GR

ORIGINAL SUBMISSION

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Nestlé USA

800 NORTH BRAND BLVD
GLENDALE, CA 91203
TEL (818) 549-6000

Nestleusa com

REGULATORY / NUTRITION / LABELING

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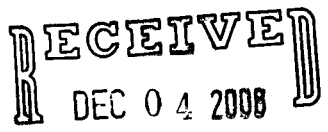

November 17, 2008

Robert L. Martin, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notice for Ferrous Ammonium Phosphate

Dear Dr. Martin:

On behalf of Nestlé USA, Inc., 800 North Brand Boulevard, Glendale, CA 91203-1244, I am submitting this notice of a claim for exemption from premarket approval requirements of the Federal, Food, Drug and Cosmetic Act in accordance with proposed 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized As Safe (GRAS) determination. 62 FR 18938 (17 April 1997)].

The subject of this notice is ferrous ammonium phosphate, which, as characterized and under the conditions of use as a food ingredient specified in the enclosed documents, we believe to be GRAS on the basis of scientific procedures. The enclosed documents set forth the basis of the GRAS determination, and include a comprehensive summary of the data available and reviewed by an independent panel of experts. The *curricula vitae* demonstrating the qualifications of the members of the panel of experts who made the determination of safety are also enclosed.

I trust that the enclosed Notice is acceptable. Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

Mark F. Nelson, Ph.D.
Director of Scientific & Regulatory Affairs
Nestlé USA, Inc.
800 North Brand Boulevard
Glendale, CA 91203-1244
+1 (818) 549-5530
mark.nelson@us.nestle.com

Encl.

RECEIVED
DEC 04 2008
BY:_____

FERROUS AMMONIUM PHOSPHATE GRAS NOTICE

Prepared for: Robert L. Martin, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Prepared by: Nestlé USA, Inc.
800 North Brand Boulevard
Glendale, CA 91203-1244

November 17, 2008

FERROUS AMMONIUM PHOSPHATE GRAS NOTICE

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I. GRAS EXEMPTION CLAIM

A. Claim of Exemption From the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.30(c)(1) [62 FR 18938 (17 April 1997)] (U.S. FDA, 1997)

As defined herein, ferrous ammonium phosphate, an iron salt, has been determined by Nestlé USA, Inc (Nestlé) to be Generally Recognized as Safe (GRAS) for use in a variety of traditional food products. This determination is based on scientific procedures, as described in the following sections, under the conditions of their intended use in food. Therefore, consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act,* the use of ferrous ammonium phosphate in food as described below is exempt from the requirement of premarket approval.

Signed,

17 NOV 08

Mark F Nelson, PhD
Director Regulatory Affairs US and Canada
Nestlé USA, Inc.
800 North Brand Boulevard
Glendale, CA 91203-1244

Date

B. Name and Address of Notifier

Nestlé USA, Inc.
800 North Brand Boulevard
Glendale, CA 91203-1244

C. Common Name of the Notified Substance

Ferrous ammonium phosphate

D. Conditions of Intended Use in Food

Nestlé intends to use ferrous ammonium phosphate as a food ingredient in various traditional food products intended for sale on the United States (U.S.) market. The intended food uses include beverages and beverage bases, grain products and pastas, milk products, and soups and soup mixes. Ferrous ammonium phosphate is proposed as a direct replacement for other iron fortificants currently in use in existing categories of fortified foods in the U.S. and will not be used in products specially formulated or processed for use by infants or children less than 4 years of age. Fortification will be in accordance with the Fortification Policy (21CFR §104.20)

RACC) sources of iron where the DV is currently 18 mg/person/day for adults and children 4 or more years of age. Fortification will be in accordance with the Fortification Policy (21CFR §104.20) outlined in the FDA's Nutritional Quality Guidelines for Foods (21CFR §104) (U.S. FDA, 2008). Ferrous ammonium phosphate is only proposed as a direct replacement for other iron fortificants currently in use in existing categories of fortified foods in the U.S. and will not be used in products specially formulated or processed for use by infants or children less than 4 years of age.

E. Basis for the GRAS Determination

Pursuant to 21 CFR § 170.30, ferrous ammonium phosphate has been determined by Nestlé to be GRAS on the basis of scientific procedures (U.S. FDA, 2008). This GRAS determination is based on data generally available in the public domain pertaining to the safety of iron, ammonium, and phosphate salts, as discussed herein, and on a consensus among a panel of experts[1] [who are qualified by scientific training and experience to evaluate the safety of ferrous ammonium phosphate as a component of food [see Appendix A, entitled, "**EXPERT PANEL CONSENSUS STATEMENT REGARDING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF FERROUS AMMONIUM PHOSPHATE FOR USE IN FOODS**"].

F. Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. Food and Drug Administration (FDA) upon request, or will be available for review and copying at reasonable times at the offices of:

Nestlé USA, Inc.
800 North Brand Boulevard
Glendale, CA 91203-1244

Should the FDA have any questions or additional information requests regarding this notification, Nestlé will supply these data and information.

---

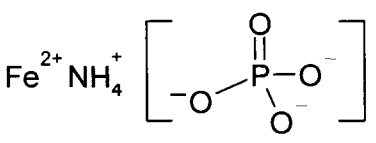
[1] The panel of experts consisted of Prof Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University, School of Medicine), Prof Robert Nicolosi, Ph.D. (University of Massachusetts Lowell), and Prof. John Thomas, Ph D. (Indiana University, School of Medicine).

II. DETAILED INFORMATION ABOUT THE IDENTITY OF THE SUBSTANCE

A. Identity

Ferrous ammonium phosphate is an inorganic salt of iron(II), ammonium, and phosphate ions (see Figure 1) and occurs as a grey/green fine powder. The relative percentages (w/w/w) of iron, ammonia, and phosphate are consistent with a 1:1:1 inorganic salt associated with a small amount of water ($FeNH_4PO_4.xH_2O$ where x = 1 or less typically). The percentage iron(II), ammonia, and phosphate are restricted to well defined ranges by the product specification that ensure the product is always consistent with this formula. During the manufacture of ferrous ammonium phosphate, approximately 5% of the iron(II) ions are oxidized to iron(III) (ferric ions). The total amount of iron(III) permitted in the final product is restricted to no more than 7% by the product specification.

