence the solubility of $FePO_4$, which may help explain the variable RBV in the literature.

**To my parents, William and Evelyn Malone**

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AOAC | Association of Official Analytical Chemists |
| ATP | Adenosine Triphosphate |
| Aw | Water Activity |
| B.E.T. | Brunauer, Emmett and Teller (Theory) |
| DRI | Dietary Reference Intakes |
| DVS | Dynamic Vapor Sorption |
| EAR | Estimated Average Requirement |
| FAO | Food and Agricultural Organization of the United Nations |
| $FeP0_4$ | Ferric Orthophosphate |
| FFC | Food Chemical Codex |
| HgB | Hemoglobin |
| HRE | Hemoglobin Regeneration Efficiency |
| ICP | Inductively Couples Plasma |
| IDA | Iron Deficiency Anemia |
| INACG | International Nutritional Anemia Consultative Group |
| MPS | Mean Particle Size |
| MOST | The USAID Micronutrient Program |
| PS | Particle Size |
| PSD | Particle Size Distribution |
| RBV | Relative Biological Value |
| RDA | Recommended Daily Allowance |
| RH | Relative Humidity |
| RTE | Ready-to-Eat (cereal) |
| SA | Surface Area |
| SUSTAIN | Sharing United States Technology to aid in the Improvement of Nutrition |
| USAID | United States Agency for International Development |
| WHO | World Health Organization |
| WIC | Women Infant and Children Special Supplemental Nutrition Program |

# INTRODUCTION

Anemia remains the most prevalent micronutrient deficiency in the world. Iron deficiency anemia (IDA) accounts for a large percentage of the total problem affecting 600 – 800 million people worldwide with women, infants and young children most at risk (INACG 2001; Turner 2002). The World Health Organization (WHO) estimates that approximately 50% of children, 42% of women and 26% of men are affected in developing countries, while 2-38% of the population is at risk in developed countries (Nalubola and Nestel 2000; Benoist 2001; Martinez-Navarrete and others 2002; Miret and others 2003). In May of 2000, the WHO and the U.S. Agency for International Development through the International Nutritional Anemia Consultative Group (INACG) reexamined the nature and magnitude of the public health problem at the Belmont Conference (Stoltzfus 2001b). The participants of the Belmont Conference undertook a multidisciplinary critical review process to redefine the nature of the public health problem of IDA on the basis of its link to functional outcomes in human populations. Conclusions drawn from the conference proceedings found that in the majority of cases, the cause of IDA is diet related. Yet despite diet-intervention strategies implemented in almost all countries, IDA remains endemic throughout the world and new strategies are needed to address the problem (Stoltzfus 2001b).

In developing countries, government intervention programs focus on fortifying cereal staples for poorer segments of the population. Industrialized countries fortify a variety of foods, including cereal staples such as wheat flour, corn meal and rice, ready-to-eat (RTE) cereal and products targeted at infants and young children. Foods are

enriched by law or are voluntarily fortified by food manufacturers. Elemental iron powders are the most commonly used iron fortificants worldwide because they are affordable, cause the least problems with food quality, and have good consumer acceptance. However, conclusions drawn by experts at the Monterey Workshop, held in Monterey, Mexico in 2000 to address conflicting data on absorption of elemental iron, found that not enough is known about the extent to which elemental iron powders are absorbed by the body (Hurrell 2002a; Turner 2002). As a result of the Monterey Workshop, the 'Sharing United States Technology to Aid in the Improvement of Nutrition' or SUSTAIN group commissioned a review of the literature published over the past 45 years and found variable iron bioavailability[1] results. In addition, little information was available on the precise types of iron used in these intervention programs and nutritional studies.

Iron is a very reactive element and there are various types of iron, some of which are used as fortificants, including elemental iron and various ferric and ferrous iron salts. The different iron forms have different degrees of reactivity, which generally impair the organoleptic properties and shelf life of foods, thus limiting the iron forms used in foods to those that are less reactive. Unfortunately, the physical properties that make an iron source less reactive also are thought to make it less well absorbed. These challenges make the prevention of iron deficiency through food fortification difficult {Fairweather-Tait, 2002 #66; Hurrell, 2002 #132; SUSTAIN Task Force, 2001 #29; Turner, 2002 #133}. Consequently, experts believe that food stability limitations and the conflict or

[1] For the purposes of this research, bioavailability is defined as the ability of the human body to digest, absorb, and use an iron source for metabolic functions (Wienk KJH, Marx JJ, Beynen AC. 1999. The concept of iron bioavailability and its assessment. Eur J Nutr 38:51-75.

lack of literature information on bioavailability have hindered the successful worldwide implementation of cereal enrichment programs. These barriers raise questions about the efficacy of iron fortification (Lynch 2000; Hurrell 2002a; Turner 2002).

The Recommended Daily Intake (RDI) for iron is 18 mg/day (FDA 1999). Most RTE cereals cannot be fortified with elemental iron above 25% RDI per serving. Empirical experience at the Kellogg Company has shown that oxidatively sensitive cereal such as whole-grain, non sugar-coated cereals often can not exceed 10% RDI even when less reactive iron powders are chosen; however, certain forms of ferric orthophosphate have been found to have minimal negative effects on product quality even at high fortification levels (>40% RDI/serving). Ferric orthophosphate is an affordable alternative iron source that is used when color, density, and stability issues prevent the use of elemental iron powders. Historically, nutritional experts have considered the bioavailability of ferric orthophosphate to be poor (Shah and others 1977; Hurrell 1985; Forbes and others 1989; Hallberg and others 1989; Kellogg 1997; Willis and Allen 1999; Nalubola and Nestel 2000; SUSTAIN Task Force 2001; Dary and others 2002). Although there is less research data available on ferric orthophosphate, a review of the literature suggests that the bioavailability information is as variable as that for elemental iron powders. And unlike elemental iron powders, the physical and chemical properties that influence the bioavailability of ferric orthophosphate are not well understood. In order to better understand the factors influencing bioavailability, the first aim of this research was to determine the bioavailability of commonly used sources of ferric orthophosphate that on visual inspection appeared to have different physical characteristics.

Six types of commercially available food-grade, ferric orthophosphate powders were selected after screening for visual differences in color, appearance, flow properties and supplier-specified mean particle size. Examination of these sources by polarized light microscopy revealed marked differences in microstructure and degree of crystallization and presence of amorphous material. Ferric orthophosphate is a semi-crystalline salt. The presence of amorphous structure in semi-crystalline materials has been given increasing attention in the pharmaceutical sciences over the past ten years and it is now recognized that even relatively low levels of amorphous material (<10%) affect stability and dissolution characteristics (Buckton and Darcy 1999; Mackin and others 2002; Burnett and others 2006). Therefore, the hypothesis of this research is that the amorphous content of ferric orthophosphate is a critical determinant of bioavailability due to its greater capacity to absorb water and, therefore, increase solubility of this material in the gut.

In addition to the six sources of ferric orthophosphate, this study also included ferrous sulfate as the standard of comparison that by definition has a relative bioavailability (RBV) of 100%, hydrogen-reduced iron used commercially to fortify RTE cereal, and encapsulated hydrogen-reduced iron that can be used at higher fortification levels due to its greater oxidative stability. Bioavailability was determined using the AOAC Rat Hemoglobin Repletion Bioassay, which is a widely accepted method for predicting bioavailability in human subjects and has shown excellent agreement with human clinical studies (Forbes and others 1989). This method was suitable for consistently screening a relatively large number of iron sources in one study.

The second aim of this research was to measure the physicochemical properties of the iron sources used in the bioavailability study. Besides chemical composition,

properties such as particle size, surface area, porosity and microstructure all influence important physicochemical properties, such as solubility. There is a great deal of variation in the literature on the methodology used to measure the solubility of iron fortificants and the outcome of these methods is strongly influenced by pH, temperature and shear. This variability makes inter-laboratory comparisons difficult. For this research, the solubility method of Shah (Shah and others 1977) was modified to decrease assay variation by performing carefully controlled experiments in a dissolution apparatus used to determine the solubility of dietary supplements in the gastrointestinal tract. A range of dilute HCl concentrations that simulated differences in human gastric juice was studied as a function of time to understand the effect of pH on solubility.

Particle size distributions were determined using laser light diffraction, and surface areas were measured by nitrogen adsorption. Both these methods are widely used to evaluate and specify elemental iron powders. Several techniques have been developed to measure amorphous content, including dynamic (gravimetric) vapor sorption (DVS), which measures the higher moisture uptake of amorphous versus crystalline forms of the same material. DVS was chosen because of its sensitivity for very low levels of amorphous content, and because it allows a direct measure of the moisture-induced changes affecting the dissolution properties of ferric orthophosphate. Moisture uptake is the main cause of food quality loss in RTE cereals. Solubilization of iron is a prerequisite for absorption in the small intestine and is, therefore, critical to iron bioavailability.

The final aim of this research was to determine the relationship between the physicochemical properties of ferric orthophosphate and their combined effect on

solubility as predictors of bioavailability and also on the organoleptic properties of a standard RTE cereal. Regression analyses was used to determine how well solubility predicts bioavailability and also what physical properties most impact solubility and bioavailability.

This dissertation is organized into a series of chapters that build on information from the previous chapter using the same lots of ferric orthophosphate throughout all the experiments. Each chapter has a specific introduction, materials and methods, results and discussion sections. In chapter 2, the bioavailabilities of six commercially available food-grade ferric orthophosphate powders were determined in comparison with three other iron sources. An *in vitro* solubility method was optimized in Chapter 3, and particle size distributions and surface area were measured for the iron sources. Moisture uptake was also measured by DVS in Chapter 3 to estimate the amorphous content of the ferric orthophosphate samples. Finally, in Chapter 4, a standard RTE cereal was fortified at 45% RDA with each of the iron sources and the sensory attributes of the test cereals evaluated. Sections common to the entire dissertation include the initial abstract, introduction, literature review, final conclusions, future research and the list of references.

# CHAPTER 1

## LITERATURE REVIEW PART I

## Biological Importance, Chemistry, Absorption and Regulation, and Bioavailability

### 1.1 BIOLOGICAL IMPORTANCE OF IRON

Iron is an essential nutrient to all living organisms whose cells rely on iron to play a key role in a plethora of biochemical processes, which include electron transfer reactions, gene regulation, binding and transport of oxygen and regulation of cell growth and differentiation. Iron enables the final energy-yielding steps of the electron-transport chain, a complex metabolic system that functions in the terminal oxidation of nutrients to form water and adenosine triphosphate (ATP). In addition, enzymes that catalyze the production of amino acids, hormones and neurotransmitters require iron (Whitney and others 1998b; IOM 2001; Beard 2006).

The body's iron storage pool can contain as much as 1 to 4 g of iron. The majority of iron is bound to three proteins in the human body: almost two-thirds is found in the hemoglobin of circulating red blood cells, twenty-five percent is in the storage protein, ferritin, and another fifteen percent is in muscle myoglobin. Iron bound to tissue ferritin serves as a reservoir to meet the body's requirements of iron not provided by the diet. Nearly all cells contain ferritin, although 60% of the body's ferritin is found in the liver and spleen. Approximately 95% of the iron stored in the liver is bound to ferritin in the heptocytes and approximately 5% is bound to hemosiderin in the Kupffer cells. The remaining 40% is found in muscle tissues and the cells of the reticuloendothelial system. Iron mobilized from tissue is transported by the iron transporter, transferrin. Transferrin

comprises a small pool of about 5 mg labile iron, and is normally 25-50% saturated with iron. When iron stores are depleted, the saturation of transferrin will decline to less than 15%, resulting in reduced transport of iron to iron-binding proteins that take part in essential reactions. The chemical reactions in which iron participates are classified as oxygen transport and storage, electron transfer, and substrate oxidation-reduction. These reactions are an integral part of metabolism and occur in all cells (Beard 2006). There are four major classes of iron-containing proteins that play critical roles in metabolic reactions: 1) The iron-containing, non-enzymatic hemoproteins (hemoglobin, myoglobin) that function as ligands to bind and transport oxygen; 2) The iron-sulfur enzymes, flavoproteins and heme-flavoproteins, that participate in single electron-transfer reactions, primarily in energy metabolism; 3) The heme-containing enzymes associated with various cofactors, which participate in electron-transfer reactions such as cytochrome P450 complexes; and 4) the other iron-activated enzymes that lack a porphyrin ring structure or iron-sulfur complex (Whitney and others 1998a; IOM 2001; Lynch 2002; Beard 2006).

## 1.2 IRON CHEMISTRY

Iron is the fourth most abundant metal in the Earth's crust and is a member of the transition elements on the Periodic Table of the Elements. The most important property of the transition metals is their ability to exist in different oxidation states and the fact that they can easily move between reduced and oxidized states. This unique redox chemistry is due to the gradual filling of the d electron orbitals and allows iron to take part in electron transfer reactions. This property also allows iron to act as a chelator and

reversibly bind ligands such as oxygen, nitrogen and sulfur in biological processes (Hider and Singh 1993). The electronic spin state and biological redox potential can vary significantly depending on the protein and ligand. This gives iron a great deal of versatility to participate in a large number of reactions (Winterbourn 1991; Beard 2006).

In nature, iron can exist in oxidation states ranging from –2 to +6 (Murphy and Rousseau 1969; Keenan and Wood 1971; Arora 1997). The ferric form, $Fe^{+3}$, and ferrous form, $Fe^{+2}$ (and transient ferryl +4 states involved in biochemical oxidation reactions) are the only forms that occur naturally in living tissues (Silver 1993). Elemental iron, $Fe^{0}$, is not found naturally (except in rare instances), but is a by-product of several manufacturing processes and is purified for use as a fortificant in foods. Many common reducing agents such as ascorbic acid will reduce ferric to ferrous iron with the addition of an electron, and exposure to oxygen will quickly oxidize ferrous iron back to the ferric iron form. Iron's ability to cycle between oxidation states and to reversibly bind ligands form the basis for its two most important chemical properties that are critically important to all life forms: 1) The redox potential between the two common oxidation states of iron, $Fe^{+3}$and its reduced form $Fe^{+2}$, allows oxidation processes centered on iron to be readily coupled to metabolic processes; and 2) Iron's high affinity for oxygen. Iron-containing proteins widely utilize these two properties for many life-sustaining functions (Silver 1993; Beard and Dawson 1997; Miret and others 2003; Beard 2006). In contrast, the properties of iron also allow it to catalyze and propagate deleterious reactions involving oxygen and nitrogen making it a potential toxicant as well as an essential nutrient (Dianzani 1991).

Very little free iron is present in living tissues since it readily participates in redox reactions, which is the basis of iron's inherent toxicity. Iron is most toxic when it is non-specifically bound to the surface of proteins and membranes, as is the case in iron overload (poisoning) and certain disorders of iron metabolism. In the presence of molecular oxygen, weakly bound iron can cycle between its two oxidation states and this can result in the production of the highly reactive $OH^{\bullet}$ radical from the Haber-Weiss-Fenton reaction shown below (Dianzani 1991; Hider and Singh 1993; Symons and Gutteridge 1998; Beard 2006).

$$Fe^{+2} + O_2 \rightarrow Fe^{+3} + O_2^-$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

$$Fe^{+2} + H_2O_2 \rightarrow OH^- + {}^{\bullet}OH^- + Fe^{+3}$$

The potential toxicity of iron requires tight physiological homeostasis control to regulate iron uptake, incorporation into cells and proteins, storage, release and transport (Dianzani 1991).

### *1.2.1 Solubility and Formation of Ligands*

In acidic solutions (pH <7), ferrous ions are rapidly oxidized to ferric ions. Ferric ions quickly form hydrates in acidic environments, e.g. $Fe(H_2O)_6^{+3}$ and $Fe(H_2O)_6^{+2}$. As the pH increases (becomes more alkaline) and more hydrogen ions are removed, water molecules give up protons to form the corresponding iron hydroxides, $Fe(OH)_2$ (ferrous) and $Fe(OH)_3$ (ferric). Iron hydroxides decrease in solubility as the pH becomes more alkaline and eventually will precipitate out of solution. Ferrous hydroxide will precipitate out of an aqueous solution as a pale green gel having a solubility of about $10^{-1}$ M at pH 7

(Symons and Gutteridge 1998; Miret and others 2003). Ferric hydroxide is much less soluble ($10^{-16}$ M) at pH 7 and precipitates out of solution as a dark brown powder (rust). The formation of sparingly soluble hydroxides (and especially ferric hydroxides) has nutritional significance, as the solubility of iron is the necessary first determinant of bioavailability.

In the acid environment of the stomach, soluble iron is hydrated. The hydrated iron passes into the small intestine where bile and other digestive secretions increase the pH to a slightly alkaline environment (pH 7). At this pH, iron hydroxides would eventually precipitate out of solution. However, other components are present in foods, called ligands, that act to keep iron complexed and soluble in the small intestine (Symons and Gutteridge 1998; Miret and others 2003; Beard 2006).

In foods, iron forms soluble ligands with organic molecules such as amino acids, peptides, carboxylic acids (e.g. citric and ascorbic acids), polyols (sugars), phosphates, and with food-additive sequestrants like ethylenediaminetetraacetic acid, or EDTA. Recent work by Huh and others (2004) found that the active fractions in cooked fish that enhanced iron absorption were highly enriched with carbohydrate and contained negligible amounts of protein or amino acids. The authors speculate that these enhancing fractions may originate from glycosaminoglycans of muscle tissue. Depending on the ligand, one iron atom can form from one to six bonds with the ligand. The stability of the complex increases as the number of ligand bonds to the iron atom increases. A ligand that forms more than two bonds, as in the case of EDTA, is called a polydentate ligand. A metal complex formed with a polydentate ligand is very stable and is referred to as a chelate. Soluble ligands naturally present in food are able to form weak chelates by

binding only a few of the six possible coordination sites of iron. These unstable ligands keep gastric acid-solubilized ferric ions in solution at the pH in the duodenum and serve as iron donors to mucin, which enhance reduce ferric iron to ferrous iron for absorption. Certain other dietary constituents are able to form stable chelates or precipitates with iron. Constituents that may form precipitates, such as oxalates, carbonates, phytates, and tannates, may interfere with the binding of iron and mucin. The formation of chelation complexes during digestion may be in part responsible for the conflicting research on the biological availability of iron from foods (Lee and Clydesdale 1979; Benito and Miller 1998). Analogous to the binding of iron to food ligands during digestion, the endogenous biological ligands for iron are oxygen, nitrogen and sulfur atoms in organic molecules like hemoglobin, myoglobin, chlorophyll and ferritin (IOM 2001). The chemistry of ligand formation controls the absorption and regulation of iron in living systems and prevents non-specific binding of iron.

## 1.3 DIETARY IRON

There are four sources of iron in the diet: animal origin; plant origin; fortification iron; and contamination iron (e.g. dirt, minerals in water, iron cooking utensils). Most of the world's population relies on foods of plant origin and many populations do not have access to fortified foods. The main plant-based food staples include wheat, rice, maize, potatoes and beans. The endogenous iron in these foods has moderate to low bioavailability (Morck and Cook 1981). The typical American diet contains about 50% of its iron from grain products in which the iron concentration is typically between 0.1

and 0.4 mg of iron per serving. On the other hand, some fortified cereal products have been found to contain more than 18 mg of iron in a single serving (Beard 2006).

### *1.3.1 Heme and Non-Heme Iron*

There are two fundamental forms of iron in the diet: heme and non-heme iron. Heme iron comes exclusively from animal-based food sources and non-heme iron is supplied from inorganic iron compounds present in animal and plant-based foods. Heme iron is well absorbed and only slightly influenced by dietary factors while the absorption of non-heme iron is strongly influenced by its solubility and interaction with other meal components during digestion. The two forms of iron follow different pathways for absorption from the gut and incorporation into the intestinal mucosal cells. Both have a common pathway out of the mucosal cells and into the plasma (Hallberg 1981; Benito and Miller 1998; Whitney and others 1998b; Miret and others 2003).

Non-heme iron makes up the majority of iron in the diet but its availability for absorption is very dependent on the properties of the iron compound, the meal composition and other factors operating in the lumen of the stomach and proximal small intestine. The iron status of the individual is the key determinant of the amount of iron absorbed from a meal. The rate of absorption for heme iron is relatively constant at about 23% but can be as high as 45%, depending on iron status. The rate of absorption for non-heme iron ranges from 2- 23% (Hallberg and Hulthen 2000). Dietary iron absorption is increased approximately 4-fold once iron stores are totally depleted (Lynch 2002).

### *1.3.2 Iron Absorption and Regulation*

Non-heme iron absorption depends on the solubilization of predominantly ferric iron from the acid milieu of the stomach (IOM 2001). Most food iron is already in the ferric form or it is quickly oxidized in the acid environment of the stomach. Ferric iron must be kept solubilized by components in the diet capable of chelating the ferric iron such as citrate and amino acids, and reduced to its ferrous form by compounds such as ascorbic acid or the ferrireductase enzyme present on the mucosal cell surface of the upper small intestine. This bioavailable iron is them absorbed in a 3-step process where iron is first taken up by the enterocytes across the cellular apical membrane by an energy-dependent, carrier mediated process; absorbed iron is then transported intracellularly and transferred across the basolateral cell membrane into the blood plasma. The mechanism of transport through the enterocytes is not fully elucidated. However, it is a well accepted that only soluble ferrous iron can be absorbed during digestion, and once solubilized and absorbed, ferrous iron joins a common intracellular "labile iron pool" for use by the body (IOM 2001; Miret and others 2003). The iron is available for binding by transferrin in plasma and transported via transferrin throughout the body. Substantial evidence exists to support the commonly held theory that all non-heme iron mixes together in a intracellular "iron pool" in the cells of the upper gastrointestinal tract (Beard 2006). The microenvironment in the gut provides a proton gradient directed toward the cell interior, which together with the brush border membrane will provide the necessary driving force for iron uptake (Benito and Miller 1998; Miret and others 2003).

Solubilized ferrous iron is a prerequisite to iron absorption. Iron uptake is regulated by the total amount of iron ingested, the form of iron, the composition of the

meal and iron-status of the individual. Individuals with a high iron status will absorb proportionally less of any amount of iron consumed than will an iron-deficient individual. Individuals with a lower iron status will absorb more of any dietary form of iron. This process is called "selective absorption" and is the fundamental mechanism of iron balance in humans (Beard 2006).

Ferric ions are rapidly converted to ferrous iron by a membrane-bound member of the cytochrome P450 family, called Dcyth, which is present in abundance and is not a rate-limiting factor (Beard 2006). Specialized transport mechanisms in the enterocytes at the tips of the duodenal villi control iron absorption. Non-heme ferrous iron is transported into the intestinal enterocytes by the divalent metal transporter, DMT1. Ferrous iron taken up by DMT1 and internalized via vesicle endocytosis is then either stored in association with ferritin or exported into the plasma at the basolateral membrane via transferrin. Homeostatic control of iron uptake and basolateral membrane transfer appears to by controlled by the recently discovered plasma signal-protein, hepcidin. Hepcidin is a putative plasma protein regulator of iron absorption. It is released from liver into the plasma in concentrations proportional to the amount of liver iron stores (and also cytokine regulation as part of the immune function). Hepcidin is associated with a protein on the basolateral membrane called ferroportin (MTP1) which releases iron into the plasma. Hepcidin appears to have two primary targets, the basolateral membrane of the enterocytes and macrophages. As hepcidin concentrations vary, so does the release of iron from ferroportin. This in turn regulates non-heme iron uptake from the lumen by DMTI. When physiologic iron needs are high, there is a higher concentration of DMT1 on the enterocytes.

### *1.3.3 Iron Transport*

Iron released into the plasma binds to transferrin. Transferrin's primary function is to move iron from one organ to another. Increased production of tranferrin occurs when iron stores are low resulting in decreased plasma iron concentrations. Some iron is delivered to the myoglobin of muscle cells and to other tissues while the bone marrow takes up large quantities and incorporates iron into the hemoglobin of red blood cells. The liver and spleen dismantle aging red blood cells that have a life expectancy of about 4 months. When iron in the diet is plentiful, the liver stores iron bound to ferritin and breaks it down rapidly to supply iron when the need arises. When iron is limited, hemosiderin is the main storage form of iron. Hemosiderin is a water-soluble breakdown product of ferritin that releases iron more slowly to conserve its supply (Whitney and others 1998a).

### *1.3.4 Iron Regulation: Uptake and Loss*

The human body hoards iron very effectively and has no physiological means of iron excretion. Iron absorption is the sole mechanism by which iron stores are managed. The average adult stores 1 to 4 grams of iron, approximately 70-80 % of which is found in erythroid cells such as hemoglobin and another 20-25% stored in association with ferritin. About 1-2 mg are lost per day through the sloughing of intestinal and skin cells; menstruation accounts for an average iron loss in females of about 2 mg per day. Approximately 3 mg or 0.1% of the total iron in the body circulates in the plasma as an exchangeable iron pool. Essentially all of this iron is chelated to transferrin to render the

iron soluble under physiologic conditions, prevent iron-mediated free radical toxicity and facilitate iron transport into cells (Bridges 2007).

The body efficiently conserves iron and the primary regulator of iron homeostasis is regulation of iron absorption at the enterocytes to approximate iron loss. Iron's poor solubility precludes excretion from the body as a means of maintaining iron homeostasis, which is an important regulator of other minerals in the body. The predominant route of iron loss is from the gastrointestinal tract (shed enterocytes, extravasated red blood cells and biliary heme breakdown products) and amounts to 0.6 mg/d in adult males. Urogenital and integumental losses have been estimated at 0.1-0.3 mg/d, respectively, for adult males and menstrual blood losses range for 1.5 – 2.1 mg/d (Beard 2006).

### *1.3.5 Factors Affecting Iron Absorption*

Most naturally occurring non-heme food iron is in the form of ferric iron salts and several dietary factors affect its absorption in the gut. Enhancers of absorption include amino acids, animal proteins, ascorbic acid, increased hydrochloric acid secretion in the stomach, and organic acids. Inhibitors include carbonates, calcium, egg yolk phosvitin, fiber, oxalates, phytates, plant polyphenols, some flavonoids and soy proteins (Hallberg 1981; Beard 2006).

#### *1.3.5.1 Enhancers*

Ascorbic acid is an important luminal enhancer of iron absorption. It acts as a reducing agent at acid pH and as a low-affinity ligand in the upper duodenum. Ascorbic acid maintains iron in a soluble, low molecular weight form in the slightly alkaline

environment of the upper small intestine (Hallberg and Hulthen 2000; Miret and others 2003). Ascorbic acid has also been shown to counteract the major inhibitors of iron absorption such as phytic acid and polyphenols. Iron absorption from foods containing phytates or polyphenols increases up to four-fold with the addition of ascorbic acid (Hallberg and Hulthen 2000). Several studies have shown that the absorption of non heme iron is increased significantly from meals containing ascorbic acid. The effect is greater for poorly water-soluble ferric iron sources (Forbes and others 1989; Moretti D and others 2006). Animal proteins provide amino acids and peptides to form ligands with iron that aid solubility and absorption during digestion. Organic acids maintain iron in its ferrous form and also create ligands that enhance solubility and protect iron from inhibiting factors. The low pH of the stomach acid is important for releasing iron from macromolecules in food. The action of digestive enzymes, active in an acidic environment, destroy high molecular weight ligands and create new, smaller and more soluble ligands (Miret and others 2003).

#### *1.3.5.2 Inhibitors*

Polyphenols and phytates are the major inhibitors of iron absorption (Hurrell 2002b; Lynch 2002). Polyphenols, phenolic compounds and phytates occur naturally and are widespread in foods such as cereals, vegetables, spices, wine, chocolate, coffee and tea. The lignin and phenolic constituents of insoluble fiber bind iron and the oxalates present in certain grains (wheat bran) and vegetables (spinach and rhubarb) to form highly insoluble complexes with ferric iron. Other inhibitors are present in eggs

(phosvitin) and soy protein. Some flavonoid compounds chelate iron similar to polyphenols (Beard 2006).

#### *1.3.5.3 Micronutrient Interactions*

The potential risk of interactions between micronutrients competing for absorption in the gastrointestinal tract should also be considered in a fortification program. At the naturally occurring levels of micronutrients present in foods, most micronutrients appear to utilize specific absorptive mechanisms and are not vulnerable to competitive interactions. At higher intake levels, competition between elements with similar chemical characteristics and uptake-regulated processes can occur. The interactions have clearly been demonstrated in absorption studies and to some extent been confirmed in supplementation studies (Sandstrom 2001). (Lynch 2000; Sandstrom 2001). Several researchers have shown that the potential exists for calcium to decrease the absorption of non-heme iron and this calcium fortification levels should be considered in foods fortified with both calcium and iron (Fairweather-Tait and Teucher 2002).

## 1.4 BIOAVAILABILITY

The terms bioavailability and absorption are often used interchangeably and the definition of bioavailability can vary (Wienk and others 1999). In a comprehensive review on the concept of bioavailability and its assessment by Weink and others (1999), the authors discussed the topic of bioavailability from the perspective of three scientific disciplines: nutritional science, animal nutrition and pharmacology. In human nutritional sciences the concept of bioavailability is regarded as the efficacy with which nutrients are

utilized. The view of animal scientists is similar except that it focuses on the nutritive value of a diet to support growth and maintenance. In the field of pharmacology, bioavailability is defined as the fraction of the active substance that reaches systemic circulation after an oral dose is administered. This research will follow the definition of Weink and others (1999) and use the concept of bioavailability from a nutritional perspective: ***"Bioavailability is a function of digestibility, absorbability, and the ability to use a nutrient for metabolic functions."*** The authors also make the important distinction that bioavailability must be *quantifiable* if it is to be useful and that the working definition of iron bioavailability is determined by the methods available to measure it.

Bioavailability can be thought of as occurring in three stages: First is the digestibility or the solubility of the iron source in the gut; second is how well the iron is absorbed and delivered to the circulation; and third is how well the iron is processed by the body once it reaches the circulation, target organs and cells for incorporation into measurable functional entities. Iron bioavailability measures always assess one of these three stages.

### *1.4.1 Measures of In Vitro Bioavailability*

It is a generally accepted that only soluble ferrous iron can be absorbed during digestion (Miret and others 2003). Tests to measure solubility can be simple dissolution studies in various concentrations of hydrochloric acid or more complex methods that mimic conditions during digestion. The methods that mimic digestion use a combination of a stomacher (device that simulates the mixing of food contents in the stomach) and

dialysis to simulate the transport of soluble iron into the mucosal membranes of the lumen (Forbes and others 1989). Subsequent soluble iron analysis is accomplished using chromogens ($\alpha$-$\alpha'$ dipyridyl, bathphenanthroline disulphonate, potassium dichromate and ferrozine), atomic absorption spectroscopy, mass spectroscopy (stable isotopes) or $\gamma$-counting of radioisotopes (Wienk and others 1999). The physiological approaches use a pepsin-HCl digestion step followed by neutralization using either bicarbonate or sodium hydroxide and pancreatin-bile extracts to simulate the gastrointestinal tract (Larsson and others 1997).

The greatest limitation of studies using these methods is their inability to model the physiological processes of absorption and incorporation of iron. The dialysis approach comes closer to simulating physiological conditions but cannot take into account the *in vivo* complexity of the digestive process, such as variation in transit time, roles of enzymes, pH, mucosal membranes and barriers to diffusion. Solubility studies that evaluate the dissolution of different iron sources in dilute hydrochloric acid solutions do not take into account the effects of food composition and physiological conditions. In addition, the different HCl solubility methods have not been standardized with respect to concentration (pH), incubation temperature, incubation time and method of iron analysis (Harrison and others 1976; Rasmussen and others 1977; Shah and others 1977; Forbes and others 1989; Hurrell and others 2002; Swain and others 2003). However, when any one procedure is used for the analysis of a single type of iron, these individual methods will give relative results that can be used to compare the solubilities of different forms of the same material.

The solubility of several types of iron fortificants has been analyzed using HCl solubility methods. The methods were developed for elemental iron sources, including hydrogen and carbon monoxide-reduced, carbonyl and electrolytic iron and have been applied to various iron salts such as ferric orthophosphate, ferric pyrophosphate, ferric fumarate and others. Typically a small amount of elemental or iron salt containing 50 - 100 mg iron is added to 200 – 500 ml of dilute HCl at concentrations ranging from 0.02 - 0.25 N for various times and at varying temperatures. Different mixing techniques, including the use of magnetic stir bars, water-bath shakers and wrist-action shakers have been used to mix the samples during dissolution. For example, in a study by Forbes and others (1989) using a modified method of Shah and others (1977), aliquots of electrolytic iron and ferric orthophosphate were weighed to contain 50 mg of iron and added to 250 ml of 0.02 N HCl (pH 1.7). The mixture was gently shaken for 30 minutes at a temperature of 37° C. After 30 minutes dissolution time, between 60 -75% (30-37.5 mg) of the electrolytic iron and between 1.4 – 3.4% (0.7-1.7 mg) of the ferric orthophosphate dissolved. Forbes and others (1989) also measured *in vivo* relative bioavailability of electrolytic iron and ferric orthophosphate using the AOAC Rat Hemoglobin Repletion Bioassay in two laboratories. They found RBVs from 66-78% and 24-34%, for reduced iron and ferric orthophosphate, respectively. The authors concluded that *in vitro* solubility in 0.02 N HCl underestimated the bioavailability of ferric orthophosphate in human and animal studies. Both iron sources used in the experiments were produced in the authors' laboratory (bench-scale) and information on particle size and other physical characteristics, such as surface area and density, were not given.

In contrast, Harrison and others (1976) measured the solubilities of five commercial sources of ferric orthophosphate with known mean particle sizes (MPS) in 0.1 N HCl (pH 1.0) and found that sources with smaller MPS were more soluble. Samples with a 15 μm MPS had 11% of the iron analyzed dissolve while powders with a MPS <1 μm had solubilities of between 42 – 64% (Harrison and others 1976). Swain and coworkers found two measures, solubility in dilute HCl and the surface area of reduced iron powder, to be better predictors of *in vivo* bioavailability than particle size using the rat hemoglobin repletion method (Swain and others 2003). Hallberg and coworkers studied the dissolution rate of crystalline and amorphous ferric orthophosphate in HCl at pH 1.0 (0.1 N), pH 1.5 (0.03 N), pH 2.0 (0.01 N) and pH 3.0 (0.001 N) at 30, 45 and 60 minutes and found that between 5 and 55% of the total iron in the samples dissolved in the different concentrations of HCl. Results from this study also demonstrated that both amorphous and crystalline ferric orthophosphate samples were completely soluble at pH ≥ 2.0. The authors' method for establishing the presence of amorphous material was not given (Hallberg and others 1989), and a quantitative measure of amorphous or crystalline content was not provided. Researchers at the USDA, (Willis and Allen 1999) hypothesized from their work with gypsy moth diets that the amorphous form of ferric orthophosphate was necessary for human bioavailability. These authors developed a solvent fractionation procedure to separate crystalline and amorphous ferric orthophosphate.

The literature indicates that pH, particle size, surface area and the presence of amorphous material influence solubility. However, information on the physical properties of the iron sources used in the literature was very limited. Recently, The International

Anemia Nutritional Consultative Group (INACG), at the conclusion of their 2001 Symposium on global iron fortification, concluded that not enough is known about the extent to which elemental iron powders are absorbed by the body (Hurrell 2002a; Turner 2002). As a result of the Belmont Conference proceedings, The 'Sharing United States Technology to Aid in the Improvement of Nutrition' or SUSTAIN commissioned a review of the literature published over the past 45 years and found variable iron bioavailability results. Little information was available on the precise types of iron used in the various studies and programs. Consequently, experts concluded that conflicting or lacking literature information on physicochemical properties and their relation to bioavailability have hindered the successful implementation of cereal enrichment programs worldwide and questioned the efficacy of iron fortification of foods (Lynch 2000; Hurrell 2002a; Turner 2002). Therefore, there is a need for standardized methods and better understanding of the relationship of the physicochemical properties of iron sources and solubility, which is an indicator of bioavailability.

### *1.4.2 Measures of In Vivo Bioavailability*

Endpoint measures in animals and humans currently provide the best estimate of bioavailability when solubility, absorption and the ability to use a nutrient for metabolic functions are all in question. An endpoint method gives a quantitative measure of the nutrient in a representative part of a metabolic function. In the case of iron, hemoglobin is the target biomarker of iron of bioavailability.

Iron absorption was originally studied by chemical balance techniques. It was not until the use of radio-labeled foods that absorption from individual foods was shown to

differ greatly. The concept of a common iron pool resulted from intrinsic double-radio labeling experiments. When single foods were biosynthetically labeled with an intrinsic iron tracer and mixed with an inorganic iron source labeled with a different radioisotope (an extrinsic tracer), the observation was made that the absorption of the two tracers from the doubly labeled foods was almost the same. An important implication of this method was that all the components of the meal contributed to the bioavailability of non-heme iron in the meal, including components that by themselves originally contain little or no iron. This led to the theory of a common non-heme iron pool (Hallberg 1981). With the recognition that different sources of non-heme iron form a common pool in the intestinal lumen, it was no longer necessary to use the intrinsic tracer and a more convenient method, using only the extrinsic tracer was developed. An extrinsic tracer alone may be used because the small amount of radio-labeled iron added to the meal has been shown to exchange with the intrinsic non-heme iron present, allowing determination of iron absorption by the absorption of radio-labeled iron. Although use of an extrinsic tag has allowed the study of the effect of individual components of meals on the bioavailability of non-heme iron, conditions must be chosen to assure complete exchange (Forbes and Erdman 1983). In the case of certain reduced iron powders and iron salts, like ferric orthophosphate and ferric oxide, iron exchange with the extrinsic tracer in the common iron pool may be in incomplete (Hallberg 1981). Other disadvantages of this method are exposure of human subjects to radiation and the fact that it is based on a single meal or at most two meals administered over a 30-day period (Forbes and others 1989; Benito and Miller 1998; Wienk and others 1999; Miret and others 2003). Less expensive alternatives to human studies are still needed, especially *in vitro* studies to screen and study factors

that affect bioavailability under controlled conditions that can't be duplicated in living systems.

In a comprehensive collaborative study by Forbes and others (1989) that compared human radioisotope techniques with iron solubility methods and the AOAC Rat Hemoglobin-Repletion Bioassay, they concluded that the standard AOAC rat model was the economical method of choice for predicting iron bioavailability in humans. Other reviews consider this method obsolete on the basis that the physiology of iron absorption in rats is different from that than humans (Forbes and others 1989; Benito and Miller 1998; Wienk and others 1999; Miret and others 2003). Dutra-de-Oliveira and others (1995), compared the bioavailability of ferric EDTA, ferric bisglycinate and ferric orthophosphate using the AOAC Rat Hemoglobin Repletion Assay. They measured mean hemoglobin and hematocrit levels, transferrin saturation and liver iron stores. Results showed that the iron from ferric orthophosphate was not as well incorporated into hemoglobin or stored in the liver as the iron from soluble ferrous sulfate or other soluble the ferric chelates (Dutra-de-Oliveira and others 1995).

# LITERATURE REVIEW

## PART II

## Iron Deficiency Anemia, Fortification of Foods, and Iron Sources

### 1.5 IRON DEFICIENCY ANEMIA

Nutritional anemia remains the most prevalent micronutrient deficiency in the world and iron deficiency anemia (IDA) accounts for a large percentage of the total problem. Commonly cited estimates show that IDA affects 20-50% of the poorer populations in Asia, Africa and Latin America and 2-28% in the poorer populations of developed countries (Nalubola and Nestel 2000; Hurrell and others 2002; Martinez-Navarrete and others 2002; Miret and others 2003). The most commonly affected segments of the population are pregnant women (56%), school-age children (42%), women (44%) and preschool-age children. Worldwide, the highest incidence of IDA occurs in Saharan Africa and Southeast Asia (Nalubola and Nestel 2000). Many estimates have been given on the total percentage of the world's population suffering from IDA with some estimates, according to the International Nutritional Anemia Consultative Group (INACG), being questionably high. In 2001, INACG gave a conservative estimate (extrapolated from 1985 data) of 600-800 million people suffering from IDA worldwide.

In May of 2000, scientists at the Belmont Nutritional Conference sponsored by the WHO and INACG, re-examined iron-deficiency anemia and the magnitude of the public health problem. They evaluated the strength of the causal evidence linking iron-deficiency to global health outcomes. Strong causal evidence was found to correlate

severe anemia with increased child and maternal mortality, and mild to moderate iron-deficiency anemia with impaired childhood development (delayed psychomotor development and impaired cognitive development) and decreased work productivity in adults. The outcome of the proceedings concluded that "given the widespread prevalence of mild to moderate iron-deficiency anemia and the public health importance of the outcomes in question, there is an urgent need to elucidate these potentially causal relationships" (Stoltzfus 2001b). Many intervention strategies were reported as not working as well as predicted and experts recognized that multiple new strategies were needed.

Following the Belmont Conference, a workshop was held in Monterey, Mexico in September 2000 to address the conflicting data on absorption of elemental iron and to evaluate its usefulness as a cereal fortificant. The SUSTAIN group presented their finding from a commissioned a review of the literature published over the past 45 years that found variable iron bioavailability results. Experts at the Monterey Workshop concluded that not enough is known about the extent to which elemental iron powders are absorbed by the body (Hurrell and others 2002; Turner 2002). They found little information available on the precise types of iron used in literature and in the intervention programs and identified a need to better understand the factors influencing absorption elemental iron (Hurrell 2002b).

Iron is a very reactive element and there are various types of iron, some of which are used as fortificants, including elemental iron and various ferric and ferrous iron salts. The different iron forms have different degrees of reactivity, which generally impair the organoleptic properties and shelf life of foods, thus limiting the iron forms used in foods

to those that are less reactive. Unfortunately, the physical properties that make an iron source less reactive also are thought to make it less well absorbed. These challenges make the prevention of iron deficiency through food fortification difficult (SUSTAIN Task Force 2001; Fairweather-Tait and Teucher 2002; Hurrell 2002b). The public health importance of combating anemia has been widely recognized since the 1960's and measures to control anemia are implemented in almost all countries. The World Bank has identified diet-based micronutrient interventions as among the most cost-effective of all health interventions. Despite these efforts and the improvement seen in the nutritional status of populations for other micronutrients, iron-deficiency remains endemic throughout the world (Benoist 2001).

The effectiveness of a food fortification program is dependent on both the quantity and quality of the iron sources used, access to and frequency of use of the fortified food by the at risk population and the composition of the whole diet of the target population. The bulk of iron-deficiency cases are diet-related and despite the challenges, diet-related intervention strategies remain important control measures. Although not as effective as planned, they are responsible at least in part, for the reduction in IDA in certain populations (ADA 2001; Turner 2002; FDA Department of Health and Human Services 2003).

## 1.6 IRON FORTIFICATION OF FOODS

IDA has important negative health and economic consequences in countries where it is a major health problem. For this reason, many countries are evaluating strategies to combat iron deficiency and food fortification of staple foods is considered

the most cost-effective, long-term approach (Stoltzfus 2001a; Hurrell 2002a). Cereal staples include flour made from wheat, corn, rice and staple foods made from these grains such as porridge, bread, biscuits, pasta, and corn meal. Unlike the successful initiatives to improve the intake of vitamin A, iodine and B vitamins using staple food vehicles, enrichment with iron has not been as successful (Stoltzfus 2001a; Stoltzfus 2001b; Dary 2002; Hurrell 2002a). Several reasons combine to contribute to this lack of success. One of the major reasons is that iron is a very reactive transition metal with multiple valence states that allow it to participate in oxidation and reduction reactions and act as a catalyst for undesirable reactions in food. The form of iron added to a food may change during processing, storage and even during digestion (Lee and Clydesdale 1979). The reactivity of iron, which enables iron to play a critical role in metabolic processes is associated with the deterioration of organoleptic properties and shortened shelf life of foods. Thus, the reactivity of iron limits the amount and type of iron that can be used for food fortification, usually limiting choices to iron less reactive forms. Unfortunately, the physical properties that make an iron source less reactive also make it less bioavailable. These challenges make the prevention of iron deficiency through food fortification with non-heme iron sources difficult. (SUSTAIN Task Force 2001; Fairweather-Tait and Teucher 2002).

In more developed countries, where the economy supports varied food choices, food manufacturers voluntarily fortify processed products in addition to staple foods. Government-subsidized programs help lower the purchase price of fortified foods for poorer segments of the population and make these food items more widely accessible to those who need it most. RTE cereals along with composite flour, and products targeted at infants and young children, play an important role in dietary intervention strategies.

Fortified RTE breakfast cereals are a major source of essential nutrients in many countries and are of particular importance in the U.S., U.K., Canada, Central and South America and Australia where they play a significant role in the diet and are consumed regularly. Several government-sponsored initiatives are aimed at improving the nutritional status of women and children and cereal plays an important role in these programs. In the U.S., the School Breakfast Program and the Special Supplemental Nutrition Program for Women, Infants and Children (WIC) are important initiatives to safeguard the health of low-income women and children. WIC-eligible cereal products must contain 45% of the RDA for iron per serving (270 ppm) (Oliveira and others 2002). Achievement of this mandate is technologically challenging.

### *1.6.1 The Amount and Quality of Added Iron*

The FDA recommends that producers who voluntarily fortify products follow the guiding principle of their position statement: "Nutrients may be added to a food to correct a dietary insufficiency that is recognized by the scientific community to exist and known to result in nutritional deficiency disease if sufficient information is available to identify the nutritional problem and the affected groups." However, other than the requirement that iron sources used to fortify foods be of food grade quality and compliant with the quality standards set forth in the U.S. Pharmacopoeia (USP) or the Food Chemical Codex (FCC), there are no regulations mandating the quality of iron to add from a bioavailability perspective. The nutrition community highly recommends the use of iron sources with known bioavailability and discourages the use of poorer quality forms (Turner 2002).

Hydrogen-reduced iron is the most widely used iron source for food fortification due to its relatively low cost and availability in a range of particle sizes, surface areas, porosity and purity. Its availability over a wide range of physical parameters allows food manufacturers some control over the form used for a particular ingredient or product application. As discussed earlier, bioavailability and reactivity are related to the physicochemical properties of the iron form. Currently, particle size (and purity) is the physical property most often used by food manufacturers to specify reduced iron powders. Milling and sieving are inexpensive and fast procedures used to determine the particle size fractions of reduced and electrolytic iron powders, which generally consist of dry particles with a maximum particle size of 1 mm (Hurrell 2002a). The Food Chemical Codex (FCC) guidelines require that reduced iron powders used to fortify food pass through a 100-mesh screen or sieve (particle size < 149 μm) and that electrolytic and carbonyl powders pass through a 325-mesh sieve (particle size < 44 μm) (Government 2006). Most manufacturers set their specifications for reduced iron powder at the 325-mesh screen size even though this higher quality standard is not stipulated by the FCC. The SUSTAIN Task Force on the iron fortification of cereal flours recommends a maximum 325-mesh specification. According to the SUSTAIN Task Force's 2002 report, other physical parameters such as surface area, porosity and purity, have not been sufficiently investigated to enable the formulation of clear quality guidelines for these parameters (Hurrell 2002a). Although other forms of iron, such as the iron salts and iron chelates are used to fortify foods, only reduced iron has an FCC guideline.

### *1.6.1.1 Recommended Dietary Intakes for Iron*

At the time of this writing, the Recommended Dietary Allowances (RDAs) of essential nutrients, established by the Food and Nutrition Board (FNB) of the National Academies, Institute of Medicine (IOM) are under revision. The RDAs have been re-evaluated by the FNB periodically since their inception in 1943, with the latest ($10^{th}$) edition published in 1989. Over the last few decades, the original focus of the RDAs, which was the prevention of classical nutritional deficiencies, has expanded to include fortification levels for optimal health and information that could be used for individual dietary planning as well as population assessments. In the early 1990's the FNB in collaboration with Health Canada undertook the task of revising the RDAs and Canadian Recommended Nutrient Intakes (RNIs). The outcome of this effort was a new framework that consists of multiple sets of nutrient specific guidelines collectively referred to as Dietary Reference Intakes or DRIs. The DRIs are composed of a family of four nutrient reference values: 1) Recommended Daily Allowances (RDA), 2) Estimated Average Requirements (EAR), 3) Adequate Intakes (AI), and 4) Tolerable Upper Intake Levels (UL). The DRIs are slated to replace and expand the traditional RDAs in the U.S. and Recommended Nutrient Intakes (RNIs) in Canada (Penland 2001).

The new DRIs for iron were published in 2001. The RDA for iron for boys and girls between the ages of 9-13 is 8 mg/d (UL 40 mg/d). The RDA for boys 14-18 years of age is 11 mg/d, while the RDA for girls of the same age is increased to 15 mg/d (UL for adolescents is 45 mg/d).The RDA for healthy adults between the ages of 19 and 50 was set at 8 mg/d for males and 18 mg/d for females (UL for adults is 45 mg/d). Men over the age of 50 should continue to consume 8 mg/d and women over 50 years of age (or

postmenopausal) need to decrease their iron intake to 8 mg/d (IOM 2001). The new DRIs for iron are lower for children, adolescent men and older adults as compared to the previous RDAs. However, at the time of this writing, the original 1989 RDAs remain the reference guidelines for food labeling.

The food labeling system uses Recommended Daily Intakes (RDIs) for vitamins and minerals, based on the 1989 RDAs, for moderately active young adults consuming 2000 calories per day. Daily Reference Values (DRV) are estimated for nutrient components that do not have an RDA, such as fiber, protein, total fat, saturated fat, cholesterol, sodium, potassium, and carbohydrate. The RDI for iron is 18 mg/d for adults and children (IOM 2001). Cereal products are typically fortified at between 10 – 25% of the RDI for iron or 1.8 – 4.5 mg iron per typical 30-gm serving of RTE cereal (60 to 150 ppm iron). Iron present at levels as low as 0.1 ppm can decrease the lipid oxidation induction period and accelerate oxidation rates (Nawar 1996). Sugar-coated cereals and cereals high in natural antioxidants, like wheat bran, can withstand higher levels of iron fortification but most whole grain, low sugar cereals cannot withstand levels beyond 60 ppm or 10% of the RDI for iron due to the oxidatively sensitive polyunsaturated fats inherent in the grain. A sugar-coating is also an excellent oxygen and moisture barrier for cereal, which uncoated cereals lack. The WIC program requirement is 45% RDI per serving or 270 ppm, which is an extremely challenging level of iron fortification to meet for any product.

### *1.6.2 Effect of Iron on the Oxidative Stability of Foods*

Lipid oxidation is one of main causes of food quality loss and spoilage and is of great economic concern to the food industry. Oxidation of fat in foods leads to off-odors and off-flavors (rancidity) that shorten shelf life. Oxidative reactions can decrease the nutritional quality of a food and certain products are potentially toxic (Nawar 1985). Lipid oxidation is a complex process and proceeds by a classic free-radical chain mechanism involving three discrete phases: 1) Initiation, the abstraction of a hydrogen atom from the carbon $\alpha$ to the double bond in a methylene-interrupted, unsaturated fatty acid in the presence of an initiator to form a carbon-centered lipid radical; 2) Propagation, the lipid radical reacts with molecular oxygen to form peroxy radicals and lipid hydroperoxides; and 3) termination, in which two radicals combine to form non-radical end-products that do not propagate further reactions (Nawar 1985; Arora 1997). The initiation step requires the formation of free radicals and is thermodynamically unfavorable, requiring an initiator. Transition metals that possess two or more valence states with a suitable oxidation-reduction potential between them (iron, copper, cobalt, manganese, nickel) act as major pro-oxidants. If soluble, reactive iron is present, even at concentrations as low as 0.1 ppm, it can act as a catalyst and decrease the length of the oxidation induction period and increase the rate of oxidation. When iron is used as a food fortificant, added amounts often exceed 30 ppm and may exceed 200 ppm. Iron sources used at such high levels must have properties that limit reactivity in the food but not bioavailability in the gut. Few iron sources meet this criterion, often resulting in the use of iron forms that are less bioavailable than desired in order to meet product quality standards.