Common or Usual Name:	Ferrous ammonium phosphate
Chemical Name:	Ammonium iron(II) phosphate
Chemical Abstracts Service (CAS) Number:	10101-60-7
Empirical Formula and Formula Weight:	$FeNH_4PO_4$
Molecular weight:	168.85 (anhydrous salt)
Structural Formula:	See Figure 1.0



(a small percentage of water will be associated with the salt)

Figure 1 Structure of Ferrous Ammonium Phosphate

B. Method of Manufacture

An overview of the manufacturing method is provided in Figure 2. Ferrous ammonium phosphate is manufactured consistent with current Good Manufacturing Practices (cGMP) by the reaction of ammonium hydroxide, iron powder, and phosphoric acid in demineralized water. Iron powder (21 CFR §184.1375), ammonium hydroxide (21 CFR §184.1139), and phosphoric acid (21 CFR §182.1073) (U.S. FDA, 2008) all are affirmed as GRAS for use in the manufacture of foods with no limitations other than cGMP. Any excess starting materials or insoluble salts will be removed during the purification (*e.g.*, unreacted iron powder) and spray drying (*e.g.*,

volatiles, such as ammonia) processes. The manufacturing method is straightforward and utilizes common procedures employed by the vitamins and minerals industry. A sample is taken of all packaged units and blended to form a representative batch sample which is subject to inspection by the Quality Control department to ensure it meets the required purity criteria and other product specifications. The appropriate production controls are in place to ensure the quality and consistency of the ferrous ammonium phosphate produced. The manufacturing site operates under the principles of GMP and is certified by the German Health Authorities. The production process is overseen by the Quality Assurance department and all raw materials, intermediates, and final product are monitored by the Quality Control department. The analytical data provided in Section II.C confirm that no toxicological or microbiological hazards arise during the process that would have an adverse effect on health.

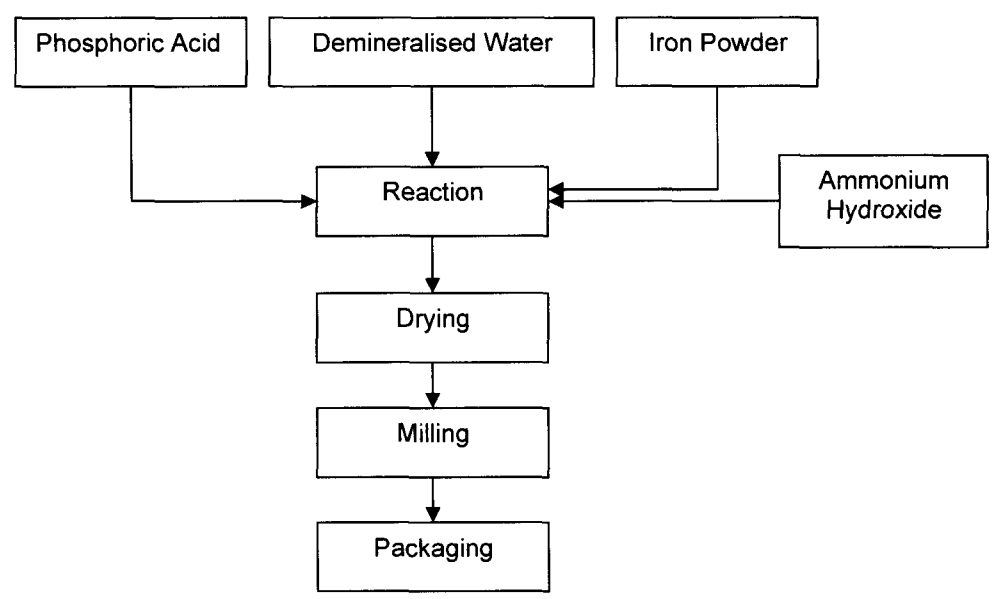


Figure 2 Schematic Overview of the Manufacturing Process for Ferrous Ammonium Phosphate

C. Specifications and Analytical Data for Food Grade Material

Ferrous ammonium phosphate is produced in accordance with cGMP, and in order to ensure consistent, safe products, Nestlé has established food-grade specification parameters for the final ingredient (see Table 1). These parameters comprise specifications for the physical appearance, identification, and purity of ferrous ammonium phosphate as well as specifications for potential chemical and microbiological impurities and contaminants. The identity of ferrous ammonium phosphate is determined by qualitative tests that detect the presence of iron(II), ammonium, and phosphate ions. The specific ranges for iron, ammonia, and phosphate defined in the product specifications ensure that the product is always consistent with a 1:1:1 (w/w/w)

combination of iron, ammonium, and phosphate as an inorganic salt associated with a small percentage of water, confirming the chemical formula to be $FeNH_4PO_4.xH_2O$ (where x = 1 or less typically). Although no international purity standards are currently available for this material, the parameters included in the product specification are typical of that of analogous inorganic salts. For example, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) Monograph for ferrous sulfate includes conventional identification tests for iron and sulfate in addition to restrictions on the levels of lead and mercury (JECFA, 2006). In addition, small amounts of iron(III) are generated during production of ferrous ammonium phosphate; however, the total amount of iron(III) permitted in the final product is restricted to no more than 7% by the product specification and is monitored by the Quality Control department.

Analyses of three commercial batches of ferrous ammonium phosphate confirm that the material produced by the manufacturing process is consistent and complies with these product specifications. The analytical data also demonstrate the absence of microbiological contamination. The complete analyses for these batches are presented in Appendix B. Where available, all analytical procedures follow established International/European methods.

Table 1 Product Specifications for Ferrous Ammonium Phosphate

Parameter	Specification	Method of Analysis
Appearance		
Description	Fine powder	Visual
Color	Grayish green	Visual
Identification		
Iron(II) salts	Positive	FCC, Appendix III, A Identification Tests
Ammonium (NH_4^+) salts	Positive	FCC, Appendix III, A Identification Tests
Phosphate (PO_4^{3-}) salts	Positive	FCC, Appendix III, A Identification Tests
Properties		
pH (5% suspension)	pH 6 8-7 8	Ph Eur. *2.2.3*
Purity		
Iron(II) Assay	22-30% w/w	Ph Eur [Monograph 'Ferrous Fumarate']
Ammonia (NH_3) Assay	5-9% w/w	Distillation of Ammonia
Phosphate (PO_4^{3-}) Assay	49-55% w/w	Photometry (Lange LCK 348)
Iron(III) Assay	Max 7% w/w	FCC [Monograph 'Ferrous Fumurate']
Total Iron	Min. 28.0% w/w	Sum of Iron(II) and Iron(III)
Water (H_2O)	Max. 3.0% w/w	Ph. Eur. *2.5.32* (Karl Fischer)
Impurities		
Sulfate (SO_4^{2-})	Max. 0.2% w/w	BPC 1973 [Monograph. 'Iron Phosphate']
Aluminum (Al)	Max. 30 mg/kg	Ph. Eur. *2.2.23*, Method I (AAS)
Arsenic (As)	Max 3 mg/kg	FCC [Monograph: 'Ferric Phosphate']
Lead (Pb)	Max 10 mg/kg	Ph Eur. *2.2.23*, Method I (AAS)
Cadmium (Cd)	Max. 3 mg/kg	Ph. Eur. *2.2.23*, Method I (AAS)

Table 1 Product Specifications for Ferrous Ammonium Phosphate

Parameter	Specification	Method of Analysis
Nickel (Ni)	Max. 50 mg/kg	Ph Eur. 2.2.23, Method I (AAS)
Fluoride (F^-)	Max. 50 mg/kg	Nestlé (in-house validated) LI-00.855
Microbiology		
Aerobic mesophilic count	Max. 500.0 cfu/g	Nestlé (in-house validated) LI-00.701-2

Abbreviations: AAS, Atomic Absorption Spectrometry; BPC, British Pharmacopeia Codex, FCC, Food Chemicals Codex; Ph. Eur., European Pharmacopeia.