Other initiators of lipid oxidation are singlet oxygen ($^1O_2$), ultraviolet light, and high temperatures (Simic and others 1992). Lipid degradation in foods may be propagated by multiple reactive species, which include hydrogen peroxide ($H_2O_2$), lipid hydroperoxide radical (HOO·), superoxide anion ($O_2^{\cdot -}$), hydroxyl radical (·OH), peroxy radical (ROO·), and the highly reactive alkoxyl radical (RO·). Several mechanisms for metal catalysis are postulated:

***1) Acceleration of hydroperoxide decomposition to form alkoxyl radicals***

$$M^{n+} + ROOH \longrightarrow M^{(n+1)+} + OH^- + RO\cdot$$

$$M^{n+} + ROOH \longrightarrow M^{(n-1)+} + H^+ + ROO\cdot$$

***2) Direct reaction with unoxidized substrate***

$$M^{n+} + RH \longrightarrow M^{(n-1)+} + H^+ + R\cdot$$

***3) Activation of molecular oxygen to give singlet oxygen and peroxy radicals***

$$M^{n+} + O_2 \longrightarrow M^{(n+1)+} + O_2^- \xrightarrow{-e^-} {}^1O_2$$

$$O_2^- \xrightarrow{+H^+} HO_2\cdot$$

(Nawar, 1985;(Fennema 1996))

One of the simplest free radical-generating reactions involving iron is:

$$O_2 + \text{Fe (II)} \leftrightarrow \cdot O_2^- + \text{Fe (III)}$$

The oxygen radical formed in this reaction, superoxide anion, is a potent initiator of lipid oxidation. The superoxide radicals recombine in the following reaction:

$$2\,\cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide generated via this reaction becomes a source of other radicals and reacts with free metal ions like iron and copper, as well as some of their complexes, thereby generating highly reactive free radicals. These are the Haber-Weiss and Fenton reactions. (Simic and others 1992; IUPAC 2003). The Haber-Weiss cycle consists of the following reactions:

$$H_2O_2 + {}^{\cdot}OH \rightarrow H_2O + O_2^{\cdot-} + H+ \text{ and}$$

$$H_2O_2 + O_2^{\cdot-} \rightarrow O_2 + {}^{\cdot}OH + OH^-$$

The second reaction has a negligible rate constant and it is believed that iron (II) complexes catalyze the reaction via the Fenton reaction:

$$H_2O_2 + Fe(II) \rightarrow {}^{\cdot}OH + OH^- + Fe(III)$$

Many complexes of iron with a low redox potential generate the superoxide radical, $O_2^{\cdot-}$. Reducing agents, such as ascorbate, act to keep recycling ferric iron back to reactive ferrous iron via the following reaction (Simic and others 1992; IUPAC 2003):

$$Fe\ (III) + AH^- \rightarrow Fe\ (II) + {}^{\cdot}A + H^+$$

In this situation, the action of ascorbate to produce reactive ferrous ions is potentially deleterious to biological molecules and foods. Lipid oxidation by ionic iron requires the presence of ferrous iron and the subsequent production of oxygen radicals that go on to produce hydroxyl radicals. Reactive oxygen species are required to abstract hydrogen from the carbon $\alpha$ to site of unsaturation (double bond) on a lipid molecule to begin the oxidation process (Decker and Hultin 1992; Simic and others 1992).

## 1.7 SOURCES OF ADDED IRON

Even though many compounds are available for use as iron fortificants, only five compounds are commonly used: elemental iron powders, ferrous sulfate, ferric orthophosphate, ferric pyrophosphate, and ferrous fumarate (Hurrell 1997). A few other highly bioavailable sources are recommended, such as ferric EDTA, and ferric bisglycinate, but these forms are cost-prohibitive in many food applications, and limited due to their reactivity in others. Elemental reduced iron powder is the most commonly used fortificant worldwide and particularly in cereals because of its relatively low cost and low reactivity during processing and in the finished food during storage. Based on 1970 estimates that had not changed significantly as of 2002 (SUSTAIN Task Force 2001; Hurrell and others 2002), it is estimated that reduced iron powder accounts for 40-55% of the iron added to foods, followed by ferrous sulfate (25 -30%) and iron phosphates (15-25%). Other compounds, like ferrous fumarate, contribute only 1 to 5% of the iron added to foods (Lee and Clydesdale 1979; Nalubola and Nestel 2000). The relative cost of the commonly used fortificants compared to ferrous sulfate (considered the optimum fortification source for bioavailability) is given in Table 1.1 (Hurrell 1985; 1997).

Table 1.1 provides a comprehensive list of the many iron sources available for fortification purposes, categorized by their relative water solubility. The forms listed are classified as freely water-soluble, slowly water-soluble, poorly water-soluble and insoluble. In general, water-soluble iron sources score higher in relative bioavailability tests in rats and human studies because they are readily soluble during digestion. However, the more water-soluble sources are also more prone to cause off-flavor and off-

color problems due to their increased availability to catalyze and participate in undesirable reactions.

**Table 1.1 Relative cost (to ferrous sulfate) and bioavailability of iron sources used for food fortification [1]**

| Iron Source | Approximate Fe content (%) | Relative Bioavailability (RBV) [2] Rat | Human | Relative cost compared to $FeSO_4$ |
|---|---|---|---|---|
| **Freely water soluble** | | | | |
| ***Ferrous sulfate-7H$_2$0*** | ***20*** | ***100*** | ***100*** | ***1.0*** |
| Ferrous gluconate | 12 | 97 | 89 | 5.1 |
| Ferrous lactate | 19 | | 106 | 4.1 |
| Ferric ammonium citrate | 18 | 107 | - | 4.1 |
| **Slowly water soluble** | | | | |
| Anhydrous ferrous sulfate | 33 | 100 | 100 | 0.65 |
| Ferric saccharate | 10 | 92 | 74 | 5.2 |
| Ferric citrate | 17 | 73 | 31 | 4.8 |
| **Poorly water soluble** | | | | |
| Ferrous fumarate | 33 | 95 | 100 | 1.3 |
| Ferrous succinate | 35 | 119 | 92 | 4.1 |
| Ferrous tartrate | 22 | 77 | 62 | 3.9 |
| Ferrous saccharate | 10 | 92 | 74 | 5.2 |
| **Water insoluble** | | | | |
| Ferric pyrophosphate | 25 | 45-58 | 21-74 | 2.3 |
| Ferric orthophosphate | 28 | 6-46 | 25-32 | 4.1 |
| ***Elemental iron powders*** | | | | |
| Electrolytic | 98 | 44-48 | 5-100 | 0.5 |
| Carbonyl | 98 | 39-66 | 5-20 | 1.0 |
| Hydrogen-Reduced | 97 | 24-54 | 13-148 | 0.2 |
| **Protected Compounds** | | | | |
| NaFeEDTA | 14 | na | 28-416 | 6.0 |

[1] Adapted from (Hurrell 1985) and (Hurrell 1997)

[2] Percent Relative Bioavailability Value (RBV) is determined by a ratio comparison to of the iron source to ferrous sulfate; ferrous sulfate is considered to have excellent bioavailability and a RBV of 100% and is the reference standard used in most *in vivo* animal and human assays.

The differences in the cost of various iron fortificants can be quite large on a manufacturing scale. Costs of the most commonly used sources are given in Table 1.2 along with information on iron content, color and bioavailability. Iron content is an extremely important consideration as it can dramatically impact the cost of the raw material and total amount of the iron source added. Equally important are the other constituents present in the iron compound used. The sulfur, phosphate, amino acid, organic acid, etc. moieties impart flavor, affect pH, solubility, ionic strength and react with other components of food.

**Table 1.2 Cost, iron content, color and relative bioavailability of iron fortificants [1]**

| Iron Source | Amount (% Fe) | Cost ($/Kg) | Cost ($/Kg Fe) | Color | Relative Bioavailability in Man (%) [2] |
|---|---|---|---|---|---|
| Ferrous sulfate | 32 | 2.40 | 7.50 | White | 100 |
| Ferrous fumarate | 33 | 3.00 | 9.09 | Red | 100 |
| Ferric orthophosphate | 28 | 5.00 | 17.86 | Yellow – pinkish white | 24-32 |
| Reduced iron | 97 | 2.00 | 2.06 | Black | 13-85[3] |
| Electrolytic iron | 98 | 6.70 | 6.84 | Dark gray | 5-100 |
| Ferric EDTA | 13 | 8.70 | 66.92 | Dark yellow | 100 |
| Ferrous bisglycinate | 20 | 20.26 | 101.30 | Grey-green | 100 |

[1] Adapted from (Nalubola and Nestel 2000)

[2] Percent Relative Bioavailability Value (RBV) is determined by a ratio comparison of the test iron source to ferrous sulfate; ferrous sulfate is considered to have excellent bioavailability and a RBV of 100% and is the reference standard used in most *in vivo* animal and human assays.

[3] Dependent on the type and physical properties of reduced iron powder used

### *1.7.1 Elemental iron*

As mentioned previously, elemental (reduced) iron powder is the most widely used iron fortificant. Cereal fortified with reduced iron serves as the reference against which alternative iron sources like ferric orthophosphate are compared (SUSTAIN Task Force 2001). The hydrogen-reduced form is the most common food-grade iron source. It is produced by reduction of ground iron oxide with hydrogen or carbon monoxide at high pressures and temperatures. Purity is dependent on the purity of the oxide used. Most often, reduced iron is a byproduct of other industries since using mill scale produces a higher purity iron powder than iron ore and is less expensive (Patrick 1985). The physical properties of reduced iron vary widely and can range from smooth dense spheres of varying size to spiked or smooth, irregularly shaped, dense or porous particles. The surface area of the particles play a major role in the solubility rate and amount of iron dissolved during digestion.

Impurities in food-grade iron also affect solubility. The most common impurities consist of a mixture of ferrous and ferric oxides that form on the surface of iron powders or they can be trapped during manufacture in the center of the particles. The surface of all iron powders contain varying amounts of a mixture of ferrous and ferric oxides. Ferrous oxide is nutritionally beneficial since it is more soluble in gastric juice and the oxidized areas provide attack sited for particle dissolution. However, iron oxide can further react to form insoluble ferric hydroxides (rust) if exposed to humidity, which are not bioavailable and are detrimental to product quality. Reactive ferric and ferrous oxides can behave unpredictably during food processing causing negative organoleptic and oxidative instability problems in the finished product.

In general, elemental iron is less well absorbed than other more water-soluble iron sources (like ferrous sulfate) but this depends heavily on how the iron powder was produced and its resultant physiochemical properties and impurities (Hurrell and others 2002; Turner 2002). All commercial elemental iron powders must contain >96% iron. The powder characteristics that influence absorption are those that influence dissolution at acid pH in the gastric juice of the stomach. Physical properties such as particle size and microstructure (shape/density/porosity) ultimately affect the available surface area and rate of dissolution. Different manufacturing processes produce the different iron types and the subsequent grinding and sieving processes also contribute to the final characteristics of the powders. As mentioned earlier, all food grade iron powders must conform to the Food Chemical Codex (FCC) specifications that require the material to pass a 100-mesh (149 μm) sieve. Food companies in the United States and Europe mainly use smaller particle size powders that are < 50 μm in an effort to reduce organoleptic issues (black specks in the product and graininess) and improve bioavailability (Hurrell and others 2002; Turner 2002).

As discussed, reduced iron is not a single entity but varies widely depending on its method of manufacture. Historically, reduced iron powder has been classified into three categories of iron based on their method of manufacture and resultant physical properties. A fourth category, atomized iron, has become a recognized type of iron in the last ten years (Table 1.3). The four categories are briefly described (Hurrell and others 2002), as follows:

1) **Hydrogen-reduced iron and carbon monoxide-reduced (CO-reduced) iron powders** are made at elevated temperatures and pressures by using either hydrogen or

carbon monoxide to reduce iron oxide to its elemental state. The iron oxide can be either naturally-occurring iron ore, $Fe_2O_3$ (hematite) or mill scale, $Fe_3O_4$ (magnetite), which is a by-product of the steel industry. These forms have the lowest purity of the food-grade iron powders at > 96% iron. Impurities can be mixtures of carbon, magnesium, aluminum, silicon, phosphorous, sulfur, chromium, manganese, nickel and copper, usually present in the form of acid insoluble oxides and therefore would be expected to reduce solubility. The particles typically have an irregular but smooth exterior surface and porous internal structure. Milling and sieving is done under inert gas to reduce surface oxidation (the greatest source of impurities of any iron powder) and produce powders with a range of particle sizes, typically less than 150 μm.

2) **Electrolytic iron** is produced by electrolytic migration of iron from a pure iron anode through a ferrous sulfate solution onto a stainless steel cathode. The thin brittle sheets produced by this process are ground into powders having fine particle sizes. Electrolytic iron powders contain significantly fewer impurities than reduced iron powders at > 99% iron and are composed of irregularly shaped particles with high surface areas that are considered to have good to excellent bioavailability.

3) **Carbonyl iron** is made directly from reduced elemental iron or from scrap iron. The iron source is reacted with carbon monoxide under heat and pressure to produce iron pentacarbonyl, which is then decomposed under controlled conditions to yield iron powder and carbon monoxide gas. A second reduction with hydrogen is done to reduce the carbon impurities to produce powders containing > 98% iron. Carbonyl iron consists of dense spheres with extremely small particle sizes (2-10 μm) whose structure is characterized by concentric shells of iron arranged in an onion-like fashion. The particles

have a smooth outer surface less prone to oxidation than reduced and electrolytic iron types.

**4) Atomized iron** is a recently introduced hydrogen-reduced iron powder that is manufactured by an "atomizing" process that gives the iron particles higher surface area due to a highly irregular and "spiked" surface structure. This iron is available in a range of particle sizes, depending on the milling process, similar to H-and CO-reduced iron. Quebec Metal Powders developed this relatively new process that can be found in the Handbook of Powder Metal Technologies and Applications (1998).

Hydrogen-reduced iron and electrolytic iron are provided as well-characterized powders that should differ only slightly from batch to batch. Carbon-monoxide-reduced iron powders can have variable composition and particle sizes ranging from the least expensive $< 150$ μm product commonly used in developing countries to the higher quality $< 45$ -50 μm used in the US and Europe. The greatest advantage of using elemental iron powder over other iron fortificants is the relative stability of the material. However, experience fortifying with reduced-iron powders at the Kellogg Company has found that H-reduced iron powders having a surface area $> 10$ $m^2/g$ often produce product defects associated with oxidative deterioration that increases with increasing surface area in sensitive products.

**Table 1.3 Manufacturers and forms of food-grade elemental iron[1]**

| Powder Type | Manufacturer | Particle Size |
|---|---|---|
| H-reduced | North American Höganäs (formerly Pyron) | 325-mesh[2] |
| Reduced (atomized)[3] | Quebec Metal Powders (QMP), Canada International Metal Powders (IMP), India | 325-mesh |
| CO-reduced (sponge)[4] | Höganäs AB, Sweden | 325-mesh<br>300-mesh<br>100-mesh |
| Electrolytic | OMG Americas, USA (Glidden, A131) | 325-mesh<br>(39% <10 μm, 25% 10-20 μm, 16% <20-30 μm, 8% 30-44u\μm) |
| Carbonyl | BASF, Germany | 325-mesh |
| Carbonyl | International Specialty Products (ISP), USA | 325-mesh (mean particle size 5 μm) |

[1]Adapted from Hurrell and others 2002

[2]325-mesh means that ca. 95% of the particles are < 45 microns; 300-mesh means that ca. 95% of the particles at < 50 microns; 100-mesh means that ca. 95% of the particles are < 150 microns.

[3]Sponge iron is smooth on the surface and has a porous sponge-like interior with high surface area and an RBV typically greater than 50 (Kellogg, 2006)

[4]Atomized iron has an irregular highly spiked surface that gives the particles a comparable surface area to sponge iron even though its center is not porous (Kellogg, 2006)

### *1.7.2 Ferric Orthophosphate*

Elemental iron powder and ferric orthophosphate (Figure 1.2) are the most widely-used iron sources for the fortification of ready-to-eat (RTE) cereals because of their low cost and limited negative effects on product quality. Hydrogen-reduced iron powder (<50 μm) costs approximately one to two dollars per pound and is approximately one-half to one-third the cost of ferric orthophosphate. Food-grade elemental iron contains a minimum of 96% iron compared to the 26% iron content of ferric orthophosphate. This makes the cost of ferric orthophosphate six to seven times more expensive than elemental iron to attain the same level of iron fortification (Kellogg 2006). Due to cost and usage level, hydrogen-reduced iron in the U.S. and both hydrogen-reduced and carbon monoxide-reduced iron outside the U.S. continue to be the most commonly used forms of iron. However, for specific applications, ferric orthophosphate is preferred over reduced iron due to its lighter color and lower density (2.87 $FePO_4$ g/cm$^3$ versus 7.8 g/cm$^3$, respectively) (Budavari and others 1996). Ferric orthophosphate is also preferred for oxidatively sensitive foods and can be used at higher levels than reduced iron powders, although its effect on product quality can be mixed depending on the processing involved.

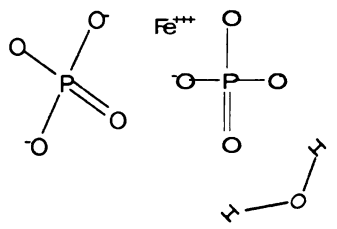


**Figure 1.1 Ferric orthophosphate** ($FePO_4 \cdot x\ H_2O$) is an odorless, yellow to buff to pinkish-white powder composed of one Fe (III) atom complexed to from one to three phosphate groups and hydrated with one to four molecules of water. The iron content typically ranges from 26- 32%. The monobasic, dihydrate is most often used for food fortification. It is prepared by reaction of sodium phosphate with ferric chloride or ferric citrate (Madison Chemicals 1996; IUPAC 2003).

As mentioned previously elemental iron powder exhibits a range of reactivity and bioavailability depending on its physical properties, such as particle size and microstructure (shape/density/porosity). Bioavailability can vary significantly with iron powders (RBV = 2 to greater than 80%). The elemental iron powders with a mean particle size < 50 μm are generally thought to have approximately ½ the bioavailability of ferrous sulfate (RBV = 100%) (Waddell 1974; Harrison and others 1976; Hallberg 1981; Patrick 1985; Forbes and others 1989; Beard and Dawson 1997; Wienk and others 1999; Nalubola and Nestel 2000; Dary and others 2002).

The chemical properties that determine the reactivity and bioavailability of iron phosphates are even less well understood. Although the volume of literature on the bioavailability of ferric phosphates is much less extensive, RBV in animal and human studies also vary widely with literature values from 6 to 100%. Because less is known about the factors influencing the bioavailability of ferric orthophosphate, the nutritional quality is thought to be unpredictable and considered by many experts to be poor (Hurrell 1985; Forbes and others 1989; Hallberg and others 1989; Beard and Dawson 1997; Willis and Allen 1999; Nalubola and Nestel 2000; SUSTAIN Task Force 2001; Dary and others 2002; Moretti D and others 2006).

Despite its uncertain nutritional quality, ferric orthophosphate is often the only choice for beverages, light colored and oxidatively sensitive applications. The use of ferric orthophosphate in RTE cereal applications has had mixed success (Kellogg 2006). Certain RTE cereal applications require exposure to high temperatures and pressures during prolonged cooking, tempering, drying and toasting processes. Product quality

problems include off-colors, off-flavors and oxidative instability in finished products. Unpredictable quality and bioavailability have limited the use of this fortificant.

### *1.7.3 Bioavailability of Iron Forms used in RTE Cereal*

Ferrous sulfate is the highly soluble iron form used as a relative standard in methods to assess bioavailability of iron sources in both animals and humans (Forbes and others 1989; Wienk and others 1999). Ferrous fumarate also is considered to have good bioavailability as are the chelated iron forms, ferric EDTA and bisglycinate. The reported bioavailability of ferric EDTA is 1 to 1.5 times that of ferrous sulfate but the cost of this ingredient (35 times the cost of hydrogen reduced iron) has limited the use of this fortificant. Ferric bisglycinate is another form of chelated iron that has excellent bioavailability, however it is expensive and inherently less stable than elemental iron due to the reactivity of its amino acid component in certain applications where browning reactions are likely to occur (Hurrell 1997; Nalubola and Nestel 2000; Kellogg 2006).

Due to its manufacturing process, electrolytic iron is > 98% pure and is less variable batch to batch than other elemental iron forms. When its mean particle size is < 20 μm, electrolytic iron has been shown to have a RBV > 70%. However, it is approximately three times more expensive than reduced iron and so less often used. Ferric orthophosphate is referenced to have poor bioavailability according to Nalubola and Nestel (2000), Forbes and others (1989) and Harrison (1976), averaging 30% the bioavailability of ferrous sulfate. These authors recommend that three times the amount of ferric orthophosphate should be added to compensate for its lower bioavailability but state that its bioavailability is less variable than some elemental forms of iron Typical

RBVs in animal and human studies for electrolytic iron and ferric orthophosphate fortificants are shown in Table 1.4. Table 1.5 gives the RBVs for different particle size fractions of elemental iron as summarized by Hurrell and others (2002c).

**Table 1.4 Literature percent Relative Bioavailability[1] Values (%RBV) for human, rat and *in vitro* studies [2,3]**

| Model and Method Used | Relative Bioavailability Values (RBV) | | | |
|---|---|---|---|---|
| | Electrolytic Iron | | Ferric Orthophosphate | |
| | $^{55}$Fe | Fe | $^{55}FePO_4$ | $FePO_4$ |
| Human<br>Double isotope, extrinsic tag | 0.75 | | 0.25 | |
| Rat<br>Hemoglobin depletion-repletion assay | | | | |
| AOAC | | 0.66 | | 0.25 |
| AOAC | | 0.77 | | 0.33 |
| HRE (35 mg Fe/kg diet) | | 0.78 | | 0.58 |
| HRE (20mg/kg diet) | 0.80 | 0.86 | 0.60 | 0.61 |
| | .48 | | .26 | |
| Rat | .69 | | .26 | |
| Double isotope, extrinsic tag | .47 | | .22 | |
| In Vitro | | .75 | | .04 |
| Solubility | | .60 | | .03 |
| | | .75 | | .28 |
| In Vitro | | .58 | | .27 |
| Dialysis | | .71 | | .46 |

[1]Relative Bioavailability = test Fe sample/$FeSO_4$
[2] Data are given from four laboratories participating in collaborative study
[3] Adapted from Forbes, and others 1989

**Table 1.5 Influence of particle size on the bioavailability of elemental iron powders [1]**

| Production Method | Particle Size (μm) | RBV |
|---|---|---|
| Electrolytic 1 | 7-10 | 64 |
| | 27-40 | 38 |
| Electrolytic 2 | 0-10 | 76 |
| | 10-20 | 75 |
| | 20-40 | 48 |
| | >40 | 45 |
| H-reduced | 10-20 | 54 |
| | >40 | 34 |
| CO-reduced | 6-10 | 36 |
| | 14-19 | 21 |
| | 27-40 | 13 |
| Carbonyl | <4 | 69 |
| | 4-8 | 64 |

[1] Adapted from Hurrell and others (2002c)

## 1.8 PHYSICOCHEMICAL PROPERTIES AND THEIR MEASUREMENT

The physical attributes of iron sources used in food fortification, such particle, porosity size and surface area, influence their solubility during digestion and ultimately determine how well an iron source is available for absorption. There are many techniques available to measure the physical properties of iron sources. However, careful consideration needs to be given to choose the correct method for the material in question. In the case of iron fortificants, methods need to be cost effective, relatively fast and reproducible as well as accurate.

### *1.8.1 Particle size analysis*

Particle size is a very important physical property that influences the solubility, reactivity and organoleptic properties of elemental iron fortificants and is considered

important for iron phosphate powders as well (Oetker 1992). It is often used as a quality criterion for various iron fortificants (Budenheim 1995; Wienk and others 1999; SUSTAIN 2001; Hurrell and others 2002). Controlling particle size will ensure that particles do not exceed the limit for desired appearance nor impart undesirable organoleptic attributes (dark specs, grainy mouth feel and metallic taste). Iron is a reactive substance and particle size is a major determinant of the surface area available for chemical reactions, and in particular, dissolution in a solvent such as water or dilute hydrochloric acid. Particle size measurements have been found to be potential predictors of relative bioavailability for reduced iron powders (Forbes and others 1989; Swain and others 2003). However, there is limited information in the literature about the affect of particle size on the solubility of ferric orthophosphate powders (Hallberg 1981; Swain and others 2003). Supplier information suggests that a similar relationship between particle size and solubility exists (Budenheim 1995).

There are several techniques used to measure particle size distribution, with the most common being microscopy, image analysis, sedimentation, sieving, electrozone sensing (Coulter Counter), and laser diffraction. Several factors should be taken into consideration when choosing a technique: the size range of the test material (minimum and maximum), precision required, sample size and number of particles measured, analysis time, and the media of choice for optimum sample dispersion (air or liquid, aqueous or organic, electrolytic). It is also important to consider whether the technique requires standards for calibration and whether knowledge of other properties of the material are required, such as refractive index and density. Each type of analysis produces a single value that describes mean particle size using the theory of equivalent

spheres (Rawle 2003). Low angle laser light diffraction has the capability to measure the needed particle size distribution range (0.1-100) for iron sources and can analyze iron powders in the organic dispersants needed to prevent agglomeration. Obtaining a representative sample is always challenging and laser light scattering allows the analysis of the entire sample within minutes so many samples can be practically tested. The measurement does not require standards and is not temperature (viscosity) dependent.

Light scattering measures the volume of a "sphere" of particles passing by a series of detectors set at different angles using the volume moment mean calculation (Malvern 2000; 2003). The volume mean is equivalent to the mass mean, which is also equivalent to a weight mean if the density of the sample remains constant. The volume property tells you where the mass of the distribution lies as opposed to a number mean that gives you a distribution based on the number of particles. These two aspects of a size distribution each give valuable information and light scattering allows data collected as the volume mean to be transformed to a number mean (using the Hatch-Choate equations) with an acceptable increase in experimental error. This has the advantage of providing reliable volume and number particle size distributions with one measurement (Malvern 2003). In addition to particle size, the particle shape and porosity [2] of a material contribute to the amount of available surface area (Lowell and Shields 1991). For this reason, surface area measurements may provide more comprehensive information if particle structure is complex.

[2] Porosity is defined as the surface flaws, which are deeper than they are wide Lowell S, Shields JE. 1991. POWDER: Surface Area and Porosity. Third ed. New York: Wiley and Sons Inc. 250 p..

### *1.8.2 Surface area analysis*

Surface area estimates can be calculated from particle size measurements. However, these values will at best establish the lower surface area limit because the fundamental assumption of equivalent spheres does not take into account the highly irregular nature (shape and porosity) of real surfaces (Lowell and Shields 1991). Most surfaces are not smooth and the irregularities can exist in any number of topographies, such as hills, valleys and surface fissures. Holes, or pores, in the surface of a particle may be shallow indentations or extend deep into the particle giving it a sponge like appearance that creates a high surface area due to the additional exposed surface.

Because of the limitations of particle size methods for surface area measurement, gas sorption techniques are used. Gas sorption probes surface irregularities by enveloping a powder sample in thin layer of physically adsorbed gas molecules, called the adsorbate. This technique is based on the tendency of solid surfaces to physically attract gas molecules (physisorption). Surface area measurements rely on kinetic theories that predict the number of molecules required to cover the surface of a solid with a monolayer of a gas. However, a complete monolayer surface is never actually formed due to different potential energies at different sites on a solid.