D. Stability of Ferrous Ammonium Phosphate

The recommended storage conditions for ferrous ammonium phosphate (in bulk) are:

Temperature:	5 to 10°C
Humidity:	20 to 60%
Packing Material:	25 kg paper bags, stored sealed and dry
Re-Test Period:	12 months

The proposed storage conditions are supported by stability experiments performed on the material retained in its original packaging. Representative real-time stability data over typical storage conditions and accelerated stability experiments at elevated temperature/humidity have been conducted in accordance with the International Conference on Harmonisation guidelines (ICH, 2005) to confirm the storage requirement and re-test period. A clear indicator of degradation is measurement of the iron(III) content which increases on oxidation of iron(II) over prolonged periods, high temperatures, or increased humidity (note the total iron content is the sum of iron(II) and iron(III) and will therefore remain constant). The results of these studies confirmed that the iron(III) content remains within specification throughout the shelf-life of the bulk product.

Ferrous ammonium phosphate also has been shown to be suitable for use in proposed food products in which maintenance of the organoleptic properties is a concern. The addition of soluble iron salts to tea, chocolate milk, or banana-containing products may result in an interaction between the metal ion and iron sensitive components, such as polyphenols, in the food matrix. The resulting iron/phenol complex is an intense dark grey or black color causing the product to become discolored, particularly when a powdered beverage formulation is reconstituted with water or milk at high (boiling) temperatures. Additionally, the addition of soluble iron sources to fat containing products, in particular products high in unsaturated fatty acids, can result in lipid oxidation and lead to changes in the flavor and nutritional quality of the product. Therefore, the choice of iron compound used for fortification is typically that which demonstrates the highest bioavailability without provoking unacceptable organoleptic characteristics during production, storage, or typical conditions of use. For example, in products sensitive to taste, odor, and color changes, such as infant cereals, chocolate drinks, and milks

containing long chain polyunsaturated fatty acids, an iron salt, such as ferric pyrophosphate, that has low reactivity (*i.e.*, greater stability) in the finished product is used. Although ferric pyrophosphate exhibits desirable organoleptic properties, its bioavailability is low. In contrast, ferrous sulfate is established to be highly bioavailable, but also results in discoloration, off-flavor, and fat oxidation in these types of food products, which limit its practical application. Ferrous ammonium phosphate has been developed as an alternative with the advantage that it has high stability at neutral pH in formulated foods but readily dissociates under the low pH conditions of the stomach to become nutritionally available. It is intended to be used in products where low reactivity similar to ferric pyrophosphate and high bioavailability similar to ferrous sulfate is required, in particular those containing polyphenols or which have a high fat content.

All food products fortified with the ferrous ammonium phosphate should demonstrate the appropriate levels of organoleptic and nutritional stability prior to marketing using the appropriate test methodology. Experiments to evaluate discoloration both visually and by colorimetry have been performed in a range of beverages fortified with ferrous ammonium phosphate under typical formulation and reconstitution conditions and compared with controls containing either no supplemental iron salts or the common fortificant, ferrous sulfate [note that the intellectual property from a number of these studies has been patented (Sher *et al.*, 2001; Rekhif *et al.*, 2002)]. In general, no color changes were observed in tea, cocoa powders, or milk products under conditions typical of use. In all the majority of cases, ferrous ammonium phosphate was more effective than ferrous sulfate at retaining product color and was often comparable to the product without iron supplementation. Additionally, accelerated stability studies have been conducted with fish oils fortified with ferrous ammonium phosphate in order to mimic the potential effect of fortifying foods high in unsaturated fats (Sher *et al.*, 2001, Rekhif *et al.*, 2002). The time taken for the fish oil to undergo oxidation when fortified with ferrous ammonium phosphate was comparable to the control (without iron supplementation) and significantly slower than for oils fortified with ferrous sulfate. Unlike fish oils, food products will typically only contain a percentage of fat rather than being comprised entirely of fat and these studies also provide strong evidence that ferrous ammonium phosphate does not decrease the nutritional quality of the food as a result of fat oxidation. Furthermore, accelerated and real-time storage studies in cereals and a range of milk-based powders indicate that these foods, fortified with ferrous ammonium phosphate, are stable over prolonged periods (representative of shelf-life) and that product quality is improved if exposure to air is minimized. Typically, the stability of foods fortified with ferrous ammonium phosphate was comparable or greater than those fortified with ferrous sulfate or ferric pyrophosphate (Sher *et al.*, 2001).

III. SELF-LIMITING LEVELS OF USE

Although ferrous ammonium phosphate has more favorable organoleptic properties than other sources of iron, the use of ferrous ammonium phosphate at levels significantly higher than those proposed will result in unfavorable color, odor, and taste qualities of the food, thus making it

unpalatable. The levels of use of ferrous ammonium phosphate are, therefore, limited by quality and organoleptic considerations.

IV. BASIS FOR GRAS DETERMINATION

A. Documentation to Support the Safety of Ferrous Ammonium Phosphate

The determination that ferrous ammonium phosphate is GRAS is on the basis of scientific procedures, and the information supporting the general recognition of the safe use of ferrous ammonium phosphate includes:

- dissociation of ferrous ammonium phosphate to iron(II), ammonium, and phosphate ions when subject to low pH conditions;
- background consumption of iron, ammonium, and phosphate salts as added or natural components of the diet;
- data pertaining to the identity, intended use, and estimated intake of ferrous ammonium phosphate; and
- the entirety of preclinical and human studies assessing the safety of iron, ammonium, and phosphate salts.

Moreover, these data were reviewed by a panel of experts, qualified by scientific training and experience to evaluate the safety of ingredients as components of food, who concluded that the proposed uses of ferrous ammonium phosphate are safe and suitable and would be GRAS based on scientific procedures [see Appendix A, entitled, **"EXPERT PANEL CONSENSUS STATEMENT REGARDING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF FERROUS AMMONIUM PHOSPHATE FOR USE IN FOODS"**]. A summary of these data is presented herein.

B. Estimated Intake of Ferrous Ammonium Phosphate

The individual proposed food-uses and use-levels for ferrous ammonium phosphate, and the resulting use-level for iron, are summarized in Table 2. The serving sizes were determined using the RACC per eating occasion, according to Section §101.12 of the Code of Federal Regulations (CFR, 2007a) or, where indicated, using data provided by Nestlé. Food codes representative of each proposed food-use were chosen from the National Health and Nutrition Examination Survey (NHANES) 2003-2004 (CDC, 2006; USDA, 2007). Food codes were grouped in food-use categories according to Title 21, Section §170.3 of the Code of Federal Regulations (CFR, 2007b). Product-specific adjustment factors were developed based on data provided in the standard recipe file for the Continuing Survey of Food Intakes by Individuals (CSFII) 1994-1996, 1998 survey (USDA, 2000).

Table 2 Summary of the Individual Proposed Food-Uses and Use-Levels for Ferrous Ammonium Phosphate and the Corresponding Use-Levels of Iron in the U.S.

Food Category	Proposed Food-Use	Serving Size (g or mL)[a]	Ferrous Ammonium Phosphate		Iron	
			Use-level (mg/serving)	Use-level (%)	Use-level (mg/serving)	Use-level (%)
Beverages and Beverage Bases	Non-Milk Based Meal Replacements	240	18	7.50×10^{-3}	5.4	2.25×10^{-3}
	Powdered Chocolate and Malt Beverages	15[b]	13 6	$9\ 06 \times 10^{-2}$	4.1	$2\ 73 \times 10^{-2}$
	Sport Beverages	240	18	7.50×10^{-3}	5.4	2.25×10^{-3}
Grain Products and Pastas	Fortified Cereal Bars[c]	40	18	4.50×10^{-2}	5.4	1.35×10^{-2}
	Instant Noodles[d]	30[b]	14	$4\ 67 \times 10^{-2}$	4.2	$1\ 40 \times 10^{-2}$
	Meal Replacement Bars	40	18	4.50×10^{-2}	5.4	1.35×10^{-2}
Milk and Milk Products	Milk-Based Meal Replacements	240	18	7.50×10^{-3}	5.4	2.23×10^{-3}
	Powdered Milk and Milk Beverages	32.5[b]	10 9	$3\ 35 \times 10^{-2}$	3 3	$1\ 01 \times 10^{-2}$
Soups and Soup Mixes	Bouillon Cubes/Powders[e]	2[b]	10.9	5.45×10^{-1}	3.3	1.65×10^{-1}

[a] RACC = Reference Amounts Customarily Consumed per Eating Occasion (21 CFR §101.12 - CFR, 2007a).
[b] Serving size provided by Nestlé.
[c] Food codes representative of fortified cereal bars were not identified; thus, food codes representative of unfortified cereal and granola bars were used as surrogates.
[d] Due to the low number of food codes representative of instant noodles identified, food codes representative of canned ready-to-serve noodle soups and canned pastas were used as surrogates.
[e] And all equivalent products

On an all-user basis, the mean intake of ferrous ammonium phosphate by the total U.S. population from all proposed food-uses was estimated to be 13 mg/person/day or 0.2 mg/kg body weight/day. The heavy consumer (90th percentile) all-user intake of ferrous ammonium phosphate by the total U.S. population from all proposed food-uses was estimated to be 29 mg/person/day or 0.6 mg/kg body weight/day. Ferrous ammonium phosphate has a maximum iron content of 30%, and therefore, the corresponding mean intake of iron by the total U.S. population from all proposed food-uses of ferrous ammonium phosphate was estimated to be 3.8 mg/person/day or 0.074 mg/kg body weight/day. The heavy consumer (90th percentile) all-user intake of iron by the total U.S. population from all proposed food-uses of ferrous ammonium phosphate was estimated to be 8.8 mg/person/day or 0.181 mg/kg body weight/day.

While higher intake estimates of 1.7 mg ferrous ammonium phosphate/kg body weight/day (0.505 mg iron/kg body weight/day or 5.5 mg iron/person/day) were obtained in infants on an all-

user basis from all proposed food-uses of ferrous ammonium phosphate at the 90th percentile, it should be stressed that the specified food-uses for ferrous ammonium phosphate are not intended to be marketed to infants, nor does Nestlé intend on marketing or formulating their products for consumption by children under 4 years of age. Thus, the actual infant consumption of ferrous ammonium phosphate-enriched food products is expected to be highly limited. Furthermore, the consumption estimates presented herein are largely based on a use-level for ferrous ammonium phosphate that provides 30% of the DV for iron (*i.e.*, 5.4 mg iron/RACC), whereas in most instances, the aim is to add ferrous ammonium phosphate to foods to provide iron at levels within 10 to 20% of the DV (*i.e.*, 1.8 to 3.6 mg/RACC). Therefore, although an estimate of the consumption of ferrous ammonium phosphate and the resulting intake of iron on a body weight basis in infants from all-proposed food uses has been included for completeness of the data, it is considered to be a gross over-estimate of the actual expected intake of ferrous ammonium phosphate in infants from its addition to food. Moreover, in the event that this population group does consume products fortified with ferrous ammonium phosphate, their intakes would remain well below the Tolerable Upper Intake Level (UL) of 40 mg/person/day established for young children (0-13 years) by the Institute of Medicine (IOM) (IOM, 2001). Importantly, ferrous ammonium phosphate is only proposed as a direct replacement for other iron fortificants currently in use in existing categories of fortified foods in the U.S. and will not be used in products specially formulated or processed for use by infants or children less than 4 years of age.

C. Metabolic Fate of Ferrous Ammonium Phosphate

The advantage of ferrous ammonium phosphate compared to many other analogous iron(II) (*i.e.*, ferrous) salts is the high solubility at low pH (acidic conditions) but poor solubility at higher pH (neutral conditions). The material exhibits little reactivity in finished products therefore, until subject to the low pH conditions (between 2 and 3) of the stomach whereby dissociation to the respective iron, ammonium, and phosphate ions is observed. The solubilization process involves dissociation of ferrous ammonium phosphate to the individual ions which become hydrated and essentially "free" in solution. Solubility is therefore directly related to bioavailability and critical to the efficacy of ferrous ammonium phosphate as a fortificant in foods.