In order to overcome this, the most widely used procedure for determining surface area, the Brunauer, Emmett and Teller (B.E.T) theory, enables an experimental prediction of the number of molecules required to form a theoretical monolayer. The B.E.T. theory assumes that a dynamic equilibrium exists between the first adsorbed layer and upper vapor layers and so that the actual location of the surface sites covered with one, two or more layers might vary but the number of molecules in a layer remains constant and can

be estimated (Lowell and Shields 1991; Quantachrome 1997). The BET theory requires the use of the B.E.T equation.[4]

A linear plot of the B.E.T equation terms $1/W((P_0/P)-1))$ versus the relative pressure $P/P_0$, is required. The linear range of the adsorption isotherm for nitrogen is restricted to a narrow range of relative pressure (0.05 – 0.30) for most solids. The equations for the slope and the y-intercept are combined and the estimated weight of a monolayer of adsorbed nitrogen is determined by solving for $W_m$.[5] In the B.E.T equation, C is the B.E.T constant and represents the magnitude of the interaction between the adsorbate and the solid surface. Nitrogen is the most widely used adsorbate because of its intermediate C constant values of 50-250 for most surfaces. This acceptable range of B.E.T constants for nitrogen makes it possible to calculate its cross-sectional area, which is needed for the final surface area calculation (Lowell and Shields 1991; Quantachrome, 1997).

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[4] **BET equation = $(1/W((Po/P)-1) = (1/W_mC) + (C-1/W_m C)(P/Po)$**

**Where, at 77.3° K using nitrogen:**

**W = weight of nitrogen gas molecules adsorbed at relative pressure $P/P_0$**
**$W_m$ = weight of nitrogen gas molecules adsorbed in estimated monolayer**
**P = equilibrium vapor pressure, in torr**
**Po = saturated vapor pressure, in torr**
**C = BET constant = (s/i) = 1**
**s = slope of the BET plot, $1/W[(Po/P) - 1)]$ versus (P/Po)**
**i = intercept of BET plot**

[5] **$Wm = [(P/Po) - 1][(1/C) + ((C-1)/C)(P/Po)] = 1/(s+I)$**

Multiplying the weight of the gas molecules, $W_m$, times the adsorbate cross-sectional area times Avogadro's number and then dividing this quantity by the molecular weight of the adsorbed gas gives the total surface area of the sample.[6] The specific surface area is obtained by dividing this number by the weight of the sample. The BET theory continues to be the most universally applied surface area measurement because of its simplicity and accuracy (Lowell and Shields 1991).

Surface area can be determined using either a multipoint or a single point BET method. One data point alone is sufficient to calculate surface area and is called the single point BET method. This simplified approach is rapid and less expensive and often results in little loss of accuracy (Lowell and Shields 1991). Multipoint methods require a minimum of three data points for surface area measurements or as many as 40 data points for in depth analysis of adsorption and desorption isotherms. Isotherms provide additional information on the shape of pores and the pore size distribution.

### *1.8.3 Microstructure and moisture absorption*

The physical state, or phase, of a food substance is an important characteristic that influences its behavior over time and under different conditions, such as during processing. There are three basic physical states: solid, liquid and gaseous. Most food materials exist in either a solid or liquid state and in almost any combination of the two.

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**[6] Surface area = $W_m N A_{cs}/M$**

**Where:**
**$W_m$ = weight of the adsorbed nitrogen gas molecules**
**N = Avogadro's Number = $6.023X10^{23}$ molecules/mol**
**$A_{cs}$ = cross-sectional area for nitrogen gas at 77.3° K = 16.2 Å$^2$**
**M = molecular weight of nitrogen**

The physical state of a solid is extremely sensitive to the presence of water, temperature and pressure. Chemically pure compounds in foods, such as water and many organic and inorganic compounds have exact temperatures where they transition between phases, called phase transition temperatures. Solids may exist in molecularly ordered structures described as crystalline or disorganized structures referred to as amorphous, or a combination of both. Certain substances crystallize as hydrates and the presence of hydration water affects phase transition temperatures, for instance, the temperature at which the crystals melt. Materials that are at equilibrium do not change their physical state over time and remain stable at a given temperature, pressure and water content. A change in conditions, such as a temperature or humidity, may introduce a driving force (non-equilibrium) and a change of phase results (Bhadeshia 2002).

Microscopic techniques are extremely useful in studying the physical states of food materials, especially crystalline morphology. The crystalline state is the most ordered state of molecular arrangement (Roos 1995). Transmission light microscopy using polarized light if often used to explore crystal structure. Crystals display a phenomenon called birefringence, also called anisotropy, when they are exposed to two different refractive indices of plane-polarized light from two directions. A sequence of interference colors will appear depending on the thickness and morphology of the crystal structure. The colors yield information about the degree of crystallization and type of crystal present. Ferric phosphate salts are in a class of molecules termed "rigid molecules" and represent a large group of substances consisting of aggregates held together by strong molecular forces, e.g. covalent, ionic, and polar covalent bonds. This class is made up of metals, oxides, salts, ceramics, silicate glasses and also rigid solids

that have a strongly associated, ordered network, such as diamonds. In order to melt this class of molecules (phase change from solid to liquid) strong bonds must break and this requires sufficient heat and pressure and/or the presence of hydration water. The melted version of crystals form an unorganized association of aggregates termed amorphous. Birefringence is lost as crystals become amorphous (Wunderlich 1990; Roos 1995; Genck and Bayard 1997).

Amorphous content refers to regions of molecular disorder within a semi-crystalline solid that is formed during crystallization or typically during subsequent processing, like milling and spray-drying; it is seldom a deliberate or controlled event. Process-induced amorphous content is thought to be mostly at the powder surface. A small amount (by weight) of amorphous surface structure can increase the surface area significantly (Buckton and Darcy 1999). Even relatively low levels of amorphous material (<10%) are known to influence stability and dissolution characteristics (Mackin and others 2002). Amorphous regions in crystalline material will absorb water to a greater degree than crystalline regions and are often thermodynamically unstable. These areas of molecular disorder and higher moisture content are subject to physical transitions and chemical degradations. If exposed to the changes in humidity and temperature, amorphous material may undergo spontaneous glass transition to its more stable crystalline state. The presence of amorphous ferric orthophosphate may have important mechanistic properties that determine its bioavailability and stability in food products (Buckton and Darcy 1999; Mackin and others 2002; Burnett and others 2006).

# CHAPTER 2

# COMPARISON OF THE RELATIVE BIOAVAILABILITY OF SIX TYPES OF FERRIC ORTHOPHOSPHATE POWDERS IN READY-TO-EAT CEREAL

## 2.1 INTRODUCTION

Anemia remains the most prevalent micronutrient deficiency in the world and iron deficiency anemia (IDA) accounts for a large percentage of the total problem. Approximately 600 – 800 million people are affected worldwide with women, infants, and young children the most at risk (INACG 2001; Turner 2002). In the majority of cases, the cause of IDA is diet related, yet despite diet-intervention strategies implemented in almost all countries, IDA remains endemic throughout the world and new strategies are needed to address the problem (Stoltzfus 2001b).

There are many forms of iron used for fortification; however, elemental iron, ferrous sulfate, ferric orthophosphate, ferric pyrophosphate and ferrous fumarate are the five forms used almost exclusively on a large scale. Elemental iron powders are the most commonly used iron fortificant because they are affordable, cause the least problems with food quality, and have good consumer acceptance. The different iron forms have different degrees of reactivity, which generally impair the organoleptic properties and shelf life of foods, thus limiting the iron forms used in foods to those that are less reactive. Unfortunately, the physical properties that make an iron source less reactive also are thought to make it less well absorbed. These challenges make the prevention of iron deficiency through food fortification difficult (SUSTAIN 2001; Fairweather-Tait and Teucher 2002; Hurrell and others 2002). Experts believe that food stability limitations

and the conflict or lack of literature information on bioavailability have hindered the successful implementation of cereal enrichment programs worldwide (Hurrell and others 2002; Lynch 2002; Turner 2002). In the U.S., the Special Supplemental Nutrition Program for Women, Infants and Children (WIC) is an initiative to safeguard the health of at-risk populations. WIC-eligible cereal products must contain 45% of the RDI for iron per serving or 8.1 mg (> 27 ppm) and delivery of this level while maintaining food quality is a technological challenge.

Ferric orthophosphate is an affordable iron source that has had limited use as an iron supplement in ready-to-eat cereal due its variable bioavailability. It is often preferred for beverages (due to its low density), light-colored foods (e.g. rice) and oxidatively sensitive food applications, such as RTE cereals for the WIC program. RBVs for ferric orthophosphate reportedly range from as low as 2% to greater that 80%. Due to this largely unexplained variability, nutritional experts consider ferric orthophosphate to be poor source for iron fortification (Shah and others 1977; Hurrell 1985; Forbes and others 1989; Hallberg and others 1989; Willis and Allen 1999; Nalubola and Nestel 2000; SUSTAIN Task Force 2001; Dary and others 2002). Interestingly, the bioavailability of elemental iron, which is the most widely used iron fortificant in food in the world, also varies significantly. RBVs for reduced iron range from less than 13% to 100% (Waddell 1974; Harrison and others 1976; Hallberg 1981; Patrick 1985; Forbes and others 1989; Wienk and others 1999; Nalubola and Nestel 2000; Dary and others 2002; Swain and others 2003; Beard 2006).

The official method of the Association of Official Analytical Chemists (AOAC) for determining bioavailability is the rat hemoglobin repletion bioassay/slope ratio

technique. This method provides a relative measure of bioavailability, called the Relative Biological Value[2](RBV). The physiochemical properties of elemental iron vary according to its method of manufacture and evidence indicates that physiochemical properties affect bioavailability (Swain and others 2003). However, few bioavailability studies on reduced iron and even fewer on ferric orthophosphate have addressed the physiochemical basis for the inherent variability of these materials. In 2002, an expert panel concluded that incomplete and inconsistent information exists on the bioavailability and physiochemistry of elemental iron powders used by the food industry (Swain and others 2003). Limited evidence in the literature indicates that this is also true of ferric orthophosphate powders. Better understanding of the mechanism underlying the variable bioavailability of the iron sources would provide more consistent and potentially improved nutritional value for consumers.

The objective of this study was to compare the bioavailabilities of a variety of sources of commercially available food grade ferric orthophosphate powders with other sources of iron commonly used in food products. The ferric orthophosphate powders were chosen to cover a range of powder types, differing in color, particle size distribution and solubility in dilute hydrochloric acid. The study included, as references, ferrous sulfate which has an assigned RBV of 100, hydrogen-reduced elemental iron, and encapsulated hydrogen-reduced elemental iron. The relative bioavailability of the iron powders was determined using the AOAC Rat Hemoglobin Repletion Bioassay/slope ratio method. A modification of the AOAC method, the Rat Hemoglobin Regeneration

[2]The Relative Bioavailability Value is determined using animal and human test methods. The most commonly used techniques are the AOAC Rat Hemoglobin Repletion Bioassay/Slope Ratio and Human Double Isotope/Extrinsic Tag methods. The bioavailability of the test iron source is compared to ferrous sulfate, a highly bioavailable standard iron fortificant with an assigned RBV of 100%.

Efficiency (HRE) Bioassay, was compared to the AOAC official method to account for diet consumption differences between animals.

## 2.2 MATERIALS AND METHODS

### *2.2.1 Materials*

The bioavailabilities of nine iron sources were evaluated in this study (Table 2.1): ferrous sulfate (Source 1), encapsulated hydrogen-reduced iron powder (Source 2), hydrogen-reduced iron powder (Source 3), and six different types of ferric orthophosphate powder (Sources 4-9). Ferric orthophosphate (FePO4·x H2O) is an odorless, yellow to buff to pinkish white powder composed of one Fe (III) atom complexed to from one to three phosphate groups and hydrated with one to four molecules of water. The iron content typically ranges from 26- 32%. All iron sources were commercially produced, food grade materials meeting Food Chemical Codex (FCC) requirements for ferrous sulfate, hydrogen-reduced iron and ferric orthophosphate. Source 1 (ferrous sulfate ($FeSO_4 \bullet 7H_2O$) was purchased from Mallinckrodt Baker, Inc. Phillipsburg, NJ, and is the AOAC Rat Hemoglobin Repletion Bioassay reference standard against which the bioavailabilities of the other sources were compared. Source 2 (encapsulated, hydrogen-reduced iron powder) was supplied by International Flavors and Fragrances, Inc. (IFF) (New York, NY) and is used to fortify oxidatively sensitive products. Source 3 (hydrogen-reduced iron powder) was from the same source of reduced iron powder used to produce the encapsulated iron powder (e.g. Source 2). The six types of ferric orthophosphate powder were > 99% pure and had iron contents ranging

from 24.6 – 30.4 % by analysis, depending on waters of hydration and phosphate content. Sources 4 -7 were supplied by Budenheim Chemische Fabrik (Mainz, Germany). Source 8 was purchased from Madison Chemicals, Inc. (Madison Township, NJ). Source 9 was obtained from Wright Enrichment, Inc. (Crawley, LA). Iron sources were stored in closed containers under ambient conditions.

**TABLE 2.1**

**Identity of test iron sources for the hemoglobin repletion diets**

| Diet | Iron Source |
|---|---|
| Source 1 | Ferrous sulfate, heptahydrate (Standard) |
| Source 2 | Encapsulated, hydrogen-reduced iron, IFF |
| Source 3 | Hydrogen-reduced iron powder, IFF |
| Source 4 | Ferric orthophosphate, Budenheim Chemische 53-80 |
| Source 5 | Ferric orthophosphate, Budenheim Chemische 53-81 |
| Source 6 | Ferric orthophosphate, Budenheim Chemische 53-82 |
| Source 7 | Ferric orthophosphate, Budenheim Chemische 53-85 |
| Source 8 | Ferric orthophosphate, Madison Chemicals |
| Source 9 | Ferric orthophosphate, Wright Enrichment |

### *2.2.2 Methods*

Bioavailability was measured using the AOAC Rat Hemoglobin Repletion Bioassay (AOAC 1990). The animal study was conducted at Covance Laboratories Inc. in Madison, Wisconsin.

#### *2.2.2.1 Depletion Diet*

The composition of the low-iron animal diet supplied by Harlan Teklad (Madison, Wisconsin) is given in Table 2.2. The animals also received ad-libitum a deionized water

source that was analyzed for iron content at the start and periodically throughout the study.

**TABLE 2.2**
**Composition of iron depletion diet [1]**

| Ingredient | Amount (g/kg) |
|---|---|
| Casein, high protein | 200.0 |
| DL-methionine | 3.0 |
| Corn starch | 150.0 |
| Sucrose | 554.3 |
| Corn oil | 50.0 |
| Mineral mix (TD 81062) | 35.0 |
| Vitamin mix, AIN-76A (TD 40077) | 10.0 |
| Choline bitartrate | 2.0 |
| Ethoxyquin (antioxidant) | 0.01 |

[1] Diet TD 0396 purchased from Harlan Teklad, Madison, WI

*2.2.2.2 Repletion Diets*

All iron sources were added to a flaked, corn-based RTE cereal product before the pressurized cooking step at the point during processing when reduced iron powder is typically added. The cereal was manufactured in approximately 90-kg batches (Kellogg Company, Battle Creek, Michigan) and was used as the vehicle to deliver the iron source to the test animals during the hemoglobin repletion study. The cereal was made from whole corn grits, malted barley flavor, high fructose corn syrup, sugar, salt and the following added vitamins per 30 g cereal: A (500 IU), C (6 mg), D (40 IU), B-12 (1.5 μg), B-1 (0.4 mg), B-2 (0.4 mg), B-6 (0.5 mg), niacin (5 mg), and folate (0.1 mg). Nine cereal batches were formulated to each contain one of the nine iron sources at 8 mg iron/30 g cereal. A tenth batch of cereal was made with no added iron and was considered a low-

iron cereal due to the presence of approximately 4 mg of naturally occurring iron coming from the malt flavoring.

The iron repletion diets were formulated to contain 60% iron-free rat basal diet and 40% cereal (Table 2.3). Iron-fortified cereal and the low-iron (no added iron) cereal were blended to contain 0, 6, 12, 24 or 48 mg iron per kg repletion diet. The iron content of each resultant test diet was measured at Covance Laboratory using an inductively coupled plasma (ICP) method (AOAC International 2000) (Table 2.4).

**TABLE 2.3**
**Composition of iron repletion diet [1]**

| **Ingredients** | **Amount (g/kg)** |
|---|---|
| **60% Iron Free Basal Rat Diet** | |
| Casein, high protein | 333.3 |
| DL-methionine | 5.0 |
| Sucrose | 499.9 |
| Corn oil | 83.3 |
| Mineral mix, iron deficient as per depletion diet | 58.3 |
| Vitamin mix, as per depletion diet | 16.7 |
| Choline bitartrate | 3.3 |
| Ethoxyquin (antioxidant) | 0.017 |
| **40% Test Cereal** | |
| Fortified cereal | 0, 6, 12, 24 or 48 mg supplemental iron/kg repletion diet |
| Unfortified cereal | To equal 40% of the diet |

[1] Diet was prepared in 1 kg batches to contain 60% (600 g) Iron-Free Diet and 40% (400 g) mixture of fortified and unfortified cereal to equal target iron doses

**TABLE 2.4 Iron Content of Diets, by Analysis [1] (mg/kg Diet)**

| | Target Iron Dose Levels (mg) | | | |
|---|---|---|---|---|
| **Diet (Iron Source)** | **0** | **6** | **12** | **24** |
| **Diet 1 (Source 1, Standard)[2]** | 4 ± 0.04 | 10 ±1 | 16 ± 2 | 25 ± 1 |
| **Diet 2** | 4 | 11 | 17 | 30 |
| **Diet 3** | 4 | 11 | 15 | 27 |
| **Diet 4** | 4 | 11 | 17 | 27 |
| **Diet 5** | 4 | 11 | 16 | 28 |
| **Diet 6** | 4 | 11 | 16 | 29 |
| **Diet 7** | 4 | 10 | 16 | 28 |
| **Diet 8** | 4 | 10 | 14 | 25 |
| **Diet 9** | 4 | 10 | 16 | 27 |

[1]Cereal contains approximately 4 mg of naturally occurring iron from malted barley syrup.
[2] Diet 1 had the following number of replicate analyses per dose level: 2 (dose 0), 4 (dose 6 and 12) or 5 (dose 24); Diets 2-9 were analyzed singly.

*2.2.2.3 Animals*

Male Hsd: Sprague Dawley® SD® rats were procured at 21-days of age from Harlan Sprague Dawley, Inc., Madison, Wisconsin. They were single-housed in wire-bottomed, stainless steel cages under a 12-hour light/12-hour dark cycle. Upon arrival, the rats were immediately fed *ad libitum* the depletion diet for 15 days. Blood was collected from the jugular vein at the end of the depletion period and analyzed for hemoglobin concentration. The animals were then randomly assigned to test diet groups at 36 days of age with 10 animals per group. The repletion diets were fed *ad libitum* during a 2-week repletion period. At the end of the repletion period, the animals were bled and the final hemoglobin levels determined. The animals were fed their respective diets from clear glass jars to allow easy inspection on the amount and condition of the food. Fresh food was offered on day 1 and day 8. Individual animal body weight data were recorded on days 1, 8 and 15 and individual food consumption data were recorded weekly during the test period.

## 2.3 STATISTICAL ANALYSIS

### *2.3.1 Hemoglobin Repletion Bioassay/Slope Ratio Modeling*

Linearity of the regression curves was determined for each iron source separately and a multiple regression model was used to determine the slopes of the nine iron sources compared to a no-iron-added control diet. A model-based Residual Variance Method with an AIC Fit Statistic was used to determine if the y-intercepts were simultaneously equal (response at dose 0) to the reference Diet 9 and a contrast test was performed to see which diets were significantly different in terms of the change in hemoglobin repletion values. The following equation (Forbes and others 1989) was used to calculate the relative per cent biological values (RBV)[3] of the diets:

$$\% \text{ RBV} = \frac{[\text{Slope of Test Diets}_{(2\text{-}9)}]}{\text{Slope of Standard Diet}} \text{ X } 100$$

The equation used for the standard error was:

$$\frac{1}{q}\sqrt{SE_q^2 + R^2 SE_p^2},$$

where $q$ is the slope of the standard diet, and $p$ is the slope of the test diet. The standard errors of p and $q$ are shown as $SE_p$ and $SE_q$. The relative bioavailability is $R$ and $R = p/q$, calculated using the SAS output.

---

[3] Where,
Slope of Standard Diet 1 (Ferrous Sulfate) = 0.62
Slope Change Due to Test Diet Iron Treatments 2-9 = -0.35, -0.35, -0.01, -0.14, -0.30, -0.10,-0.25,-0.19, respectively

### *2.3.2 Hemoglobin Regeneration Efficiency*

The hemoglobin regeneration efficiency (HRE) ratio is determined by taking body weight and food consumption into consideration in the following formula (Forbes and others 1989):

$$HRE = \frac{[\text{HGB-Fe (mg)}_{final} - \text{HGB-Fe (mg)}_{initial}]}{\text{Total Iron Consumed (mg)}} \times 100$$

Where,

$$\text{HGB-Fe (mg)} = \text{Body Weight Test Animal (kg)} \times \left[\frac{0.075\ \text{Blood (L)}}{\text{Body Weight (kg)}} \times \frac{3.35\ \text{Fe (mg)}}{\text{HGB (g)}} \times \frac{\text{HGB (g)}}{\text{Blood (L)}}\right]$$

HGB = hemoglobin
HGB-Fe = hemoglobin iron

## 2.4 RESULTS

The objective of this research was to compare bioavailabilities of different sources of ferric orthophosphate that had different physiochemical properties to other sources of iron fortificants (reduced iron and ferrous sulfate). A RTE flaked-corn cereal was fortified with different iron sources and added to a standard rat diet in varying amounts to achieve the desired fortification levels. The differing amounts of cereal in the rat diet and the food quality differences due to the physiochemical properties of the different ferric orthophosphate types had the potential to influence animal acceptance of the diet. The HRE model and AOAC standard repletion method were compared to account for any differences in animal diet consumption.

Hemoglobin repletion study data (target iron dose, analyzed iron content, animal weight, food intake, hemoglobin gain) and RBV results are summarized in Table 2.5. Initial hemoglobin values averaged 5.0 ± 0.51 g/dl. Hemoglobin Regeneration Efficiency and RBV results are compared in Table 2.6. RBVs are graphed in Figure 2.1. The unfortified cereal had approximately 4 mg endogenous iron from the malt flavoring (AOAC International 2000). Therefore, 4 mg of iron was subtracted from the analyzed iron content of all diets. A linear lack-of-fit test was performed on the Diets 1-9 (SAS model-based, Fixed Effects Standard Error Method) comparing the iron content of the diet versus the change in hemoglobin at the end of the repletion study. Significant lack-of-fit occurred at $p \leq 0.05$ when the highest test dose (e.g. 48 mg iron/kg diet) was included in the regression analysis. When the highest iron dose was excluded, linearity was achieved. As a result, statistical analysis was performed on the data minus the highest dose and the no-iron- added dose level was included to increase the statistical power of the test.

After adjusting for endogenous iron, the SAS Residual Variance analysis of the iron content versus the change in hemoglobin found the y-intercepts of the diets to be simultaneously equal. RBVs were found to be significantly different for all diets except diets 2 and 3 (encapsulated and non-encapsulated reduced iron powders, respectively); Diets 1 and 4 (standard ferrous sulfate and Budenheim ferric orthophosphate powder 53-80, respectively); Diets 5 and 7 (Budenheim ferric orthophosphate powders 53-81 and 53-85, respectively). RBVs for the ferric orthophosphate powders ranged from 51 to 100. Reduced iron and encapsulated reduced iron powders both had an RBV of 43, thus indicating that encapsulation had little effect on bioavailability. Ferrous sulfate was

established as the standard with an RBV of 100; it was not statistically different from ferric orthophosphate Source 4 (RBV 99).

Comparison of the RBV and HRE values (Table 2.6) indicated that the two techniques gave similar results; however, the RBV method was able to detect more differences between the diets and there was more overlap in the HRE data. Diets 1, 4, 5 and 7 had the highest HRE values and were not statistically different from each other; Diets 2, 3, 6 and 8 had the lowest HRE values and also were not statistically different from each other.

**Table 2.5. Hemoglobin repletion study data and Relative Bioavailability Value (RBV) results**

| Diet[1] (Iron Source) and Animal Data[2] | Targeted Dose[3] (mg/kg diet) 0 | 6 | 12 | 24 | RBV±SD[4] |
|---|---|---|---|---|---|
| **Diet 1 (Source 1, standard)** | | | | | 100 [a] |
| Analyzed iron content [2] (mg/kg Diet) | 4 ± 0.04 | 10 ± 1 | 16 ± 2 | 25 ± 1 | |
| **Animal Data** | | | | | |
| Body Weight Gain (g) | 65 ± 9 | 85 ± 9 | 97 ± 9 | 102 ± 7 | |
| Food Intake (mg) | 165 ± 11 | 191 ±15 | 210 ± 21 | 224 ± 13 | |
| Hemoglobin gain (g/l) | -21 ± 5 | -2 ± 9 | 22 ± 11 | 44 ± 11 | |
| **Diet 2 (Source 2, encap red iron)** | | | | | 43±5 [d] |
| Analyzed iron content (mg/kg Diet) | 4 | 11 | 17 | 30 | |
| **Animal Data** | | | | | |
| Body Weight (g) | 65 ± 9 | 86 ± 8 | 95 ± 5 | 103 ± 7 | |
| Food Intake (mg) | 172 ± 10 | 174 ± 15 | 193 ± 17 | 222 ± 12 | |
| Hemoglobin gain (g/l) | -21 ± 5 | -11 ± 4 | -2 ± 6 | 15 ± 6 | |
| **Diet 3 (Source 3, red iron)** | | | | | 43±5 [d] |
| Analyzed iron content (mg/kg Diet) | 4 | 11 | 15 | 27 | |
| **Animal Data** | | | | | |
| Body Weight (g) | 65 ± 9 | 74 ± 6 | 79 ± 8 | 92 ± 6 | |
| Food Intake (mg) | 172 ± 10 | 174 ± 15 | 193 ± 17 | 222 ± 12 | |
| Hemoglobin gain (g/l) | -21± 5 | -13 ± 4 | -6 ± 5 | 11 ± 5 | |
| **Diet 4 (Source 4)** | | | | | 99±9 [a] |
| Analyzed iron content (mg/kg Diet) | 4 | 11 | 17 | 27 | |
| **Animal Data** | | | | | |
| Body Weight (g) | 65 ± 9 | 87 ± 7 | 94 ± 7 | 100 ± 7 | |
| Food Intake (mg) | 172 ± 10 | 194 ± 14 | 220 ± 30 | 225 ± 13 | |
| Hemoglobin gain (g/l) | -21 ± 5 | -3 ± 6 | 16 ± 6 | 53 ± 6 | |
| **Diet 5 (Source 5)** | | | | | 78±7 [ab] |
| Analyzed iron content (mg/kg Diet) | 4 | 11 | 16 | 28 | |
| **Animal Data** | | | | | |
| Body Weight (g) | 65 ± 9 | 79 ± 5 | 91± 6 | 97 ± 6 | |
| Food Intake (mg) | 172 ± 10 | 197 ± 12 | 203 ± 7 | 212 ± 13 | |
| Hemoglobin gain (g/l) | -21 ± 5 | -5 ± 3 | 6 ± 5 | 40 ± 6 | |

[1] Mean ±SD of 10 animals per study group per Source tested

[2] Diet 1 was analyzed in 2 (dose 0), 4 (dose 6 and 12) or 5 (dose 24) replicates; Diets 2-9 were analyzed singly.