The amount of dissolved iron in solution has been measured at pH 1 and 1.7 by Inductively Couple Plasma – Atomic Emission Spectroscopy (ICP-AES) from ferrous ammonium phosphate, ferrous sulfate, and ferric pyrophosphate source materials. At pH 1.7 the amount of iron dissolved (*i.e.*, dissociated) from ferrous ammonium phosphate after 10 minutes of exposure to the acidic conditions was shown to lie midway between that from ferrous sulfate and ferric pyrophosphate. Specifically, the amount of iron dissolved from ferrous sulfate, ferrous ammonium phosphate, and ferric pyrophosphate were 100%, 55%, and 10%, respectively. Upon decreasing the pH further to pH 1, greater than 95% of iron from ferrous ammonium phosphate was dissolved after 30 minutes, which was comparable to that from ferrous sulfate

(100% of iron was dissolved) and far greater than that from ferric pyrophosphate (less than 20% of iron was dissolved). The solubility product (K_{sp}) for ferrous ammonium phosphate was estimated to be 1.8×10^{-11} at pH 2 based on the concentration of free iron in solution. Similar results were observed when ferrous ammonium phosphate was subjected to a standard HCl dissolution test (Nestlé Research Center, 2007a). Ferrous ammonium phosphate, in an amount providing 20 mg of iron, was added to 250 mL of a diluted HCl solution at pH 1.93 at 37°C. Iron content was measured by atomic absorption spectroscopy. After approximately 30 minutes, ~50% of ferrous ammonium phosphate had dissolved in solution, and by 3 hours, 67% of ferrous ammonium phosphate had dissolved. Dissolution of ferrous ammonium phosphate also was assessed in an *in vitro* iron release study (Nestlé Research and Development, 2000). Ferrous ammonium phosphate solutions were prepared in de-ionized water at 37°C containing 100 ppm of ferrous ammonium phosphate. The pH was adjusted to 2.0 or 3.5 using HCl to mimic the conditions of infant and adult stomachs, respectively, and the level of free iron in solution was measured by ionic high-performance liquid chromatography (HPLC). Under the conditions of this test, the maximum amount of free iron released was 97% of the total iron from ferrous ammonium phosphate. Together, the solubility and stability data on ferrous ammonium phosphate demonstrate that at neutral pH, ferrous ammonium phosphate is relatively insoluble, and therefore, does not react significantly with components in the food matrix. However, at the low pH conditions of the stomach, ferrous ammonium phosphate dissolves well, releasing iron(II), ammonium, and phosphate ions.

The data above also show that the solubility of ferrous ammonium phosphate is within the range of other iron fortificants, indicating an acceptable bioavailability of iron from this compound. Indeed, bioavailability data on ferrous ammonium phosphate demonstrate that the iron compound is a good source of iron in humans (Nestlé Research Center, 2007a,b). The bioavailability study on ferrous ammonium phosphate consisted of 2 randomized, double blind, 2-period crossover trials conducted in parallel in which test meals containing ferrous ammonium phosphate, ferrous sulfate, or ferric pyrophosphate were consumed by healthy female volunteers between the ages of 18 to 30 (n = 38). Test meals consisted of 250 mL of reconstituted full cream instant milk powder supplemented with [^{57}Fe]-ferrous ammonium phosphate, [^{58}Fe]-ferrous sulfate, or [^{57}Fe]-ferric pyrophosphate at a fortification level of 2.5 mg of iron per serving. In the first trial, subjects consumed 2 servings of either ferrous ammonium phosphate or ferrous sulfate test meals on Day 1 and then 2 servings of the other test meal on Day 2. In the second trial, subjects consumed 2 servings of either ferric pyrophosphate or ferrous sulfate test meals on Day 1 and then 2 servings of the other test meal on Day 2. The total intake of iron from each iron supplement was 5 mg. Blood was drawn on Day 16 of the study for the determination of isotope-labeled iron incorporation into erythrocytes. The geometric mean of fractional iron absorption from ferrous sulfate, ferrous ammonium phosphate, and ferric pyrophosphate were 10.4, 7.4, and 3.3%, respectively. Thus, the amount of iron absorbed from the 5 mg provided by each iron supplement was 0.52 mg, 0.37 mg, and 0.17 mg, respectively. These differences in iron absorption between ferrous ammonium phosphate and

ferrous sulfate or ferrous ammonium phosphate and ferric pyrophosphate were statistically significant; the absorption of iron from ferrous ammonium phosphate was significantly greater than that of ferric pyrophosphate ($p<0.0001$), but significantly less than that of ferrous sulfate ($p = 0.0002$). The absorption values of the different iron formulations, therefore, correlate well with their respective solubilities under acidic conditions as discussed above. Importantly, the results of this study demonstrate that ferrous ammonium phosphate dissociates *in vivo*, resulting in the release and absorption of iron.

For comparative purposes, the results of iron absorption studies examining the bioavailability of iron from foods fortified with ferrous sulfate in healthy population groups were reviewed. The absorption and bioavailability of iron from a wide variety of food types fortified with ferrous sulfate have been extensively studied in humans. A review of this literature indicates that the absorption of iron from ferrous sulfate-fortified cereal-based foods and milk-based products, foods typically fortified with iron, ranges from 2 to 11% and is consistent across all age groups (infants, children, adolescents, and adults) (Rios *et al.*, 1975; Martínez-Torres *et al.*, 1979; MacPhail *et al.*, 1981; Stekel *et al.*, 1986; Davidsson *et al.*, 1997, 1998, 2001, 2002, 2005; Hurrell *et al.*, 2000; Layrisse *et al.*, 2000; Mendoza *et al.*, 2001, 2004; Fidler *et al.*, 2003; Pérez-Expósito *et al.*, 2005). Higher iron absorption values were frequently the result of concomitant ascorbic acid supplementation of the food vehicle, while very low values are sometimes observed in foods with high polyphenol and/or phytic acid content. In these studies, the level of iron provided by ferrous sulfate in each food preparation was between 2 to 10 mg. Thus, the iron absorption value of 10.4% from a ferrous sulfate-fortified milk product reported here is consistent with the high-end range reported in the published literature. Based on this measurement as well as on the *in vitro* solubility data, it can be expected that the absorption of iron from ferrous ammonium phosphate-fortified cereal-based foods and milk-based products will be approximately 7%, somewhat less than that consistently reported for ferrous sulfate.