[3] Cereal contains approximately 4 mg of naturally occurring iron from malted barley syrup.

[4] Means with different letters are statistically different (95% CL).

**Table 2.5 (continued)**
**Hemoglobin repletion study data and Relative Bioavailability Value (RBV) results**

| Diet[1] (Iron Source) and Animal Data[2] | Targeted Dose[3] (mg/kg diet) 0 | 6 | 12 | 24 | %RBV±SD[4] |
|---|---|---|---|---|---|
| **Diet 6 (Source 6)** | | | | | 51±5 [cd] |
| Analyzed iron content[2] (mg/kg Diet) | 4 | 11 | 16 | 29 | |
| Body Weight (g) | 65 ± 9 | 77 ± 4 | 84 ± 6 | 95 ± 7 | |
| Food Intake (mg) | 172 ± 10 | 184 ± 8 | 193 ± 6 | 211 ± 13 | |
| Hemoglobin gain (g/l) | -21± 5 | -10 ± 4 | -1 ± 6 | 22 ± 9 | |
| **Diet 7 (Source 7)** | | | | | 83±7 [ab] |
| Analyzed iron content (mg/kg Diet) | 4 | 10 | 16 | 28 | |
| Body Weight (g) | 65 ± 9 | 82 ± 6 | 93 ± 5 | 101 ± 7 | |
| Food Intake (mg) | 172 ± 10 | 182 ± 7 | 207 ± 12 | 223 ± 18 | |
| Hemoglobin gain (g/l) | -21± 5 | -5 ± 4 | 9 ± 7 | 40 ± 5 | |
| **Diet 8 (Source 8)** | | | | | 60 ± 6 [bcd] |
| Analyzed iron content (mg/kg Diet) | 4 | 10 | 14 | 25 | |
| Body Weight (g) | 65 ± 9 | 74± 6 | 81 ± 9 | 93 ± 9 | |
| Food Intake (mg) | 172 ± 10 | 180 ± 15 | 186 ± 9 | 202 ± 8 | |
| Hemoglobin gain (g/l) | -21± 5 | -10 ± 3 | -3 ± 5 | 20 ± 6 | |
| **Diet 9 (Source 9)** | | | | | 72 ± 7 [abc] |
| Analyzed iron content (mg/kg Diet) | 4 | 10 | 16 | 27 | |
| Body Weight (g) | 65 ± 9 | 82 ± 2 | 84 ± 3 | 98 ± 6 | |
| Food Intake (mg) | 172 ± 10 | 194 ± 10 | 199 ± 12 | 226 ± 13 | |
| Hemoglobin gain (g/l) | -21± 5 | -8 ± 5 | 2.5 ± 3 | 34 ± 6 | |

[1] Mean ±SD of 10 animals per study group per Source tested
[2] Diet 1 was analyzed in 2 (dose 0), 4 (dose 6 and 12) or 5 (dose 24) replicates; Diets 2-9 were analyzed singly.
[3] Cereal contains approximately 4 mg of naturally occurring iron from malted barley syrup.
[4] Means with different letters are statistically different (95% CL).

**Table 2.6 Mean[1] Hemoglobin Regeneration Efficiency (HRE) Ratios compared to Mean Relative Bioavailability Values (RBV)[2]**

| Diet (Iron Source) | % RBV ± SD | HRE ± SD |
|---|---|---|
| Diet 1 (Source 1, standard) | 100 [a] | 100 [a] |
| Diet 2 (Source 2, encapsulated reduced iron) | 43 ± 5 [f] | 45 ± 3 [de] |
| Diet 3 (Source 3, reduced iron) | 43 ± 5 [f] | 35 ± 3 [e] |
| Diet 4 (Source 4, ferric orthophosphate) | 99 ± 9 [a] | 93 ± 5 [a] |
| Diet 5 (Source 5, ferric orthophosphate) | 78 ± 7 [b] | 76 ± 4 [ab] |
| Diet 6 (Source 6, ferric orthophosphate) | 51 ± 5 [e] | 48 ± 8 [bcd] |
| Diet 7 (Source 7, ferric orthophosphate) | 83 ± 7 [b] | 82 ± 5 [ab] |
| Diet 8 (Source 8, ferric orthophosphate) | 60 ± 6 [d] | 55 ± 4 [cd] |
| Diet 9 (Source 9, ferric orthophosphate) | 72 ± 7 [c] | 65 ± 4 [bc] |

[1] Mean ±SD of 10 animals per study group per Source tested

[2] Means within a column with different letters are significantly different ($p<0.05$)

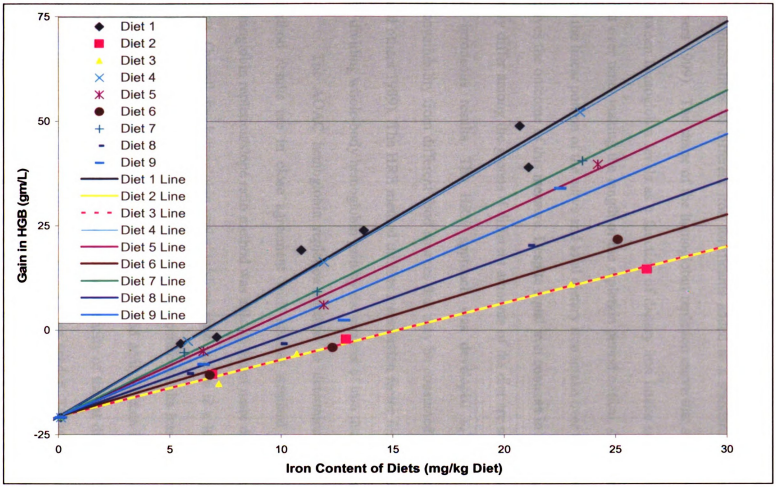


**Figure 2.1** Dose-response of hemoglobin repletion in rats using nine different sources of iron. 2.5

## DISCUSSION

### *2.5.1 Hemoglobin Regeneration Efficiency versus Hemoglobin Repletion*

The AOAC Rat Hemoglobin Repletion method was designed to compare the bioavailabilities of different iron fortificants added to a standardized diet (Wienk and others 1999). The shape of the hemoglobin repletion curve that shows that relationship between hemoglobin levels as a function of dietary iron intake is sigmoidal, becoming flat over time, making the length of the repletion period critical. Comparisons are made on the linear portion of the curve and total dietary iron intake over the course of the study drives the outcome of this measure. Despite equal iron contents in the diet, dietary intake may differ among the groups if different amounts of the diet are consumed, contributing to erroneous results. The HRE approach was developed to study intrinsic iron bioavailability from different food sources that may be consumed preferentially (Forbes and others 1989). The HRE method utilizes a correction factor for different iron intakes by dividing whole-body hemoglobin gain by the amount of iron that is consumed.

The AOAC hemoglobin repletion method to determine RBV and the HRE method results are in close agreement with similar standard errors. However, the hemoglobin repletion/slope ratio method was able to detect more differences between the diets. Overall, the hemoglobin repletion/slope ratio model is a less complex technique with fewer measurements and appears to give accurate results less influenced by animal and food intake measurement error. Variable food analysis data indicate that analytical error may have been introduced by non homogeneity of the rat diets and that improved sample blending and more replicate analyses were needed. Target levels of iron in the diets were 0, 6, 12, 24 and 48 mg/iron per kg diet (excluding 4 mg/kg endogenous iron).

After grouping the analytical results for all iron sources by target level, standard deviations were ± 0.78 (n=12), 1.2 (n=12), 2.0 (n=13) and 7.4 (n=13), respectively. The HRE takes into account animal growth and food consumption. However, the potential error in these measurements can outweigh the benefits of this added information.

In a similar study by Forbes and others (1989) for the International Nutritional Anemia Consultative Group ((INACG), the standard AOAC hemoglobin repletion/slope ratio method was compared with the HRE model for both ferric orthophosphate and electrolytic elemental iron. Both techniques were compared to results obtained by double isotope studies in humans. The AOAC Hemoglobin Repletion assay was run in two laboratories. RBVs of electrolytic iron were 66% (± 2%) and 78% (± 3%) and ferric orthophosphate were 25% (± 2%) and 34% (± 3%), respectively. The HRE assay performed in one laboratory showed values of 78% (± 4%) and 58% (± 3%) for electrolytic iron and ferric orthophosphate, respectively. When compared to ferrous sulfate in a farina-based meal fed to humans, the double isotope, intrinsic tag method found RBVs 75% for electrolytic iron and 25% for ferric orthophosphate. Forbes and others (1989) concluded that the standard AOAC hemoglobin repletion method served as a reliable predictor of iron bioavailability in man.

Forbes and others (1989) also looked at the effect of vitamin C on iron absorption from the farina meals in both humans and rats using the double isotope method. Ascorbic acid caused more than a threefold increase in the absorption of ferric orthophosphate. Slightly less of an increase was seen in electrolytic iron and ferrous sulfate. The authors hypothesized that ferric orthophosphate benefited more from the presence of vitamin C than the soluble ferrous sulfate and elemental iron because ascorbic acid, a reducing

agent, caused an increase in the Fe (II) concentration by reducing the Fe (III) content of ferric orthophosphate. However, the relative absorption of ferrous sulfate to ferric orthophosphate and electrolytic iron was the same. The authors concluded that the nature of the meal had little influence on bioavailability measurements in humans and so the theory of absorption of iron from a common non heme iron pool in the double-isotope study was viable.

In contrast, in a recent double-isotope study by Moretti and others (2006) that studied the effect of subject iron status, food matrices, processing and ascorbic acid on RBV. An experimental micronized form of ferric pyrophosphate was compared to ferrous sulfate in a wheat-milk infant cereal with and without ascorbic acid and in a processed and unprocessed rice meal. Ascorbic acid (molar ratio of 4:1 to iron) increased iron absorption more than twofold. As expected, iron status was a highly significant predictor of the RBV of ferric pyrophosphate ($P < 0.001$). RBV varied from 15 to 62% and the authors concluded that assigning a single RBV to a poorly soluble iron source, like ferric pyrophosphate, may be of limited value in evaluating their suitability for food fortification (Harrison and others 1976; Swain and others 2003; Moretti D and others 2006).

### *2.5.2 RBV of Iron Treatments*

The RBV of the iron sources used in this study were determined on reduced iron and ferric orthophosphate powders that were added to RTE cereal grains prior to processing. During manufacture, the iron sources were exposed to high pressure cooking at temperatures greater than 240 °F for longer than 60 minutes and then exposed to

forming, drying and toasting processes. The RTE cereal was fortified with vitamins and minerals including 10% DV of vitamin C, which meant the repletion diets used in this dissertation, including the standard ferrous sulfate diet, each contained 80 mg vitamin C per kg animal feed (Materials, Repletion Diets 2.2.2.2).

The powders were chosen based on visual differences, such as varying color and flow properties, which indicate physical property differences that may influence how they behave during food processing. Properties such as surface oxidation, particle size and surface area are known to influence the solubility and reactivity of elemental iron, causing oxidative instability in foods that affect taste, texture, appearance and shelf life. Factors that influence the behavior of ferric orthophosphate during processing include particle size, surface area and crystal structure. The same physiochemical properties that influence food quality during manufacture and over shelf life also influence the physiochemistry of the iron sources during digestion, which ultimately influences bioavailability. Results of this study show that all the ferric orthophosphate powders tested had bioavailabilities equal to or better than a typical reduced iron powder commonly used to fortify foods worldwide.

A potential mechanism explaining the physiochemical variability of ferric orthophosphate powders may be the presence of amorphous material, which occurs to varying degrees as a result of differing manufacturing practices. Amorphous materials are known to have higher solubility and dissolution rates and decreased chemical and physical stability as compared to their more crystalline counterparts (Williams, 2003). The presence of amorphous material may result in increased reactivity during cereal processing. Soluble iron is a prooxidant that catalyzes lipid oxidation and other

deleterious oxidative reactions such as the fading of natural colors and degradation of oxidatively sensitive vitamins. Conversely, it may also favorably influence bioavailability. Differences in the physiochemical properties of the ferric orthophosphate powders used in this study, such as particle size, crystal structure and amorphous content, are thought to have influenced the bioavailability outcome by altering solubility. Therefore, the next steps in this research project were to characterize the physiochemical properties of the powders and study their effect on the solubility of ferric orthophosphate in dilute hydrochloric acid solutions to better define the factors influencing bioavailability.

# CHAPTER 3

# PHYSICOCHEMICAL PROPERTIES OF FERRIC ORTHOPHOSPHATE INFLUENCING ITS SOLUBILITY IN DILUTE HCl

***Particle Size, Surface Area and Amorphous Content***

## 3.1 INTRODUCTION

Bioavailability of iron can be thought of as consisting of three components: first is the solubility of the iron source in the gut; second is how well the iron is absorbed and delivered to the circulation; and third is how well the iron is processed by the body once it reaches the circulation, target organs and cells for incorporation into measurable functional entities. Bioavailability must be *quantifiable* and the working definition of iron bioavailability is often relative as determined by the methods available to measure one of these three stages. Endpoint measures currently provide the best estimate of bioavailability when all three aspects of bioavailability, e.g. solubility, absorption and utilization, need to be considered. However, *in vitro* methods that give a good estimate of the bioavailability obtained from endpoint measures and that are economical and avoid the use of animals and people are desirable. Non-heme iron absorption depends on the solubilization of ferrous, ferric iron, or elemental from the acid milieu of the stomach. depending on the form of iron in the food (IOM 2001). Ferric iron must be reduced to its ferrous form by reducing agents such as ascorbic acid or ferrireductase enzyme present on the mucosal cell surface of the upper small intestine. This bioavailable iron is then absorbed in a 3-step process by the enterocytes, transported intracellularly and transferred into the plasma. It is well accepted that only soluble ferrous iron can be absorbed during digestion, and once solubilized and absorbed, ferrous iron joins a common intracellular

"iron pool" for use by the body (Miret and others 2003). Therefore, *in vitro* methods that measure the solubility of fortificants are promising for use as estimates of bioavailability and to gain an understanding of the properties that influence solubility.

Several researchers have demonstrated that the RBV of ferric orthophosphate is highly variable (Harrison and others 1976; Swain and others 2003; Moretti D and others 2006). The same is true of reduced iron, which is the most commonly used iron fortificant worldwide (Waddell 1974; Harrison and others 1976; Hallberg 1981; Patrick 1985; Forbes and others 1989; Beard and Dawson 1997; Wienk and others 1999; Nalubola and Nestel 2000; Dary and others 2002). The SUSTAIN group commissioned a review of the literature published over the past 45 years and concluded that little information was available on the precise types of iron used in the literature and that not enough is known about the extent to which different elemental iron powders (and other iron fortificants with low solubility) are absorbed by the body (Hurrell 2002a; Turner 2002).

The physical attributes of mineral sources used in food fortification, such as particle size and surface area, influence their solubility during digestion. Tests to measure solubility can be simple dissolution studies performed at various concentrations of hydrochloric acid, or more complex methods that simulate the digestive environment (Harrison and others 1976; Shah and others 1977; Forbes and others 1989; Swain and others 2003; Wortley and others 2005). Using the same procedure for a single type of iron, a given method will yield relative results that can be used to compare the solubility of different forms of the same material. However, even within the same laboratory, results have been variable (Forbes and others 1989; Swain and others 2003; Kellogg

2006; Moretti D and others 2006). Standardized methods to measure solubility and other physicochemical properties such as particle size and surface area for intra-laboratory collaborative studies are needed. For the affordable iron sources, most of the research on the physical properties influencing bioavailability has been done on elemental iron with much less work done to investigate the factors affecting the bioavailability of ferric salts, like ferric orthophosphate. It is important that the factors influencing the bioavailability of the widely used, affordable iron fortificants, like reduced iron and ferric orthophosphate, are well elucidated. In a recent study by Moretti and others (2006), the relative bioavailability of a micronized, dispersible ferric pyrophosphate varied markedly (15 – 62 % RBV) with the food matrix and iron status of the human test subjects. The authors concluded that assigning a single RBV to poorly soluble iron compounds may be of limited value in evaluating their suitability for food fortification (Moretti D and others 2006).

Two physical parameters, particle size and surface area, are thought to be important factors in the solubility of iron fortificants (Harrison and others 1976; Hurrell 2002b; Swain and others 2003) Therefore, one objective of this study was to measure these attributes and their relationship to the solubility. A third physical parameter, amorphous content, is known to play an important role in the bioavailability of ferric orthophosphate in plants and insects, and is hypothesized to be an important factor for efficacy in humans (Willis and Allen 1999). Amorphous content refers to regions of molecular disorder within a semi-crystalline solid. Amorphous regions in crystalline material will absorb water to a greater degree than crystalline regions and are often thermodynamically unstable. These areas of molecular disorder and higher moisture

content are subject to physical transitions and chemical degradations. The presence of amorphous ferric orthophosphate may have important mechanistic properties that determine its bioavailability and stability in food products (Buckton and Darcy 1999; Mackin and others 2002; Burnett and others 2006).

To date, little information is available on the amorphous content of ferric orthophosphate powders and its relationship to bioavailability in man. Quantification of amorphous content is analytically difficult, especially for contents less than 10%. Over the past ten years, several techniques have been developed that have improved sensitivity to measure low amounts of amorphous material. Bulk analytical techniques, such as differential scanning calorimetry, and powder X-ray diffraction, which measure a properties of the entire sample, are less sensitive than techniques that preferentially measure properties of the amorphous content only. In bulk measurements, the amorphous content becomes a small part of the total signal, and consequently it is difficult to quantify with confidence (Buckton and Darcy 1999). The detection limits for amorphous content with bulk techniques will generally have a lower cut off of 5–10%. In addition, techniques which preferentially investigate the properties of the powder surface, where amorphous material may predominate, are more sensitive. A powerful way of investigating surface phenomena is by dynamic (gravimetric) vapor sorption (DVS), which preferentially probes amorphous (over crystalline) regions. DVS is the preferred means in the pharmaceutical industry for measuring low levels of amorphous material (Buckton and Darcy 1999). This technique measures the mass change due to preferential solute (water) uptake by an amorphous phase under controlled conditions (Burnett and others 2006). A small amount of amorphous material on the surface of a particle (by

weight of the sample) can have a very substantial surface effect and, in the case of iron, cause solution behavior that is very different from material in the crystalline state (Buckton and Darcy 1999). These differences in amorphous content behavior can significantly alter the solubility of the iron source.

In the previous chapter, substantial differences in the bioavailability of different sources of ferric orthophosphate were observed. Solubility is known to play an important role in bioavailability. Iron sources with low water-solubility must first be solubilized in digestive juices before they can enter the common iron pool for absorption. This chapter investigates the *in vitro* solubility of the five of the sources of ferric orthophosphate studied in Chapter 2. The method of Shah and others (1977) was adapted to evaluate the solubility of ferric orthophosphate over the range of hydrochloric acid concentrations found during normal human digestion (0.02 – 0.10 N HCl). Although this method was originally developed for elemental iron powders (Shah and others 1977; Hallberg and others 1989; Swain and others 2003; Hoppe and others 2006), it has been applied to various non-heme iron sources, like ferric phosphates (Forbes and others 1989; Hallberg and others 1989).

There is wide variability in the data in the literature on the physicochemical properties and RBV for ferric orthophosphate, and standardized methods are needed to allow comparable results between laboratories. Thus, the influence of particle size, surface area and amorphous content on the solubility of the ferric orthophosphate samples was investigated in this study. Due to the potentially important effects of the presence of amorphous material on moisture uptake and, therefore, solubility and reactivity during cereal processing and over shelf life, a sensitive technique to measure amorphous content

was needed. RTE cereals area dry products and moisture uptake is their main mode of shelf-life failure along with oxidative rancidity. DVS was chosen as a sensitive measure of amorphous content and also because it directly measured the effects of exposure to moisture uptake at different relative humidity.

## 3.2 MATERIALS AND METHODS

### *3.2.1 Materials*

Solubility, particle size, surface area and isotherm measurements were made on the same lots of ferric orthophosphate powder that were used in Chapter 2, identified as Sources 4 – 8 (Table 3.1). New lots of these materials were also analyzed for Sources 4, 5 and 6 to determine lot-to-lot process variation. Different production lots were not available for Source 7 and 8. Iron sources were commercially produced, food grade materials meeting Food Chemical Codex (FCC) requirements for ferric orthophosphate. The ferric orthophosphate powders (ferric (III) –orthophosphate ($FePO_4$ X $H_2O$, mono – tribasic, molecular weight 150.82, anhydrous) were > 99% pure and had iron contents ranging from 24.6 – 30.4 % by ICP analysis, depending on waters of hydration and phosphate content. Sources 4 -7 were supplied by Budenheim Chemische Fabrik (Mainz, Germany). Source 8 was purchased from Madison Chemicals, Inc. (Madison Township, NJ). Iron sources were stored in closed containers under ambient conditions.

**TABLE 3.1**
**Iron sources used for particle size and surface area measurements**

| Sample Identification | # Lots | Iron Source |
|---|---|---|
| Source 4 | 2 | Ferric orthophosphate, Budenheim Chemische 53-80 |
| Source 5 | 2 | Ferric orthophosphate, Budenheim Chemische 53-81 |
| Source 6 | 3 | Ferric orthophosphate, Budenheim Chemische 53-82 |
| Source 7 | 1 | Ferric orthophosphate, Budenheim Chemische 53-85 |
| Source 8 | 1 | Ferric orthophosphate, Madison Chemicals |

### *3.2.2 Methods*

#### *3.2.2.1 Solubility in Dilute Hydrochloric Acid*

The solubility method used for the iron sources was based on the method of Shah and others (1977). The method was modified to decrease assay variation by performing experiments in the dissolution apparatus described in the USPC 2006 Official Method for Dietary Supplements #2040 *Disintegration and Dissolution of Dietary Vitamin and Mineral Supplements* (USPC 2006). The apparatus consists of a 1000-ml low-form beaker on a basket-rack assembly, which contains the dissolution solution and a thermostat to control heating of the solutions between 35° and 39°C. Sample mixing is precisely controlled by a device that vertically rotates the beaker a set distance (not less than 5.3 cm and not more than 5.7 cm) with controlled frequency rates (rpm).

Sources 4-8 were weighed to contain approximately 2.5 mg iron/ml acid solution, e.g. approximately 1.123 g ferric orthophosphate powder was dissolved in 450 ml of standardized HCl (Fisher Scientific Catalog #SA54-4). Three acid concentrations, 0.02 N, 0.05 N and 0.10 N were used in the experiments. The acid solutions were maintained at a temperature of 37°C ± 1° and mixed in the dissolution apparatus at 200 rpm for 45 minutes. After dissolution, a 20-ml aliquot was removed and sequentially filtered through a Whatman #40 filter and a Whatman #42 filter. One ml of the filtrate was transferred to a 25-ml volumetric flask and diluted to volume with more dissolution solution. The amount of dissolved iron was determined by inductively coupled plasma (ICP) at Covance Laboratory in Madison, WI. (AOAC International 2000). Ferric chloride (1 mg iron/ml) was dissolved in the same concentration acid solution as the sample and used as the standard stock solution. Standard concentrations were prepared to cover the range of

0.2 to 10 μg iron/ml and a standard curve was used to quantitate the amount of iron in the samples.

### *3.2.2.2 Polarized Light Micrographs*

Ferric orthophosphate Sources 4-8 were examined by polarized light microscopy at a magnification of 360X. Photomicrographs were taken under optical conditions of crossed polarizing filters and first order red compensation that resulted in a light red background. The polarization phenomena of the crystals were displayed for the two principal vibration directions, using an optical shift of 0.576 um. The crystals showed a sequence of interference colors depending on their thickness, birefringence, and orientation relative to the direction of the polarizing filters and the compensation plate (Genck and Bayard 1997)

### *3.2.2.3 Particle Size by Laser Light Diffraction*

The particle size distributions of the ferric orthophosphate samples were determined by Particle Technology Labs, Ltd. Method MM324.01 using a Malvern Mastersizer 2000 Laser Diffractor. The refractive indices (RI) of the iron sources were determined by Bayard Development Company using the Beckeline Test[4]. Results are given in the Appendix A. An average RI was used for samples having a range of refractive index values. Samples were prepared for analysis by placing an amount from the bulk sample into a scintillation vial to reach an obscuration of 10-20%, or approximately 0.003 – 0.020% volume depending on the sample. Approximately 0.5 cm of Aerosol OT (Fisher Scientific) was added to the sample. The scintillation vial was filled with 20 ml of hexane (Fisher Optima Grade), and was placed in an ultrasonic bath (Branson 3) for 2 minutes. Samples were checked for clarity and visually inspected using a microscope to verify adequate dispersion. The suspension was transferred by pipette into the Malvern Hydro 200S recirculator bath (filled with Fisher Optima grads hexane) using an amount to reach an obscuration level in the range of 10 to 20 percent. Four 12-second analyses were made at one-minute intervals and results are reported as the average of the four analyses. The analysis was repeated six times for each lot of ferric orthophosphate. Results are reported on a volume (mass) basis. Instrument and software parameters are given in Appendix I, Table 2.

[4] The Beckeline test measured the refractive indices for the sodium d line (589 nm) of the ferric orthophosphate samples based on standard immersion methods.