From the data provided above, it is expected that ferrous ammonium phosphate will dissociate to iron(II), ammonium, and phosphate ions in the stomach following oral consumption. Therefore, it is the iron(II), ammonium, and phosphate ions that are absorbed and processed by the body following consumption of ferrous ammonium phosphate. The constituent ions will undergo normal metabolic processing. The metabolic fate of iron compounds has been reviewed by a number of scientific bodies, including the International Nutritional Anemia Consultative Group (INACG, 1993), the Institute of Medicine (IOM, 2001), and the Expert Group on Vitamins and Minerals (EVM, 2002, 2003a). The IOM (1997) and the EVM (2003b) also have reviewed the metabolic fate of phosphates and phosphorus and reviews of the metabolic fate of ammonium and ammonia are provided by the International Programme on Chemical Safety (IPCS) (EHC, 1986), the World Health Organization (WHO, 2003), and Health Canada (1987). Briefly, iron, ammonium (as ammonia), and phosphate ions are absorbed from the small intestine and utilized by the body in essential physiological processes. Iron is distributed predominantly to erythrocytes and the excess stored in liver, bone marrow, and

reticuloendothelial cells. Iron is incorporated into several different types of proteins for utilization in a variety of biological functions. Proteins that require iron for functionality include hemoglobin and myoglobin for the transfer of oxygen from the lungs to tissues, cytochromes within the mitochondrial electron transport chain for cellular energy production, and cytochrome P450 enzymes for the metabolism of endogenous and exogenous compounds. Most iron from erythrocytes is recycled for incorporation into hemoglobin with only a small amount of iron excreted daily (mainly in feces). Once absorbed, ammonia is distributed to mainly to the liver where it is incorporation into urea as part of the urea cycle or utilized for protein synthesis. Ammonium ions also are present in all tissues and constitute a metabolic pool from which it can be taken up by glutamic acid to be used in various transamination and other reactions. Ammonia is excreted in the urine in the form of urea or as free ammonia to aid in the secretion of hydrogen ions for the purpose of maintaining pH balance. Finally, phosphates are distributed predominantly to bone, and also to soft tissues, teeth, blood, and extravascular fluid. Inorganic phosphate plays a critical role in skeletal development, mineral metabolism, and diverse cellular functions involving intermediary metabolism and energy-transfer mechanisms. Phosphate ions are filtered by the glomeruli in the kidney, and the majority is reabsorbed in the proximal renal tubules, with the remainder excreted in the urine.

D. Preclinical Studies Pertaining to the Safe Consumption of Ferrous Ammonium Phosphate

Studies evaluating the toxicity of ferrous ammonium phosphate in experimental animals have not been conducted. However, ferrous ammonium phosphate dissociates to iron(II), ammonium, and phosphate ions when subject to low pH conditions similar to that of the stomach. Therefore, it was considered appropriate to approach the toxicity assessment of ferrous ammonium phosphate based on the individual components. Importance of the toxicity data was placed on that pertaining to iron as the intakes of ammonium and phosphate ions from ferrous ammonium phosphate-fortified foods are negligible compared to those already consumed in the normal human diet.

Iron

The toxicity of iron in experimental animals was evaluated based on the oral administration of iron as ferrous sulfate or in elemental form (carbonyl iron). Toxicity data pertaining to ferrous sulfate are presented to provide an estimate of the toxicity of iron(II) from ferrous ammonium phosphate. The rationale for this approach is that the toxicity of iron will largely depend on the amount of iron absorbed, which is dependent on the state of iron existing within the compound (*i.e.,* ferrous *vs.* ferric). Since iron is present in ferrous form in ferrous ammonium phosphate, the toxicity of ferrous sulfate provides an indication of the potential toxicity of iron(II) as a result of oral exposure to ferrous ammonium phosphate. In addition, because the bioavailability of iron from ferrous sulfate supplementation is higher than that from ferrous ammonium phosphate supplementation, toxicity data pertaining to ferrous sulfate will provide a 'worst case scenario' of

iron(II) toxicity as a result of oral exposure to ferrous ammonium phosphate. The available toxicity data on elemental iron as carbonyl iron also is summarized.

Acute Toxicity Studies

Ferrous sulfate was reported in several studies to have relatively low acute oral toxicity in experimental animals with oral LD_{50} values of approximately 670, 1,720, 1,028, and >1,000 mg/kg body weight, equivalent to 134, 344, 206, and >200 mg iron/kg body weight, in Swiss mice, albino rats, albino rabbits, and dogs, respectively (Keith, 1957; Boccio *et al.*, 1998). In contrast, the oral LD_{50} of elemental iron as carbonyl iron is greater than 50,000 mg/kg body weight in Sprague-Dawley rats (Whittaker *et al.*, 2002). In addition, mortality rates of Wistar rats (20 to 40/sex/group) orally administered by gavage a single dose of 750 mg iron/kg body weight in the form of ferrous sulfate have been reported to be 40% for male and 80% for female prepubertal rats, 47% for male and 60% for female pubertal rats, and 95% for both male and female of adult rats (Berkovitch *et al.*, 1997). All deaths occurred within the first 24 hours of ferrous sulfate administration; no deaths occurred between 24 and 48 hours post-administration. No deaths occurred in the control groups (20 male and 20 female adult rats), which were administered distilled water under the same conditions.

It is only following large acute oral doses of iron that gastrointestinal (GI) effects are well known to occur. In the study by Keith (1957), 40 male and female albino rats (strain not specified) administered single doses of ferrous sulfate (doses not specified) by gavage and observed over a 2-week period, displayed diarrhea and depression, the intensity of which increased with increasing dose. Deaths were reported to occur within 1 to 5 hours. Convulsions were observed at the time of death and irritation was observed in the stomachs of rats that received fatal oral doses. Similarly, necrotic lesions were observed in the stomachs and villi of the proximal small intestine of male Sprague-Dawley rats (9/group) orally administered 80, 160, or 320 mg iron/kg body weight as ferrous sulfate by gavage (Nayfield *et al.*, 1976). These observations were noted at 1, 2, and 3 hours post-administration and were reported to display dose-dependence in severity. Necrotic lesions also were observed in the stomachs and villi of the duodenum of 7 male Sprague-Dawley rats orally (route not specified) administered a single dose of 200 mg iron/kg body weight as ferrous sulfate (Benoni *et al.*, 1993). The severity of the lesions was reported to be decreased at 24 hours post-administration compared to at 3 hours. At lower doses of ferrous sulfate, providing 2.0, 6.5, or 13.0 mg iron/kg body weight, cytotoxicity was not observed in the stomach or duodenum of 5 female C57BL/6J mice, although, an increase in frequency of non-specific cytotoxicity was observed in the colon of these animals (Bianchini *et al.*, 1988). In dogs (sex and strain not specified) orally (route not specified) administered ferrous sulfate at a dose of 1,000 mg/kg body weight, equivalent to 200 mg/kg body weight of iron, symptoms of toxicity were reported, including either copious diarrhea or emesis (Keith, 1957). No deaths occurred in dogs as an emetic threshold was reached before a fatal dose could be absorbed. Thus, adverse effects following acute exposure to ferrous sulfate occur at high doses of iron and are consistent among laboratory animals, consisting of GI

disturbances such as vomiting (observed in dogs only) and diarrhea and lesions of the stomach and proximal small intestine (in rodents).