The following six values, termed the mean volume statistics, were measured: mean particle size (MPS) diameter in micrometers; three cumulative percent statistics[5], 10%, 50% and 90%, which are the percentages of the total sample smaller than the indicated sizes in micrometers; uniformity statistic, which is an indicator of the normalcy of the distribution and the span, a value representative of the width of the distribution.

#### *3.2.2.4 Surface Area by Nitrogen Adsorption*

Surface area measurements were made by Particle Technology, Ltd. Method AU225.01 using a 3-point Brunauer, Emmett and Teller (B.E.T.) surface area analysis performed on a Quantachrome Autosorb-1 instrument. Approximately 2-3 grams of sample was prepared for analysis by outgassing under a helium purge at 25°C for 16 hours until the samples were thoroughly dry and free of surface contamination. Analysis was carried out using nitrogen as the adsorbate gas maintained at a temperature of 77.4 °K over a range of five B.E.T. points (0.10, 0.15, 0.20, 0.25, and 0.30). Instrument parameters are given in Appendix B, Table 3. The analysis was repeated six times for each lot of ferric orthophosphate.

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[5] The volume statistics (D[v,0.10], D[v,0.50], D[v,0.90]) are based on the volume moment mean: D[4,3] = Sum of the equivalent diameters$^4$/sum of the equivalent spherical diameters$^3$. D = spherical diameter and [4,3] refer to the power of the sum of the diameters for the numerator and denominator, respectively.

### *3.2.2.5 Moisture Uptake by Dynamic Vapor Sorption*

Dynamic Vapor Sorption (DVS) analyses on ferric orthophosphate powders were performed in the laboratory of Dr. Daryl Willams in the Department of Chemical Engineering, Imperial College, London, UK as part of a Kellogg Company-funded research project (Heng and Williams 2006). The method developed by Heng and Williams (2006) used the Dynamic Vapor Sorption DVS-1 (Surface Measurement Systems, UK) equipped with a Cahn D-100 microbalance (sensitivity 0.1 μg). Sorption/desorption analyses were conducted on the same ferric orthophosphate powders (Source 4-8) used throughout this dissertation. Approximately 100 g of fortificant was weighed in the microbalance module and pre-conditioned at 0% relative humidity (RH) for 300 minutes. Following preconditioning, the relative humidity (RH) was incrementally increased on average every 60 minutes as follows: 0, 10, 20, 30, 40, 50, 60, 70, 80 and 90% RH and then reversed to complete one sorption/ desorption isotherm cycle over approximately 2000 minutes. Two cycles were performed on each of the sources to determine if the samples underwent crystallization changes during analysis and to determine optimum cycle-time.

### *3.2.2.6 Preparation of Amorphous Ferric Orthophosphate*

A 100% amorphous ferric orthophosphate powder was not readily available from a commercial source. Therefore, an attempt was made to prepare a 100% amorphous ferric orthophosphate standard from Source 4, which appeared to be the source with the highest amorphous content based on solubility and microscopic analysis. In order to make as close to a 100% amorphous sample as possible, Source 4 was dissolved in dilute

aqueous 0.10 N HCl. This solution was spray dried using a Buchi B-290 apparatus (Buchi, Oldham, UK) and collected by a cyclone system (Heng and Williams 2006) and analyzed immediately after collection using the same experimental conditions and parameters.

## 3.3 STATISTICAL ANALYSIS

Solubility analyses were conducted in triplicate on Sources 4-8 while six replicates were performed for particle size and surface area measurements. Additional lots of material (Table 3.1) were not available for all of the ferric orthophosphate sources resulting in an unbalanced data set and reduced ability to detect differences between lots. A least squares analysis of variance was performed using the Proc Mixed Procedure in SAS. Physical properties, such as mean particle size, were considered a function of the source of the iron and therefore fixed effects while lot was considered a random effect. Multinomial decomposition of the particle size data was done using the nonlinear curve fit algorithm in Peak Fit software from Systat Software, Inc. The determination of moisture uptake (amorphous content) by DVS measurements was semi-quantitative due to the instability and hydroscopicity of the amorphous standard during analysis. Regression analysis was used to correlate median particle size, surface area and moisture uptake versus percent solubility and percent RBV. Percent RBV was correlated to solubility and $\log_{10}$ solubility and a multiple regression model was used to evaluate median particle size and moisture uptake as predictors of solubility and % RBV.

## 3.4 RESULTS

### *3.4.1 Solubility in Dilute HCl*

There was a marked difference in the solubilities of the iron sources (Table 3.2). Iron solubility was calculated as the amount (μg) iron that dissolved per gram of iron analyzed. The ferric orthophosphate samples had an average iron content of 27.8% (26.4-29.3% ± 1.09%). Approximately 0.29 mg total iron (1.12 g of ferric orthophosphate) was analyzed per sample. Each source was dissolved at three dilute hydrochloric acid concentrations, 0.02 N, 0.05 N and 0.10 N. The range of dissolved iron in the three HCl solutions was as follows: 101 μg to 4.1 mg in 0.02 N HCl; 103 μg to 24.7 mg in 0.05 N HCl; and 231 μg to 195 mg in 0.1N HCl. Expressed as a percentage of iron dissolved (weight basis), the rank solubility of the iron from the most to the least soluble was Source 4 (19.5%) > Source 7 (1.0%) > (Source 5 (0.29%) > Sources 6 and 8 (<0.05%). This trend was seen at all dilute acid concentrations tested.

**Table 3.2 Solubilities of iron sources in dilute hydrochloric acids solutions as compared to RBVs**

| Source and Lot | Acid Concentrations 0.1 N HCl Dissolved Iron (µg/g) ± SD | | % Iron Dissolved | 0.05 N HCl Dissolved Iron (µg/g) ± SD | | % Iron Dissolved | 0.02 N HCl Dissolved Iron (µg/g) ± SD | | % Iron Dissolved | Relative Bioavailability RBV ± SD |
|---|---|---|---|---|---|---|---|---|---|---|
| **Source 4** | | | | | | | | | | |
| Lot 1 (n=2) | 211,000 | ± 0 | **21.10%** | 41,400* | ± 0 | **4.14%** | 4,440* | ± 0 | **0.44%** | |
| Lot 2 (n=3) | 179,000 | ± 1000 | **17.90%** | 49,433 | ± 208 | **4.94%** | 8,127 | ± 55 | **0.81%** | |
| All Lots | 195,000 | ± 17541 | **19.50%** | 24,717 | ± 4020 | **2.47%** | 4,063 | ± 1844 | **0.41%** | 99±9[a] |
| **Source 5** | | | | | | | | | | |
| Lot 1 (n=3) | 2,880 | ± 207 | **0.29%** | 1,360 | ± 193 | **0.14%** | 653 | ± 208 | **0.07%** | 78±7[b] |
| **Source 6** | | | | | | | | | | |
| Lot 1 (n=3) | 292 | ± 17 | **0.03%** | 234 | ± 20 | **0.02%** | 121 | ± 9 | **0.01%** | |
| Lot 2 (n=3) | 400 | ± 68 | **0.04%** | 225 | ± 1 | **0.02%** | 159 | ± 5 | **0.02%** | |
| Lot 3 (n=2) | 326 | ± 40 | **0.03%** | 148 | ± 5 | **0.01%** | 102 | ± 2 | **0.01%** | |
| All Lots | 339 | ± 65 | **0.03%** | 202 | ± 40 | **0.02%** | 127 | ± 26 | **0.01%** | 51±5[c] |
| **Source 7** | | | | | | | | | | |
| Lot 1 (n=3) | 10,090 | ± 115 | **1.01%** | 4,197 | ± 31 | **0.42%** | 1,277 | ± 31 | **0.13%** | 83±5[b] |
| **Source 8** | | | | | | | | | | |
| Lot 1 (n=2) | 231 | ± 14 | **0.02%** | 103 | ± 13 | **0.01%** | 101* | ± 0 | **0.01%** | 60±6[d] |
| Pooled Variance | | 176981.9 | | | 13675.9 | | | 8587.6 | | |
| Pooled SD | | 420.7 | | | 116.9 | | | 92.7 | | |
| Pooled Mean | | 47671.4 | | | 10412.4 | | | 1881.6 | | |
| **Pooled Assay CV** | | **0.88%** | | | **1.1%** | | | **5.0%** | | |

*n=1

na =no sample available

### *3.4.2 Particle Size, Surface Area and Polarized Light Microscopy*

#### *3.4.2.1 Particle Size Analysis*

The results of the laser diffraction measurements for particle size are shown in Table 3.3 along with the % RBVs for each source (Chapter 2, Table 2.4) and surface area results. A confidence level of 95% was used. The differences between lots were found to be significant and random. Based on the PS distribution data, the sources were grouped into three statistically different distributions ($P < 0.05$).

The first group, Sources 6 and 8, had larger particle size distributions (Median PS 17.7 and 15.5 μm, respectively) and the lowest bioavailability (RBV 51 and 60%, respectively). Source 8 had the most uniform distribution and was the only source that lacked a population of fine particles. Polarized light microscopy showed Source 8 to have a uniform population of birefringent particles, indicting a much higher degree of molecular order (crystal structure).

The second group, Sources 5 and 7, had bimodal distributions of fine particles. Both Source 5 and 7 had median PS of 2 μm and intermediate RBVs (78 & 83%, respectively). Microscopic examination revealed a mixture of small, amorphous and crystalline particles with varying degrees of crystallinity.

Source 4 was distinctly different from the other two PS distribution groups and comprised the third group. This source appeared to be the most amorphous of all the sources and had a bimodal distribution with an overall median PS of 9.3 μm and an RBV of 99%. Source 4 behaved differently in dilute acid solutions, with 100 to 1000-times the solubility of the other sources (depending on pH).

Two other particle size statistics, span and uniformity are given in Table 3.3. The span parameter showed that Sources 4, 5 and 7 had the broadest particle size distribution while Sources 6 and 8 had the narrowest distribution. The uniformity parameter showed a similar trend with Sources 6 and 8 displaying a single, more Gaussian-shaped distribution than the other sources.

*3.4.2.1.1 Multinomial Curve Fitting of Particle Size Data*

Peak fitting software was used to estimate the presence of normally-distributed particle size populations within the distributions for each iron source. The MPS and the percentage of particles making up each normally-distributed subpopulation were determined for each lot of Source 4, 5, 6, 7 and 8. Results are shown in Table 3.4. Sources 4, 5 and 7 had bimodal distributions with the following MPS and percent composition for each subpopulation (percent range over lots): Source 4, MPS 6μm (33-34%) and 51μm (66-70%). %). Source 5, MPS 2 (18-43%) and 18μm (57-82%); and Source 7, MPS 2 (29%) and 17μm (72%). Source 6 had a trimodal distribution (MPS 0.8μm (0.1%), 3μm (0.3-1.8%) and 21μm (98-100%). Source 8 had a single, uniform distribution with a mean particle size of 17μm (100%).

***3.4.2.2 Surface Area Analysis***

Surface area measurements separated the five sources into two instead of three significantly different groups, which were not different within a group. The

surface area for Sources 4, 5 and 7 were approximately 10-times higher than the surface areas for Sources 6 and 8 (Table 3.3).

### *3.4.2.3 Polarized Light Microscopy*

Microscopic examination visualized the presence of different particle size populations evident from particle size distribution analysis (shown in Figure 3.1a-e with the particle size distributions). Polarized light microscopy revealed the presence of birefringent crystalline material showing different degrees of crystallinity among the sources. Source 4 (Figure 3.1a) has two populations of particles, fine particles with very small internal features and some larger entities (15 –75 μm) with a trace of birefringence. Sources 5 and 7 (Figure 3.1b and d) are composed of predominantly very small (1-5 μm) isotropic particles indicating an amorphous molecular structure. A few larger particles (10-50 μm) are intermixed and Source 5 shows a tendency to form clumps. Two populations of particles are seen in the micrograph for Source 6 (Figure 3.1c). The smaller (3-10 μm) are isotropic with very low birefringence and amorphous structure. The larger particles (15-50 μm) display more birefringence and have more defined crystal structure. Source 8 (Figure 3.1e) has a more defined crystal structure than the rest of the samples with mostly birefringent crystallites consisting of several small crystals growing radially from a common grain and a uniform particle size (10-15 μm) with a few larger (25 μm) grains.

**Table 3.3 Results of particle size distributions and surface area analysis compared to the RBV**

| Source and Lot [2] | Average MPS [3] (um) | std | Cummulation Percent Statistics [1,4] (<10%,< 50%,<90%) 10% of Total Sample is < Indicated Size (um) | std | 50% of Total Sample is < Indicated Size (um) | std | 90% of Total Sample is < Indicated Size (um) | std | Surface Area [5] ($m^2/g$) | std | Relative Bioavailability [6] (RBV) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Source 4** | | | | | | | | | | | |
| Lot 1 (n=6) | 21.5 | ± 1.1 | 1.9 | ± 0.1 | 9.3 | ± 0.4 | 59.0 | ± 2.1 | 14.4 | ± 0.0 | |
| Lot 2 (n=6) | 20.4 | ± 1.2 | 2.0 | ± 0.1 | 9.2 | ± 0.6 | 56.3 | ± 3.2 | 15.3 | ± 0.1 | |
| All Lots | $21^{a}$ | ± 1.2 | $1.9^{a}$ | ± 0.1 | $9.3^{a}$ | ± 0.4 | $57.6^{a}$ | ± 2.9 | $14.9^{a}$ | ± 0.44 | $99\pm9^{a}$ |
| **Source 5** | | | | | | | | | | | |
| Lot 1 (n=6) | 7.2 | ± 0.2 | 0.9 | ± 0.0 | 2.2 | ± 0.0 | 22.0 | ± 0.9 | 13.7 | ± 0.2 | |
| Lot 2 (n=6) | 4.0 | ± 0.4 | 0.9 | ± 0.0 | 1.8 | ± 0.0 | 11.0 | ± 0.6 | 17.6 | ± 0.3 | |
| All Lots | $5.6^{b}$ | ± 1.6 | $0.9^{ab}$ | ± 0.0 | $2^{b}$ | ± 0.2 | $16.5^{b}$ | ± 5.8 | $15.6^{ab}$ | ± 2.0 | $78\pm7^{b}$ |
| **Source 6** | | | | | | | | | | | |
| Lot 1 (n=6) | 18.6 | ± 0.2 | 8.2 | ± 0.2 | 17.1 | ± 0.1 | 31.9 | ± 0.4 | 1.5 | ± 0.0 | |
| Lot 2 (n=6) | 20.4 | ± 0.1 | 3.9 | ± 0.1 | 19.4 | ± 0.1 | 35.6 | ± 1.6 | 1.1 | ± 0.0 | |
| Lot 3 (n=6) | 18.0 | ± 0.2 | 5.4 | ± 0.3 | 16.6 | ± 0.1 | 32.5 | ± 0.3 | 3.6 | ± 0.2 | |
| All Lots | $19^{ac}$ | ± 1.0 | $5.8^{abc}$ | ± 1.9 | $17.7^{c}$ | ± 1.3 | $33.5^{bc}$ | ± 1.9 | $2^{c}$ | ± 1.1 | $51\pm5^{e}$ |
| **Source 7** | | | | | | | | | | | |
| Lot 1 (n=6) | $5.4^{b}$ | ± 0.2 | $0.8^{abc}$ | ± 0.0 | $2.0^{b}$ | ± 0.0 | $15.9^{bcd}$ | ± 0.9 | $11^{ab}$ | ± 0.1 | $83\pm5^{b}$ |
| **Source 8** | | | | | | | | | | | |
| Lot 1 (n=6) | $16.3^{ac}$ | ± 0.0 | $10.4^{b}$ | ± 0.0 | $15.5^{c}$ | ± 0.0 | $23.3^{bcd}$ | ± 0.1 | $0.75^{c}$ | ± 0.02 | $60\pm6^{d}$ |

[1] Means without letters in common are statistically different

[2] Source and Lot: Type of ferric orthophoaphate and different production lots,when available

[3] MPS: mean particle size diameter (um)

[4] The volume statistics (D[v,0.10], D[v,0.50], D[v,0.90]) are based on the volume moment mean: D[4,3] = Sum of the equivalent diameters$^4$/sum of the equivalent spherical diameters$^3$. D = spherical diameter and [4,3] refer to the power of the sum of the diameters for the numerator and denominator, respectively. (Malvern Mastersizer 2000)

[5] Surface Area: determined by nitrogen gas sorption (Quantachrome Autosorb-1)

[6] Relative Bioavailability: Relative Biologic Value (RBV) by AOAC Rat Hemoblobin Repletion Bioassay/slope ratio method

**TABLE 3.4 Particle size sub-populations by multinomial curve fitting**

| Source | Lot | Peak 1 MPS (μm) | Area (%) | Peak 2 MPS (μm) | Area (%) | Peak 3 MPS (μm) | Area (%) |
|---|---|---|---|---|---|---|---|
| **Source 4** | Lot 1 | 6 | 34 | 50 | 66 | - | - |
| | Lot 2 | 6 | 33 | 51 | 70 | - | - |
| **Source 5** | Lot 1 | 2 | 18 | - | - | 18 | 82 |
| | Lot 2 | 2 | 43 | - | - | 17 | 57 |
| **Source 6** | Lot 1 | 0.9 | 0.1 | 2 | 0.3 | 19 | 99.9 |
| | Lot 2 | 0.6 | 0.1 | 4 | 3 | 23 | 96.6 |
| | Lot 3 | 0.7 | 0.1 | 4 | 1.8 | 20 | 98.1 |
| **Source 7** | Lot 1 | 2 | 29 | - | - | 17 | 72 |
| **Source 8** | Lot 1 | - | - | - | - | 17 | 100 |

**Figure 3.1a-e.** Particle size distributions by laser light diffraction and polarized light micrographs of iron Sources 4-8, magnification 360X.

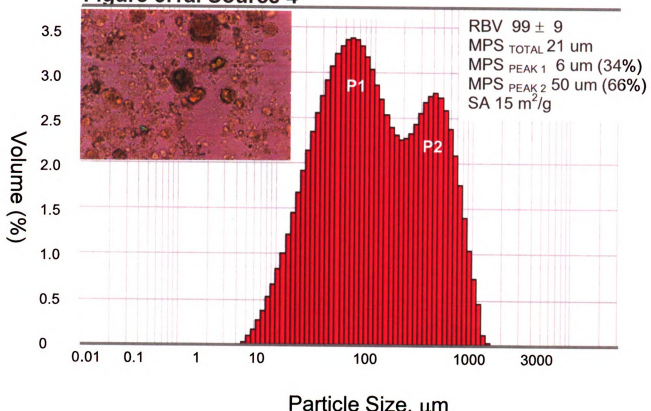


Figure 3.1a. Source 4
RBV 99 ± 9
MPS TOTAL 21 um
MPS PEAK 1 6 um (34%)
MPS PEAK 2 50 um (66%)
SA 15 m²/g
P1
P2
Volume (%)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0.01
0.1
1
10
100
1000
3000
Particle Size, μm

**Figure 3.1b. Source 5**

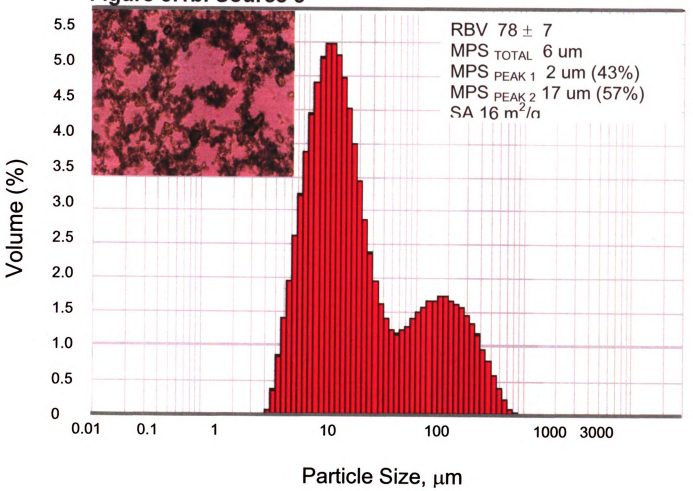


**Figure 3.1c. Source 6**

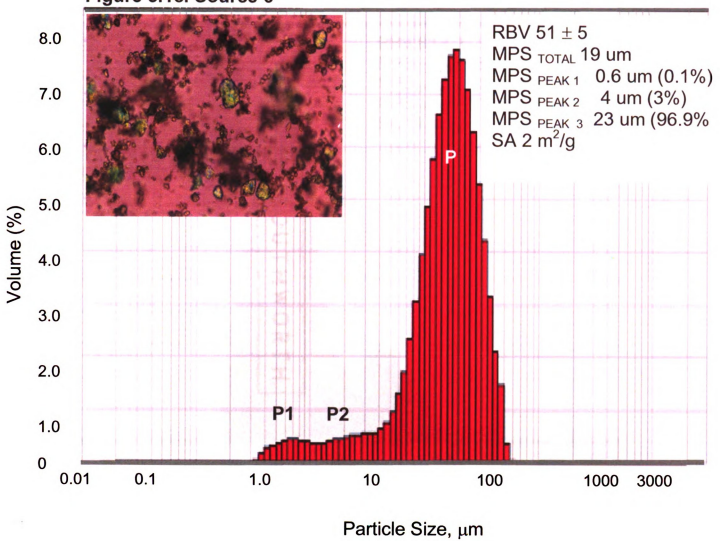


**Figure 3.1d. Source 7**

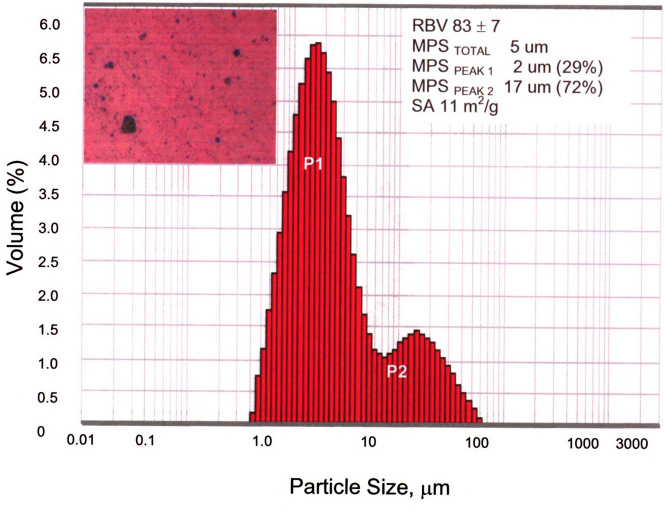
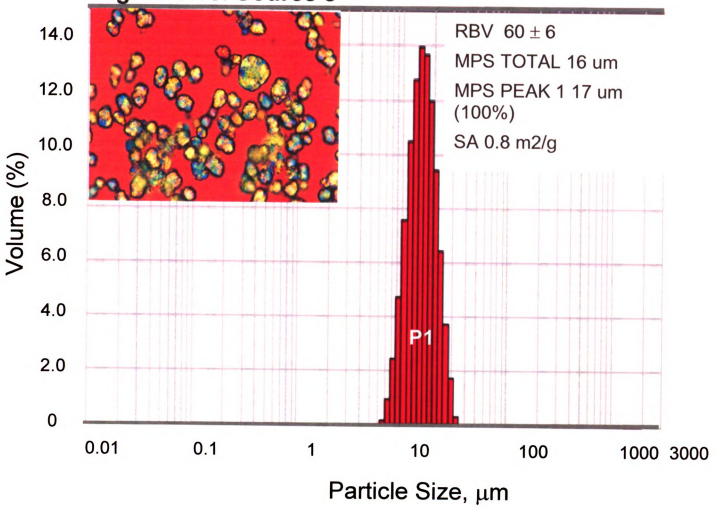


**Figure 3.1e. Source 8**



### *3.4.3 Dynamic Sorption Isotherms*

Dynamic vapor sorption (DVS) quantifies small amounts of amorphous material in an otherwise crystalline solid. The amount of moisture gain and loss in a sample is measured while relative humidity (RH) is cycled in a step-wise fashion at constant temperature, in this case from 0-100% RH, generating an isotherm for the material.

A minimum of two consecutive isotherm cycles were run on each source of ferric orthophosphate. Samples were held at each change in humidity until the sample weight (moisture gain) stabilized. Within amorphous regions, there was a substantial sorption of water vapor and increase in mass due to moisture uptake (and moisture release during desorption). Mass plots showing the percent change in mass due to moisture gain plotted against time for two isotherm cycles are shown for Source 4 in Figure 3.2. The two isotherm cycles were identical, indicating that Source 4 did not undergo any moisture induced collapse of amorphous structure and/or recrystllization with increasing humidity. These events would have caused marked differences between the two consecutive isotherms. This was true for all the sources tested, indicating that the material adsorbed moisture but would be stable when stored at different relative humidities.

Mass plots for Source 6 and Source 4 are shown in Figure 3.3, 3.4, respectively, Source 6 and Source 4 had moisture uptakes of 1.8% ± 0.8 and 11.8% ± 3.5, respectively. The biggest mass sorption increase for most of the sources occurred between 70-90% RH. Isotherm hysteresis plots (Figure 3.5) display the moisture sorption and desorption behavior and showed that the three lots of Source 4 had markedly different isotherms than the rest of the sources with a greater maximum moisture uptake and larger hysteresis. Sources 8 and 6, and Sources 5 and 7 display similar hysteresis behavior.

For Sources 4-8, the maximum moisture uptake varied from as low as 1% for one lot of Source 6 to as high as 14.3% for one lot of Source 4 (Table 3.5). The following rank order of increasing moisture uptake (mean of multiple lots) occurred for Sources 4-8: Source 8 (1.2%) < Source 6 (1.8% ± 0.8) < Source 7 (3.4%) < Source 5 (3.5% ± 0.5) < Source 4 (11.8% ± 3.5).

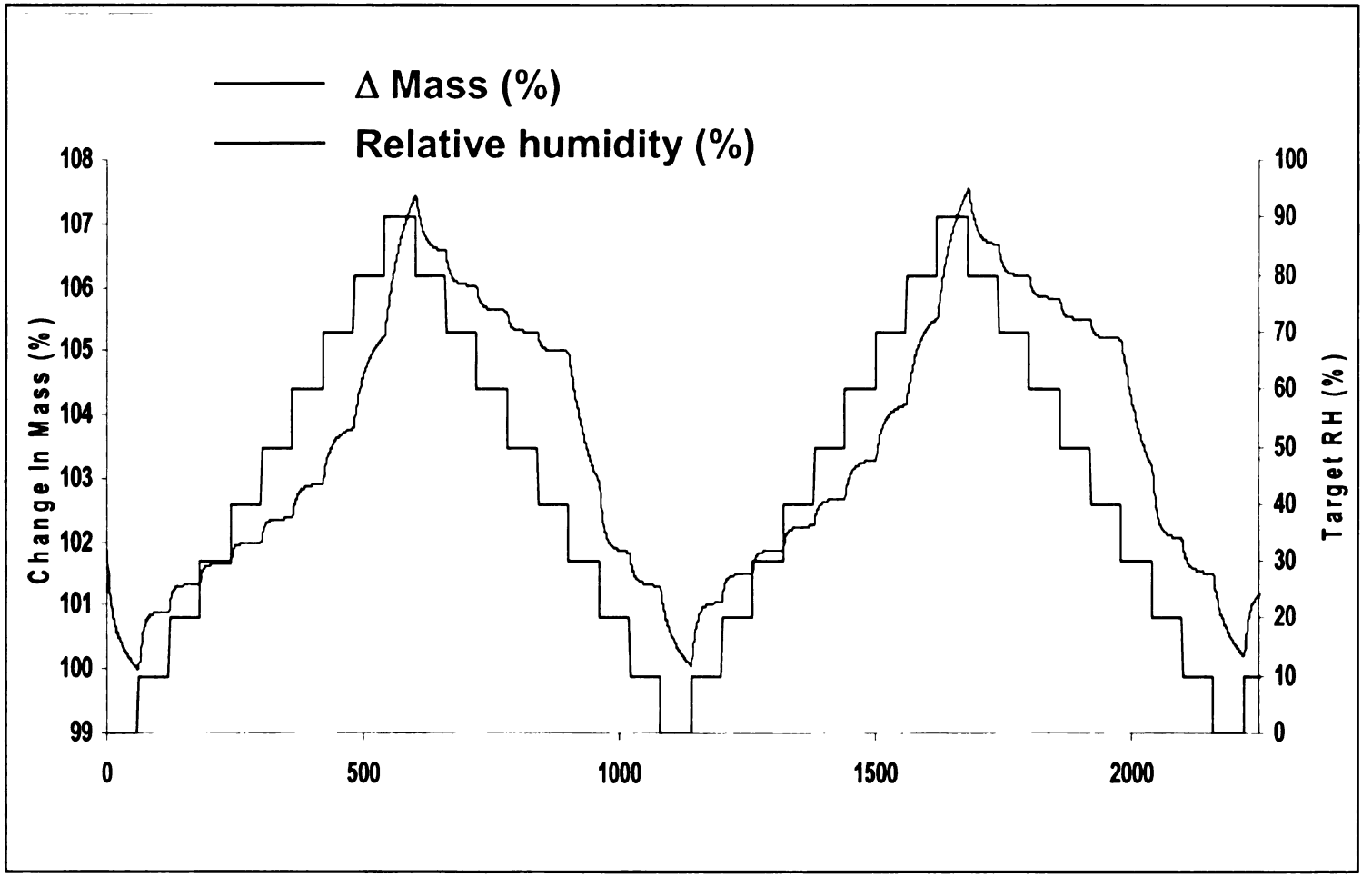



**Figure 3.2.** Source 4: Plot of the percent change in mass due to moisture uptake at incremental changes in percent RH over time for two isotherm cycles.

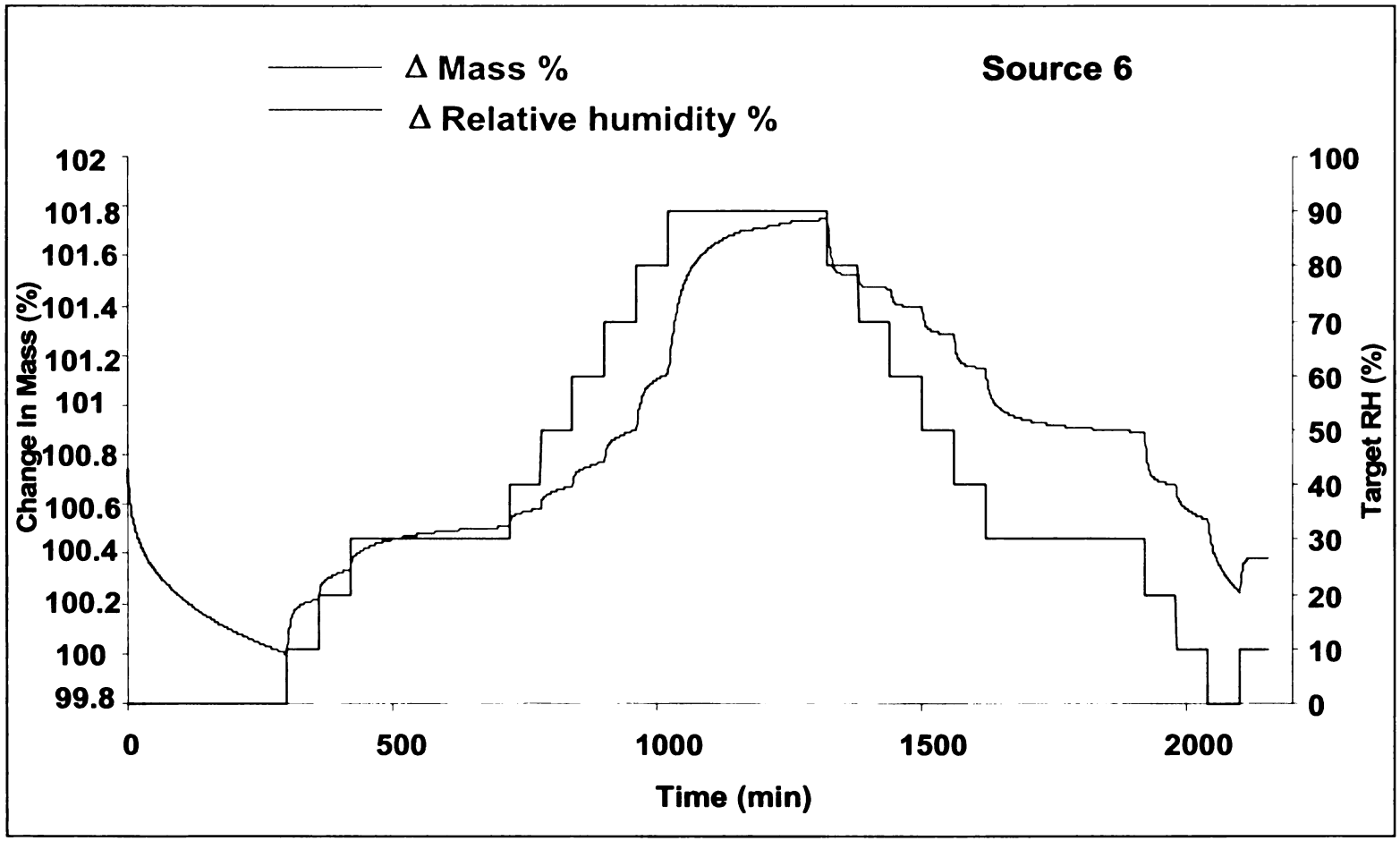



**Figure 3.3.** Source 6: Isotherm mass plot showing areas of mass increase (sorption) and mass decrease (desorption).

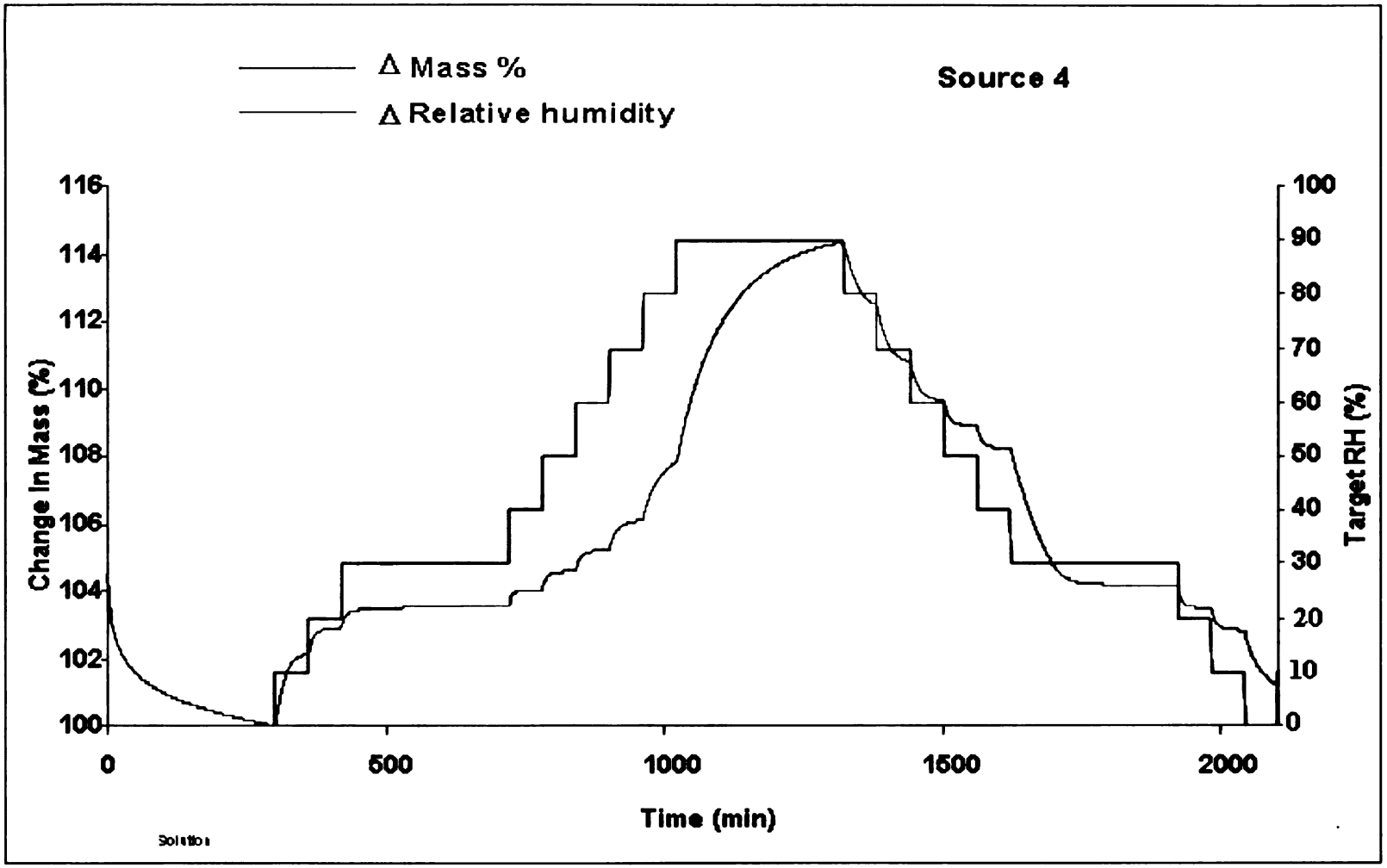



**Figure 3.4.** Source 4: Isotherm mass plot for showing areas of mass increase (sorption) and mass decrease (desorption).

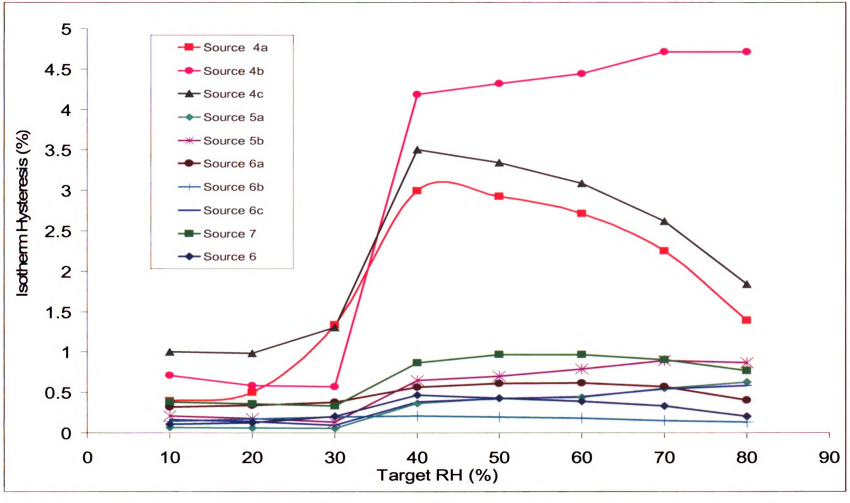



**Figure 3.5**. Sources 4-8: Isotherm hysteresis plots showing the change in mass differences between moisture sorption and desorption (moisture uptake and loss) at each incremental change in %RH.

### *3.4.3.1 Semi- Quantitation of Amorphous Content*

Repetitive isotherms are shown for the amorphous standard that was prepared from Source 4. This highly amorphous material appeared to be unstable and sublime, collecting on instrument components during measurements that accounted for a slight mass loss at the end of isotherm cycles 1 and 2 (Figure 3.6). Mass equilibration was not reached above 10% RH during the 1000-minute cycle time of each isotherm due to the hydroscopicity of the material. A semi-quantitative estimate of the amorphous content of Source 4-8 was done by ratio of the moisture content of Source 4-8 to the standard moisture content at 10% RH. Results are given in Table 3.5. The maximum moisture uptake of the spray-dried material was markedly different than Sources 4-8 and was estimated to be approximately 60% of the sample by weight.

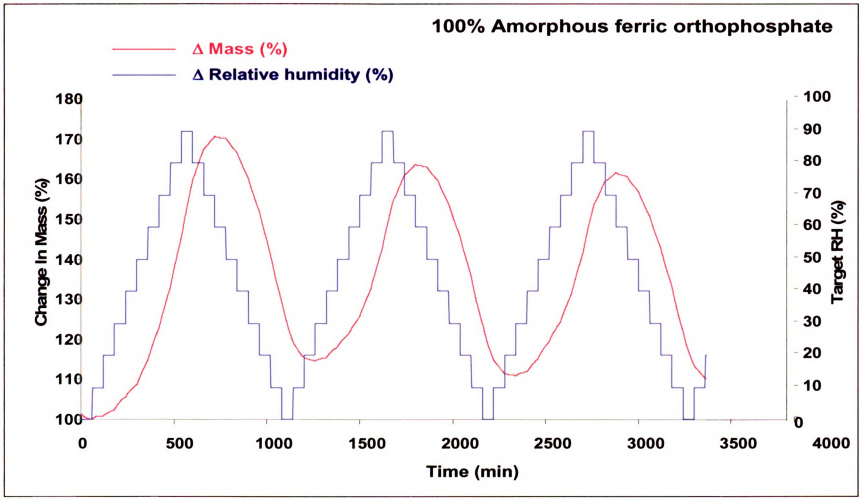



**Figure 3.6**. Mass plot showing three isotherm cycles for the spray-dried (approximately 100% amorphous material) sample of ferric orthophosphate; the mass continued to increase and did not reach a plateau during the 1000 minute cycles and a slight mass loss was seen between cycles 1 and 2. No crystal collapse or re-crystallization was observed.

**Table 3.5. Dynamic vapor sorption (DVS) results for iron sources**

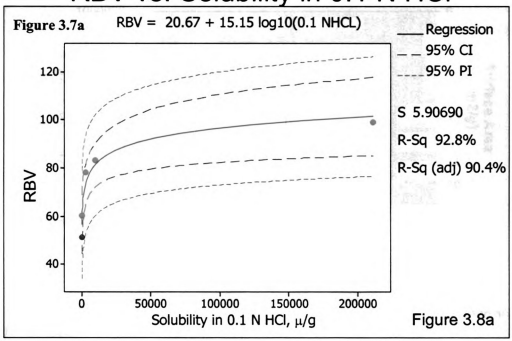
| Source Identification | Lot | Moisture Uptake (Mass %) | Estimated Amorphous Content (%) | RH % for increase | RH % for decrease |
|---|---|---|---|---|---|
| Source 4 | 1 | 14.3 | 23.8 | 70 → 90 RH% | 50 → 30 RH% |
| Source 4 | 2 | 9.3 | 15.5 | 80 → 90 RH% | 40 → 30 RH% |
| **Mean and SD** | | **11.8 ± 3.5** | **19.7** | | |
| Source 5 | 1 | 3.2 | 5.3 | 80 → 90 RH% | 90 → 70 RH%<br>90 → 80 RH% |
| Source 5 | 2 | 3.9 | 6.5 | 70 → 90 RH% | 40 → 30 RH% |
| **Mean and SD** | | **3.5 ± 0.5** | **3.3** | | |
| Source 6 | 1 | 1.8 | 3.0 | 70 → 90 RH% | 90 → 80 RH%<br>40 → 30 RH% |
| Source 6 | 2 | 1.0 | 1.7 | - | - |
| Source 6 | 3 | 2.5 | 4.2 | 80 → 90 RH% | 90 → 80 RH%<br>40 → 30 RH% |
| **Mean and SD** | | **1.8 ± 0.8** | **3.0** | | |
| **Source 7** | **1** | **3.4** | **5.7** | 70 → 90 RH% | 90 → 80 RH%<br>40 → 30 RH% |
| **Source 8** | **1** | **1.2** | **2.0** | 70 → 90 RH% | 40 → 20 RH% |

### *3.4.4 Regression Analyses and Prediction of % RBV*

Solubility results given in Table 3.2, showed that relative bioavailability increased rapidly with increasing solubility for all the iron sources at all acid concentrations. Figure 3.7a shows a regression plot of solubility versus % RBV. Due to the rapid (nonlinear) increase in % RBV, the data are presented in Figure 3.7b as a semi-log plot of % RBV versus $\log_{10}$ solubility. Solubility was found to be a good predictor of relative bioavailability ($R^2$ 90%; $P = 0.008$).

The physical properties of surface area, median particle size and moisture uptake were each plotted against $\log_{10}$ solubility in 0.1N HCl and % RBV. These relationships are shown in Figures 3.8 – 3.13. Regression analyses of moisture uptake versus $\log_{10}$ solubility or % RBV using a power law equation had the best fit of the data ($R^2$ 0.9527) followed by surface area ($R^2$ 0.8504) and particle size ($R^2$ 0.3663). The median particle size had the highest correlation of all the particle size volume statistics, however the correlation was poor.

A regression model of median particle size versus % moisture uptake was used to predict % RBV and a 2-D plot of the results for both predicted and actual %RBV is shown in Figure 3.14 and given in Table 3.6. The adjusted $R^2$ for the model was 93.0%; $P = 0.035$ (moisture uptake $P = 0.031$; median particle size $P = 0.055$). Estimated % RBVs for the five sources using the regression model were found to closely estimate the actual % RBV. Nine lots of ferric orthophosphate were tested from the 5 Sources, including lots that did not have known % RBVs.



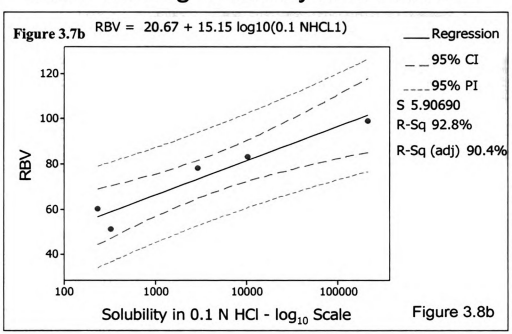




**Figure 3.7 a and b.** Curvilinear **(3.7a)** and semi-log **(3.7b)** plots of RBV versus Solubility in 0.1 N HCl;

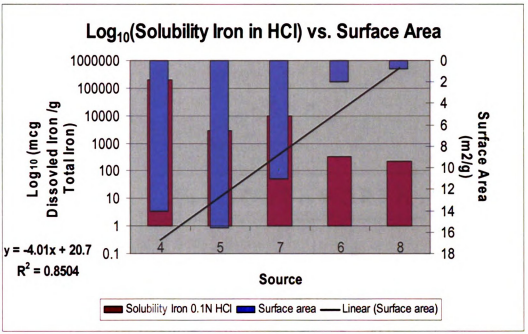



**Figure 3.8** Regression analysis of $\log_{10}$ (solubility iron in HCl) vs. surface area for Sources 4-8

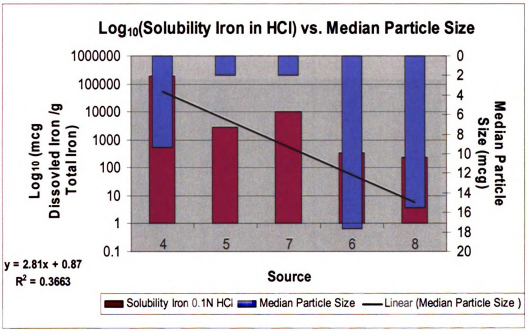



**Figure 3.9** Regression analysis of $\log_{10}$ (solubility iron in HCl) vs. median particle size for Sources 4-8

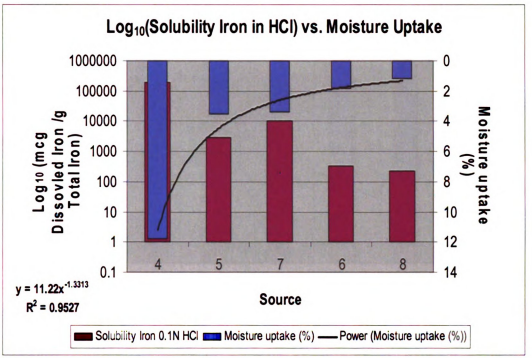



**Figure 3.10** Regression analysis of $\log_{10}$ (solubility) vs. % moisture uptake for Sources 4-8

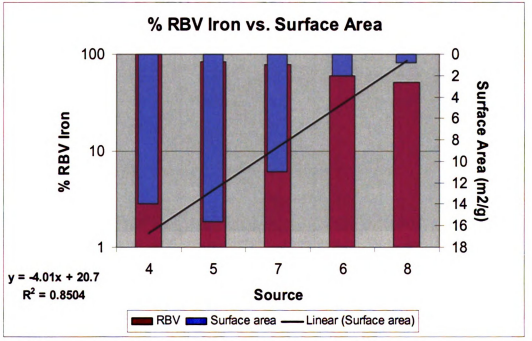



**Figure 3.11** Regression analysis of % RBV vs. surface area for Sources 4-8

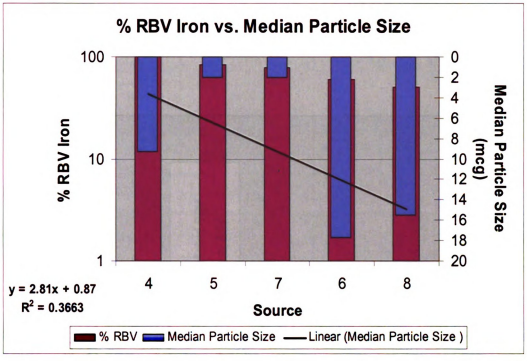



**Figure 3.12** Regression analysis of % RBV vs. particle size for Sources 4-8

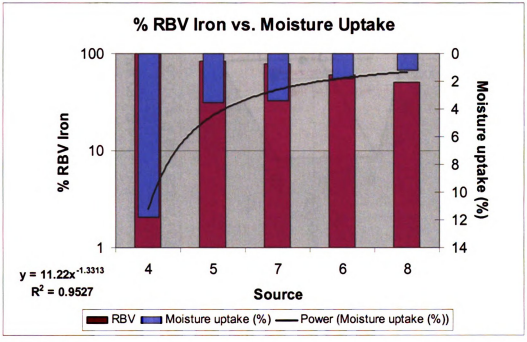



**Figure 3.13** Regression analysis of % RBV vs. % moisture uptake for Sources 4-8

Regression Model to Predict % RBV from Median Particle Size versus % Moisture Uptake

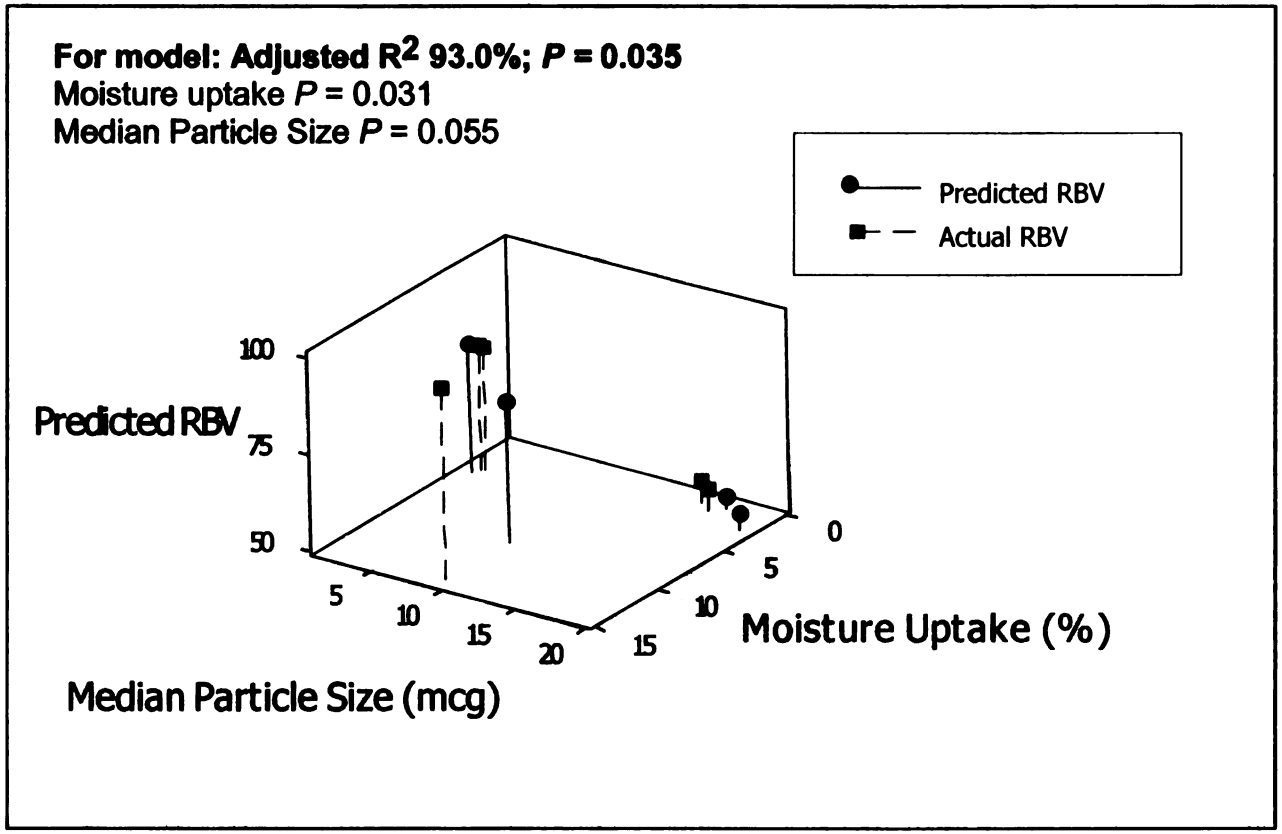



**Figure 3.14** Regression model of % moisture uptake and median particle size to predict % RBV for all lots of Sources 4-8

**Table 3.6 Comparison of Actual (By Analysis) to Predicted % RBVs for Sources 4-8**

| Source & Lot | Actual % RBV ± SD (By analysis)[1] | Predicted Regression Model % RBV | Median Particle Size (mcg)[2] | Moisture Uptake (%)[3] |
|---|---|---|---|---|
| **Source 4** | | | | |
| Lot 1 | **99±9** | 86 | **9.3** | **14.3** |
| Lot 2 | | **86** | **9.3** | **9.3** |
| **Source 5** | | | | |
| Lot 1 | **78±7** | 80 | **2.0** | **3.2** |
| Lot 2 | | **83** | **1.8** | **3.9** |
| **Source 6** | | | | |
| Lot 1 | **51±5** | 55 | **17.1** | **1.8** |
| Lot 2 | | **52** | **9.4** | **1.0** |
| Lot 3 | | **53** | **17.7** | **2.5** |
| **Source 7** | | | | |
| Lot 1 | **83±5** | 81 | **2.0** | **3.4** |
| **Source 8** | | | | |
| Lot 1 | **60±6** | 55 | **15.5** | **1.2** |

[1] RBV Determined by AOAC Rat Hemoglobin Repletion Bioassay (Chapter 2, Table 2.5)
[2] Median Particle Size, mcg (Chapter 3, Table 3.3)
[3] Moisture Uptake, % (Chapter 3, Table 3.5)

## 3.5 DISCUSSION

The results of this research demonstrate how widely the physicochemical properties of ferric orthophosphate vary and offer insight into the factors contributing to the variable bioavailability documented in the literature (Hurrell 1985; Forbes and others 1989; Willis and Allen 1999; Nalubola and Nestel 2000; SUSTAIN 2001; Dary and others 2002; Beard 2006; Moretti D and others 2006). The results of Chapter 2 demonstrate this variability for six commercially available sources of ferric orthophosphate that were found to have markedly different RBVs as determined by the

AOAC Rat Hemoglobin Repletion Bioassay. It is impractical and cost prohibitive to use *in vivo* RBV testing to study the physiochemical properties of iron sources. Therefore, in Chapter 3, experiments were performed that characterized the physical attributes thought to influence bioavailability, namely particle size, surface area, and amorphous content using an *in vitro* solubility method that was shown to provide a good estimation of *in vivo* RBV.