Subchronic Toxicity Studies

Studies in which laboratory animals were subject to repeat oral dosing with ferrous sulfate or carbonyl iron were intentionally designed to produce iron overload and hepatotoxicity particularly in mice and rats. Therefore, in many of these studies, animals were provided diets containing very high doses of iron that are above and beyond the typical levels used to fortify foods, but are summarized here to provide the complete toxicology profile on iron. Also, a no-adverse-effect level (NOAEL) could not be determined for some studies as intermediate dose levels were not included. The results of these studies indicate that very high doses of iron were required to produce adverse effects in experimental animals, demonstrating the safety of low doses of iron in the diet.

Three studies on dietary ferrous sulfate administration were identified in the available literature that were conducted in mice. In the first, groups of 5 SPF female BABL/c infant and young adult mice were iron-overloaded *via* the administration of ferrous sulfate-supplemented diets, containing 2.5 (control) or 25 mg of iron/100 g diet, equivalent to 3.75 and 37.5 mg of iron/kg body weight/day, respectively, for 1 or 2 weeks (Hirohata *et al.*, 1998). Mice were evaluated for body weight, hematocrit and blood hemoglobin concentrations, and hepatic iron content, and an electron microscopic examination of the intestinal tract also was performed. Body weight, hematocrit, and hemoglobin concentrations were unaffected by iron overloading. Hepatic iron levels increased with the duration of feeding in infant mice, but not adult mice, and were significantly different when compared to the control group after 1 week and 2 weeks of feeding. In both infant and young adult mice fed diets supplemented with 37.5 mg of iron/kg body weight/day for 2 weeks, the frequency of occurrence of secondary lysosomes in intestinal epithelial cells, intercellular junction opening between the cells, and eosinophilic leukocytes outside the basement membranes of the cells were increased compared to the control groups. The authors suggested that these changes demonstrated a change in the morphological features of enterocytes as a result of dietary iron overload.

In the second study, groups of male weanling CD-1 mice (5, 6, or 8 mice/group, depending on the parameter measured) were administered ferrous sulfate *via* the diet, providing 120 (control), 5,000, or 8,000 mg of iron/kg diet, equivalent to 18, 750, and 1,200 mg of iron/kg body weight/day, respectively, for 7 weeks (Omara *et al.,* 1993). There was no mortality reported in mice fed iron-supplemented diets. As in the study by Hirohata *et al.* (1998), hematocrit values were unaffected by iron supplementation. Water consumption and relative spleen, heart, and kidney weights also were not affected by iron supplementation. In mice fed diets containing 1,200 mg/kg body weight/day of iron, a significant reduction in body weight and a significant increase in liver weight were observed compared to control mice. In addition, a significant increase in the iron content of hepatocytes, Kupffer cells, and splenic macrophages of mice fed

iron-supplemented diets at levels of 750 and 1,200 mg/kg body weight/day was observed compared to the control group. Increases in serum markers of liver toxicity [alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activity] also were observed in mice at these dose levels compared to the control. However, no gross lesions from macroscopic examination of the liver, spleen, heart, kidney, or pancreas and no compound-related effects on serum glucose, urea, total protein, or albumin concentrations were observed in any of the groups. Similar results were observed by Omara and Blakley (1993) in a similarly designed study with inclusion of a 450 mg iron/kg body weight/day dose group. A significant increase in relative liver weight was observed in mice fed iron-supplemented diets at levels of 750 and 1,200 mg/kg body weight/day compared to the control. A significant reduction in body weight gain and elevations in serum ALT activity were only observed at the highest dose level tested (1,200 mg/kg body weight/day). However, no microscopic histopathological changes were observed at the highest dose level tested. Based on the results of these studies a NOAEL of 450 mg/kg body weight/day for iron as ferrous sulfate in CD-1 mice was determined.

One study conducted in rats was identified in the available literature, involving ferrous sulfate. Groups of 40 male Sprague-Dawley rats were fed diets containing 2.84, 5.69, or 11.54 mg/kg body weight/day of iron as ferrous sulfate for a period of 31 or 61 days (Appel *et al.*, 2001). The parameters evaluated included clinical signs, body weights, food consumption, hematology, clinical chemistry, gross necropsy, and histopathology of the liver and spleen as well as of all gross lesions. All abnormalities and signs of illness or reactions to treatment also were recorded. There were no significant differences in body weights, food consumption, and hematology parameters observed among the groups during the study period. Clinical signs of toxicity observed were considered by the authors to be common occurrences for this particular strain of rat. A dose-dependent decrease in plasma sodium was observed at 31 and 61 days and a dose-dependent decrease in plasma total iron binding capacity was observed at 61 days. A dose-dependent increase in iron concentration was observed in the spleen after 31 days and in the liver and the kidney after 61 days. No toxicologically significant gross or microscopic abnormalities were observed at any of the iron dose levels tested. Therefore, iron dose levels of up to 11.54 mg/kg body weight/day did not produce any adverse effects in rats under the conditions of this study, and therefore, the highest dose tested (11.54 mg/kg body weight/day) was determined to be the NOAEL male Sprague-Dawley rats.

Carbonyl iron has been used in many of the dietary iron feeding studies identified in the available literature. The majority of these studies were conducted in rats using 1 iron dose level exceeding 1,200 mg iron/kg body weight/day in order to induce iron overload and are discussed in brief. In studies by Stål and colleagues (Stål and Hultcrantz, 1993; Stål *et al.*, 1996), male weanling Sprague-Dawley rats (5 or 6 rats/group) were fed diets containing carbonyl iron at levels of either 0 or 1,250 to 1,500 mg iron/kg body weight/day for periods of 6 to 9 weeks. Food consumption was not affected by iron supplementation. As with high dose administration of ferrous sulfate, feeding rats high doses of carbonyl iron resulted in a reduction in body weight

gain and an increase in hepatic iron content and deposition compared to control rats. A significant increase in serum ALT activity levels at the 1,500 mg iron/kg body weight/day dose level was reported by Stål *et al.* (1996), but not by Stål and Hultcrantz (1993). Stål *et al.* (1996) also reported an increase in hepatic malondialdehyde (MDA) and glutathione (GSH) levels in carbonyl iron-fed rats compared to control rats. However, upon microscopic examination of the liver, no evidence of necrosis, fibrosis, or any other pathological feature was observed in either study. Wu *et al.* (1990) also examined the effects of feeding male Fischer 344 rats (6 to 12 rats/group) carbonyl iron at a level of 1,250 mg iron/kg body weight/day compared to no iron supplementation for 11 to 12 weeks. In both young and old male rats, significant reductions in body weight gain and food consumption were reported along with an increase in iron content of the serum, liver, spleen, and heart (old rats only). Significant increases in hepatic microsomal conjugated diene content and urinary excretion of thiobarbituric acid-reactive substances (TBARS) and prostaglandin metabolites also were reported.