Solubility in dilute HCl is an important determinant of bioavailability and an *in vitro* method that provides an accurate and reproducible measure of solubility would be useful in predicting the RBV for a variety of iron sources. Several researchers have developed and/or modified *in vitro* solubility methods and used them to predict the RBVs of iron fortificants. To date, it has been difficult to compare results between laboratories and the conclusions drawn from these studies recognized the need for further method development to reduce the assay variability for routine use (Harrison and others 1976; Forbes and Erdman 1983; Forbes and others 1989; Swain and others 2003; Moretti D and others 2006).

In the study by Forbes and others (1989), an *in vitro* method was evaluated to measure the solubility of iron (% dissolved iron by weight) of electrolytic iron and ferric orthophosphate after a 30 minute dissolution period in 0.02 N HCl at 37° C. Electrolytic iron was 60 to 75% soluble, while only 3 to 4% of the ferric orthophosphate dissolved. Swain and coworkers (2003) determined the RBVs of six elemental iron powders by the AOAC hemoglobin repletion assay and measured the surface area by nitrogen absorption. Solubility in dilute HCl at pH 1.0 and 1.7 was determined over various dissolution periods. RBVs were found to be significantly less than ferrous sulfate ($P < 0.05$). Better

RBV predictability was achieved when the method was modified to use a lower pH and less dissolution time ($R^2$ = 0.82, pH 1.0 for 30 min). Surface area measurements ranged from 90 to 370 $m^2$/kg and were highly predictive of RBV ($R^2$ = 0.80). Harrison and others (1976) separated (by nitrogen elutriation) iron powders into different particle size fractions and found that reduced iron fractions of 7-10 μm and 27-40 μm had RBVs of 68-75% and 27-29%, respectively, using the AOAC hemoglobin repletion bioassay. In the same study, the RBVs of five ferric orthophosphate samples with different average particle size ranging from 1 to 14 μm (estimated by microscopy) had RBVs between 6 and 46%. RBV was positively correlated with solubility in 0.10 N HCl (R = 0.99) and negatively correlated with mean particle size (R = 0.95). The authors concluded that reduced iron should have a mean particle size < 10 microns to achieve a RBV that approaches 50%.

In this study, the method developed by Shah and others (1977) and modified by Forbes and others (1989) was developed further to reduce the variability of the assay. The method was modified to incorporate a dissolution apparatus commonly used to measure the solubility of solid vitamin and mineral preparations (USPC 2006). This equipment carefully controls temperature, mixing shear and time, which allowed precise control of the assay parameters to allow optimization. The 250-ml volume required in the dissolution apparatus allows for larger samples sizes, which is useful for obtaining a representative sample of a nonhomogeneous material, e.g. iron fortificants with a range of particle sizes and amorphous contents. Three different HCl concentrations (0.02, 0.05 and 0.1 N), covering the normal physiological pH range found in the stomach, were tested at different shear rates and dissolution times. The 0.10 N HCl concentration was

found to give the most reproducible measure of solubility (CV < 1%), although the coefficient of variation for all the concentrations was ≤ 5% (Table 3.2). This was in agreement with the study by Swain and others (2003). The method development in this study resulted in a validated, robust technique that can potentially be used for intra-laboratory comparisons. Regression analysis found the method to be a good predictor of RBV ($R^2 = 90\%$, $P = 0.008$).

It is generally accepted that particle size plays an important role in the bioavailability of iron fortificants, particularly reduced iron powders. Less is known about the relationship of particle size and bioavailability for the other forms of iron. However, suppliers routinely specify the average particle size of their ingredients, typically using sieving techniques, for insoluble iron sources like ferric orthophosphate. A smaller particle size is thought to result in greater solubility in the gut and therefore higher bioavailability. However, results of this study did not find the particle size of ferric orthophosphate to be well correlated to either solubility or bioavailability with the median particle size giving the best fit of the particle size distribution statistics ($R^2 = 0.3663$).

Sources 6 and 8 were not statistically different by PSD results and had the largest median PS (median PS 17.7 and 15.5 μm, respectively). These sources, as expected, had the lowest bioavailability of the sources tested (RBV 51 and 60%, respectively). Sources 5 and 7 were also not statistically different and had the smallest median PS (2 μm) and higher RBVs (78 & 83%, respectively) than Source 6 and 8. Source 4 was distinctly different from all the other sources. It had a median PS of 9.3 μm and a markedly higher RBV of 99%. Multinomial curve fitting identified two normal distributions of particles within the distribution for Source 4. One population comprised 33-34% of the sample and

was made up of particles with a mean PS of 6 μm. The second population of was composed of large particles with a mean PS of 50 μm, comprising 66-70% of the sample. The high RBV was also reflected in the high solubility of Source 4, which had 100 to 1000-times the solubility of the other sources. Its drastically higher solubility could not be accounted for by particle size alone and indicated that other physical properties were playing an important role in its behavior.

Average particle size measurement is often used as a simple and inexpensive quality specification to indicate higher quality (higher solubility in the gut) iron sources, particularly for reduced iron powders, but it may be misleading when it is the only quality measure used to predict bioavailability. Surface area provides a more comprehensive measure, taking into account both surface and internal structure. It is often used in the pharmaceutical industry as a predictor of dissolution behavior of drug and supplement preparations in the body. This research found surface area ($R^2$ = 0 .8504 ) to be a better predictor than particle size ($R^2$ = 0.3366) of solubility and bioavailability. Sources 4, 5 and 7 had 10-times higher surface area than Sources 6 and 8, yet the SA of Source 4 was only slightly larger than the SA for 5 and 7 and could not completely explain the marked difference in its solubility.

Surface area is influenced by several physical properties, such as particle size and microstructure. Semi-crystalline powders, like ferric orthophosphate, can have varying amounts of disorder in their microstructures. Amorphous content refers to regions of molecular disorder within a semi-crystalline solid that is formed during crystallization, typically during subsequent processing, like milling and spray-drying; it is seldom a deliberate or controlled event (Buckton and Darcy 1999). Process-induced amorphous

content is thought to be localized mostly at the powder surface, increasing surface area. Amorphous regions in crystalline material will absorb water to a greater degree than crystalline regions and are often thermodynamically unstable. The presence of amorphous particle structure is known to be an extremely important determinant of the bioavailability of active pharmaceutical ingredients and has been given increasing attention in the pharmaceutical sciences over the past ten years (Mackin and others 2002; Burnett and others 2006). It is now recognized that even relatively low levels of amorphous material (<5%) affect stability and dissolution characteristics (Buckton and Darcy 1999; Mackin and others 2002; Burnett and others 2006). Therefore, it was hypothesized that the amorphous content of ferric orthophosphate may be an important determinant of bioavailability in humans and may also be responsible for negative effects on food.

Gravimetric vapor sorption (DVS) was chosen as the technique to measure the amorphous content of ferric orthophosphate powders. This technique is routinely used to measure amorphous contents below 5 -10% and the amount of amorphous material in the ferric orthophosphate powders was unknown. In addition, moisture uptake is the main mode of product quality failure in RTE cereal making DVS a potentially promising tool to study shelf life and stability in RTE cereal.

In order to quantitate the amorphous content of a material using DVS, an amorphous standard with known amorphous content is needed and a 100% amorphous standard is typically used. A ferric orthophosphate standard was not available commercially at the time of this work. Therefore, the standard was made using Source 4, since the physicochemical behavior of this material indicated a higher amorphous

content. The standard material made from Source 4 was found to be extremely hydroscopic and volatile during analysis. However, semi-quantitative results were possible, providing an estimate of the amorphous content of the ferric orthophosphate powders. The amorphous content was calculated directly from the moisture uptake data by a ratio comparison of the moisture uptake of the standard to the moisture uptake of the sample under identical experimental conditions. The instability of the standard was evidenced by sublimation that caused a slight decrease in mass during repeated isotherm cycles during an analysis. In addition, mass equilibration was closely approximated but not fully reached above 10% RH during the 1000-minute isotherm cycle-time used in the method. This was due to the high moisture absorption capacity of the standard. Semi-quantitative estimations of amorphous content were made using the data at 10% RH.

The amorphous contents of the samples ranged from $< 2\%$ to more than 15% (by weight). Source 4 appeared to contain at least 5 times more amorphous material than the other sources, accounting for its high solubility and resultant high bioavailability. Solubility increased rapidly as the amorphous content increased requiring a semi-log regression analysis. A comparison of the data fit for surface area, moisture uptake (e.g. amorphous content), or median particle size versus % RBV found moisture uptake to have the best correlation ($R^2 = 0.9527$) to %RBV, followed by surface area ($R^2 = 0.8504$) and particle size ($R^2 = 0.3663$).

Bioavailability is dependent on the solubility of the iron source, and solubility depends on several interdependent physicochemical factors. A 2D multiple regression model of $\log_{10}$ solubility versus surface area and moisture uptake showed these two properties to be excellent predictors of solubility ($R^2 = 0.9333$, $P = 0.001$). However, this

regression model did not meet the 95% confidence level ($R^2$ .8480, $P$=0.078) when used to predict %RBV. A possible explanation is that although surface area is highly correlated to solubility, its value is dependent on the sum of the nitrogen absorption of all the particles present. SA does not distinguish amorphous particles from the less soluble particles that are much larger in number forming the bulk of the material. A multiple regression model of median particle size and moisture uptake was found to be a better predictor of % RBV ($R^2$ = 0.9333, $P$ = 0.035) indicating the interaction between these two properties may be a more sensitive measure of the soluble particles than surface area. This model was used to predict the % RBV for four additional lots of the following sources that did not have actual % RBVs from AOAC Rat Hemoglobin Repletion data : Source 4 (1 lot), Source 5 (1 lot), Source 6 (two lots) with %RBVs of 86, 83, 52 and 53, respectively. These values were very close to %RBVs for the tested lots of the same sources.

A multiple regression model of median particle size and moisture uptake versus solubility was also a good predictor of solubility ($R^2$ = 0.9100, $P$ = 0.001). Median particle size, where 50% of the sample is less than the indicated size (μm), gave the best correlation to % RBV and solubility as compared to the mean particle size, the 90% and the 10% statistics as well as the uniformity statistics. This indicates that the amorphous material may not be uniformly distributed throughout the material and may reside in the smaller particle size fraction.

# CHAPTER 4

# SENSORY PROPERTIES OF RTE CEREAL FORTIFIED WITH DIFFERENT SOURCES OF FERRIC ORTHOPHOSPHATE

## 4.0 INTRODUCTION

Hydrogen reduced iron is the most widely used iron source for food fortification due to its relative low cost and availability in a range of particle sizes, surface area, porosity and purity. Its availability over a wide-range of physical parameters allows food manufacturers some control over the form used for a particular ingredient or product application. As discussed earlier, bioavailability and reactivity are related to the physicochemical properties of the iron form and its effect on product quality and shelf life. The different iron forms have different degrees of reactivity, which generally impair the organoleptic properties and shelf life of foods, thus limiting the iron forms used in foods to those that are less reactive. Unfortunately, this also limits the use of more bioavailable iron sources due to their negative effect on quality.

Due to the need to optimize food quality and bioavailability, the effect of the iron sources used in this research on food quality was determined. The sensory attributes of a standard RTE flaked-corn cereal made with the different iron sources were compared to the sensory attributes of cereal made with a commercially available, encapsulated reduced-iron source known to have minimal effects on food quality.

## 4.1 MATERIALS AND METHODS

### *4.2.1. Materials*

A standard corn-based, RTE-cereal was fortified with iron Sources 1-9, which included ferrous sulfate (Source 1), encapsulated, hydrogen-reduced iron (Source 2, control), hydrogen-reduced iron (source 3) and ferric orthophosphate (Source 4-9). Source 2 was encapsulated with zein (corn protein), designed to survive pressurized cooking and milling processes and to dissolve in stomach acid.

Products were produced in a research pilot plant in 200-lb batches. Cereal was fortified at the 45 % RDI level for iron for adults (29 mg iron/100g food) with each of the Sources 1-9. The cereals were also fortified with the following vitamins and minerals, typical for the standard RTE cereal:

**Table 4.1 Vitamins and minerals added to the test cereals**

| Vitamin or Mineral | RDI | Vitamin or Mineral | RDI | Vitamin or Mineral | RDI |
|---|---|---|---|---|---|
| A | 15 | $B_1$ | 25 | $B_2$ | 25 |
| $B_6$ | 25 | $B_{12}$ | 25 | C | 25 |
| $D_2$ | 10 | E | na | Fe | 45 |
| Folic acid | 25 | Niacin | 25 | Zn | 25 |

### *4.2.2. Methods*

Eleven test batches of RTE cereal were produced in a research pilot plant using a standard commercial formula and processing conditions for a flaked, corn based cereal. One lot of each iron source was used to make one 200 lb batch of test cereal. The cereal was made from whole corn grits cooked in a pressure vessel with added water, flavor, sweeteners and fortificants. All iron sources were added to the cereal cooker along with the

corn grits and other ingredients except for heat sensitive or chemically incompatible fortificants (vitamins A, $D_2$, E, $B_1$, C), which were sprayed on after cooking. Cooking temperatures and time exceeded 250°F and 50 minutes, respectively. The cereal made with encapsulated reduced-iron (Source 2) served as the control food to which all other test cereals were compared. The appearance, taste and texture of the cereals were compared to the control cereal by the six-member taste panel, who ranked the cereals by consensus on a scale of 1 –10, with 10 matching or exceeding the quality of the control product.

## 4.3 RESULTS

There were obvious food quality differences among the test cereals with regards to appearance, odor and flavor. Results are shown in Table 4.1. Sources 1 and 4 had the greatest negative effect on food quality and were ranked 1 with extreme sensory defects (off-color, gray-green spots, metallic aftertaste). Cereal made with Source 8 had equivalent product quality as the control cereal (Source 2), which had a bright yellow color, toasted corn aroma and flavor. The following rank scores for the test cereals fortified with decreasing order of quality: Source 8 (10) > Source 2 (10) > Source 6 (8) > Source 9 (7) > Source 7 (6) > Sources 3 and 5 (5) > Sources 4 and 1 (1).

**Table 4.2. Sensory scores of RTE cereal made with iron Sources 1-9**

| Iron Source | ID | Sensory Description | Sensory Score* (1-10) |
|---|---|---|---|
| Ferrous sulfate | Source 1 | Extreme presence of grayish green discoloration, metallic off-odor and metallic taste with bitter after taste | 1 |
| Encapsulated H-reduced iron | Source 2 | Control iron source used as a quality standar for comparison with other iron sources | 10 |
| H-reduced iron | Source 3 | Poor quality but less grayish green discoloration, off-odor and metallic off-flavor than Source 1 and 4 | 5 |
| Ferric orthophosphate | Source 4 | Extreme presence of grayish green discoloration, metallic off-odor and metallic taste with bitter aftertaste | 1 |
| Ferric orthophosphate | Source 5 | Poor quality but less grayish green discoloration, off-odor and metallic off-flavor than Source 1 and 4 Noticeable gray spots on flakes. | 5 |
| Ferric orthophosphate | Source 6 | Slight dullness to flakes and a few grayish areas, good initial taste and no off-odor or aftertaste | 8 |
| Ferric orthophosphate | Source 7 | Slight grayish off-color, dull color and aftertaste evident | 6 |
| Ferric orthophosphate | Source 8 | No gray spots, slight dullness, no off odor and good toasted corn flavor | 10 |
| Ferric orthophosphate | Source 9 | Slight dullness to flakes and gray off-color | 7 |

* 6 Panel Members by Consensus, Food Scores: 1 = worst quality; 10 = best quality

## 4.4 DISCUSSION

The food was packaged in high density polyethylene liners containing 0.05 lbs BHT antioxidant per ream. Sensory evaluation took place three-weeks after production, which is typical practice to allow products to equilibrate before sensory testing. Fortification with Source 8 yielded the best quality food and was a potential match to standard cereal made with encapsulated reduced iron. It was interesting that the cereal made with highly soluble ferrous sulfate was similar to that made with ferric orthophosphate, Source 4. These products had obvious product defects with grayish-

green discoloration, a strong metallic off-flavor and a bitter aftertaste. Texture, color and toasted cereal flavor of the products were diminished. In a sensory study by Lim and lawless (2006) on the detection thresholds and taste qualities of iron salts, detection thresholds for several soluble iron salts were between 20.5 – 99.2 μm. The authors concluded that the taste of iron salts can be adequately described by a combination of four basic tastes as well as metallic and astringent if only the last two terms are used by panelists.(Lim and Lawless 2006).

The RBV of the Source 1 and Source 4 were not statistically different and were the highest of the sources tested (RBV 100% and 99%, respectively). All the iron sources used in the research had RBV greater than 50, which is generally accepted practice in the cereal industry to be the lower RBV cut-off for iron fortificants. As expected, good quality scores increased as the RBV decreased. Food sensory scores below 8 were not considered acceptable for commercial production, which limited the commercially viable iron sources to Sources 8 and 6.

## SUMMARY AND CONCLUSIONS

The hypothesis of this research dissertation is that the amorphous content of ferric orthophosphate is a critical determinant of solubility and therefore its bioavailability. In order to test this hypothesis, the first objective of this research was to determine the relative bioavailability of a variety of commercially available food-grade, ferric orthophosphate powders using *in vivo* and *in vitro* methods . The *in vivo* approach used in this research was unique in that the RBVs of the of the iron sources were processed in a RTE-cereal. Typically, in the AOAC Rat Hemoglobin Repletion Bioassay, the test iron sources are added to the animal diets as supplements, and are not tested in a food so the effects of the food matrix and food processing parameters were not considered.

The *in vivo* RBV was determined for six commercial sources of ferric orthophosphate and a rapid, reproducible *in vitro* method was established to estimate RBV using the solubility of ferric orthophosphate in dilute HCl. The RBVs of the test sources were found to be variable (RBV 51-99%). However, the overall RBVs were higher that those reported in the literature. As expected, the solubilities of the sources in dilute HCl also varied (0.02 – 21%) and were found to be highly correlated with RBV ($R^2$ 90%, $P = 0.008$). The reproducibility in the *in vitro* solubility assay was excellent (CV < 1%).

The second objective of this work was to understand the physicochemical properties that influence solubility and ultimately bioavailability. Particle size and surface area are more commonly used to characterize the physicochemical behavior of materials, such as solubility. However, in the last 10-years, the importance of amorphous

content, particularly in the pharmaceutical industry, has become increasingly recognized as techniques to measure it accurately became available.

Gravimetric vapor sorption (DVS) was chosen as the technique to measure the amorphous content of ferric orthophosphate powders due to its sensitivity and underlying principle. Vapor sorption or moisture uptake is the main mode of product quality failure in RTE cereal making DVS a potentially promising tool to study shelf life and stability in RTE cereal.

Semi-quantitative measurements found the six sources of ferric orthophosphate to have low to intermediate amorphous contents (2-20%). Amorphous content (moisture uptake) had the strongest correlation to solubility followed by surface area and then particle size. However, a regression model of median particle size and moisture uptake was found to give the best estimates of RBV. Thus, the combination of median particle size and moisture uptake appeared to be a more sensitive predictor of RBV than surface area and moisture uptake. The observation might be due to the sensitivity of the median particle size statistic for distinguishing amorphous material versus surface area, which measures a bulk property. More work is needed to reproduce and confirm these results using a validated, quantitative method for determining amorphous content.

Relating the physicochemical properties that determine solubility and ultimately bioavailability to food quality, is the final aim of this research. A standard RTE cereal was fortified with the test sources of ferric orthophosphate and evaluated for food quality against a control cereal. The control was fortified with a commercially available, encapsulated H-reduced iron powder known to make excellent quality products. Food quality was determined by sensory panel testing and only Source 6 and 8 produced food

comparable to the control cereal. Product defects included grayish-green discoloration, off-odor, a strong metallic off-flavor and a bitter aftertaste. Interestingly, cereal made with highly soluble and reactive ferrous sulfate was similar to that made with ferric orthophosphate, Source 4.

In conclusion, solubility appears to be a critical determinant of the bioavailability and reactivity of ferric orthophosphate and is dependent on the interaction of particle size, surface area and perhaps most importantly, the presence of amorphous microstructure. The effect of varying amounts of amorphous material on solubility is not linear with solubility, increasing rapidly in a power law relationship as the amorphous content increases between 3 and approximately 20%. The %RBV of ferric orthophosphate was as high or higher than the H-reduced iron typically used to fortify RTE-cereal.

Ferric orthophosphate ($FePO_4$) is a promising iron source for food fortification because of its light color and oxidative stability. However, $FePO_4$ has had limited use in cereal due its variable and often reported low bioavailability. This research contributes toward a better understanding of the physicochemical properties that are determinants of its bioavailability. An understanding of the mechanisms underlying this variability may facilitate production of a $FePO_4$ source with consistent bioavailability and also offer an affordable iron source for applications where improved oxidative stability is needed.

# FUTURE RESEARCH

**Suggestions for future research:**

Further work is needed to validate the DVS technique using a well-characterized amorphous standard and test the RBV *in vitro* prediction models against new lots of ferric orthophosphate. These tools are needed to answer the following question: How closely can the amorphous content and particle size of ferric orthophosphate powders be controlled within cost and variability limitations?

One of the test sources of ferric orthophosphate (Source 4) was markedly different than the other sources and shed valuable insight on the properties influencing bioavailability. However, there were no sources available that had an amorphous content between 3 and approximately 20%. In addition, the bioavailability and food quality varied markedly at amorphous content between 1 – 3%. Can particle size and amorphous content be optimized to produce material within wider process allowances that maintains improved bioavailability?

Finally, Can the degree of amorphous material present be manipulated to optimize food quality and bioavailability? We do not know if it is a matter of the degree of disorder in all the particles present or if there is a specific soluble population that is determining bioavailability.

## APPENDIX A

### Beckeline Test Results for Refractive Indices of Ferric Orthophosphate

Analysis was performed by Bayard development Company, 1425 W. Summerdale Ave. Chicago, IL 60640, 773-728-0531

The refractive indices for the sodium d line (589 nm) were determined for the ferric orthophosphate sources using the standard microscopic immersion method.

**Table 1.0 Refractive Indices for Ferric Orthophosphate Sources**

| Iron Source | Refractive Index | Observations |
|---|---|---|
| Source 4, Lot 1 | 5%: 1.680 – 1.705<br>50 – 55%: 1.680<br>40 – 45%: 1.632 – 1.675 | Isotropic mixture |
| Source 4, Lot 2 | 3-5%: 1.680 – 1.706<br>60 – 65%: 1.680<br>30 – 35%: 1.630 – 1.675 | Isotropic mixture |
| Source 5, Lot 1 | 1.681 | Isotropic – very small fused crystallites |
| Source 5, Lot 2 | 1.680 | Isotropic – very small fused crystallites |
| Source 6, Lot 1 | 1.687 – 1.704 | Birefringent sphereulites |
| Source 6, Lot 2 | 1.674 – 1.709 | Very poorly formed sphereulites |
| Source 6, Lot 3 | 1.675 – 1.707 | Very poorly formed sphereulites |
| Source 7, Lot 1 | 1.682 | Isotropic – very small fused crystallites |
| Source 8, Lot 1 | 1.687 – 1.704 | Birefringent sphereulites |

# APPENDIX B

## 3-Point B.E.T Surface Area Analysis – Instrument Parameters

Analyses were performed at Particle technology labs, Ltd., Downers Grove, IL 6-515, 630-969-2703

Instrumentation: Quantachrome® Autosorb AS-1 Static-Pressure Analyzer

Sample weight: approximately 2.2 -2.6 grams (four decimal places)

**Experimental parameters:**

Adsorbate: Nitrogen

Cross-section area: 16.2 Angstroms $^2$ /molecule

Non Ideality: 6.580E-05

Molecular Weight: 28.135 g/mol

Outgas Temp: 25° C

Outgas Time: 16 hours

Analysis Time: approximately 38-83 minutes (sample dependent)

Bath Temp: 77.4 °K

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