Olynyk *et al.* (1995) and Plummer *et al.* (1997) both examined the hepatic effects of dietary administration of carbonyl iron from birth in rats. In the study by Olynyk *et al.* (1995), 7 male and female Wistar Furth rats were administered 1,500 mg iron/kg body weight/day for 10 weeks starting at birth followed by 11,000 mg iron/kg body weight/day for an additional 16 weeks. Mild, non-specific hepatocyte necrosis was noted and focal fibrosis was observed around iron-laden periportal macrophages, but not elsewhere in the liver. In contrast, but consistent with the findings of other studies, no evidence of fibrosis was observed by Plummer *et al.* (1997) in male or female Porton rats (3 to 6 rats/group) fed diets containing 0, 250, 500, or 1,000 mg iron/kg body weight/day for 32 weeks starting at birth despite a dose- and time- dependent increase in hepatic iron content. Rats in the 500 and 1,000 mg iron/kg body/day dose groups displayed moderate growth retardation, and therefore, the NOAEL for carbonyl iron was determined to be 250 mg iron/kg body weight/day in Porton rats under the conditions of this study.

Only 1 study, by Whittaker *et al.* (1996), was identified in which the study was conducted using a large dynamic range of doses of carbonyl iron. Male Sprague-Dawley rats (11, 10, 10, and 18 rats/group, respectively) were fed diets providing 1.75, 17.5, 175, or 1,000 mg of iron/kg body weight/day for a period of 12 weeks. Eight (8) out of 10 and 13 out of 18 rats died in the 175 and 1,000 mg/kg body weight/day groups, respectively. A dose-dependent and significant increase in hepatic and cardiac iron content and hemosiderosis (iron deposition) were observed as in other studies. Upon necropsy, an increase in the incidence and severity of cardiomyopathy was observed in rats fed diets supplemented with carbonyl iron (statistical analyses not conducted on these latter parameters). Seven (7) out of 10 (70%) and 12 out of 17 (71%) rats developed cardiomyopathy in the 175 and 1,000 mg/kg body weight/day groups compared to none in the control group and only 1 out of 10 rats (10%) in the 17.5 mg/kg body weight/day group. In rats fed 175 and 1,000 mg/kg body weight/day of iron, splenic and pancreatic atrophy of the exocrine and endocrine tissues, accompanied by apoptosis, was observed. The authors suggested that the effects observed in the liver, heart, spleen, pancreas

may be due to peroxidative injury to cell membranes caused by iron-induced oxidative stress. Based on the occurrence of death and observed organ atrophy at the 2 highest dose levels, the NOAEL for iron under the conditions of this study was 17.5 mg/kg body weight/day of iron in male Sprague-Dawley rats.

Chronic Toxicity Studies

Chronic studies in rodents ranging from 12 to 15 months in duration were identified in the available literature. All studies were focused on the hepatic effects of dietary iron-overload with carbonyl iron; no chronic studies on ferrous sulfate were identified.

Carbonyl iron in the diet was well-tolerated by male BALB/c mice (5 mice/group) at doses providing 0, 750, 2,250, 4,500 mg iron/kg body weight/day for periods of up to 12 months (Pigeon *et al.*, 1999). In high-dose animals, a significant decrease in body weight was observed compared to control mice at 2 and 8 months; however, no differences in body weight were observed at 12 months. A dose- and time-dependent increase in hepatic iron content was observed accompanied by a significant increase in relative liver weight at 12 months at the 2 highest doses tested (2,250 and 4,500 mg iron/kg body weight/day). An increase in the size of nuclei mitotic index with the presence of abnormal tripolar mitotic figures was noted in the high-dose group compared to the control group; however, no evidence of tumors was observed. Also, no evidence of fibrosis was observed.

In a study by Park *et al.* (1987), male Sprague-Dawley rats (5 rats/group) were fed diets containing 1,500 mg iron/kg body weight/day as carbonyl iron for 1 month followed by a diet containing 1,250 mg iron/kg body weight/day for an additional 12 months or a diet without supplemental iron for 13 months. Growth retardation and hepatomegaly were observed at 8 and 12 months. A time-dependent increase in iron concentration and deposition (hemosiderosis) was observed in the liver as well as in the spleen, pancreas, and kidney of iron-overloaded rats. Following the 13 months of carbonyl iron feeding in the diet, the authors observed focal hepatocellular necrosis and hepatic periportal fibrosis along with ultrastructural subcellular orgnanelle damage in iron-laded hepatocytes. In contrast, Iancu *et al.* (1987) reported that no evidence of hepatic fibrosis or cirrhosis was observed in 9 male Sprague-Dawley rats fed 1,000 mg iron/kg body weight/day as carbonyl iron for periods of up to 15 months despite an increase in hepatic iron content and deposition (hemosiderosis) compared to control rats. An increase in iron content also was observed in the small intestine, spleen, pancreas, and heart, although, microscopic examination revealed no evidence of fibrosis in these organs or any signs of cellular damage.

Developmental Toxicity Studies

Studies evaluating the developmental toxicity of iron in experimental animals indicate that iron is not teratogenic. A study conducted by Fairweather-Tait *et al.* (1984), assessed the effect of oral iron supplementation in pregnant rats fed low- or adequate-zinc diets. Pregnant Wister rats,

maintained on low- or adequate-zinc diets before pregnancy, were fed either a control diet or a diet supplemented with 567 mg/kg diet of iron as ferrous sulfate, equivalent to approximately 110 mg of iron/kg body weight/day. The mean number of resorption sites, number of fetuses in each group, and fetal dry weight were not significantly different between groups. Among the fetuses from dams fed the adequate-zinc diet, fetal wet weight was significantly decreased in the ferrous sulfate group compared to the control group. Although the low-zinc diet induced a higher incidence of fetal morbidity and uterine and fetal hemorrhages compared to the adequate-zinc diet, ferrous sulfate supplementation to either diet did not affect the incidence of these abnormalities.

In a teratology study conducted by the Food and Drug Research Lab, Inc. (FDRLI) (1974) reported in the 1983 JECFA evaluation on iron (JECFA, 1983), pregnant albino CD-1 mice and pregnant albino Wistar rats were administered ferrous sulfate by oral gavage from Gestational Day (GD) 6 through to GD 15 (rats) or GD 16 (mice). Administration of ferrous sulfate did not produce maternal toxicity or teratogenicity at dose levels up to 160 mg/kg body weight/day in mice and 200 mg/kg body weight/day in rats. JECFA also reported that ferrous sulfate, ferrous gluconate, and ferrous lactate were not teratogenic to the developing chick embryo.

Mutagenicity and Genotoxicity Studies

Results of mutagenicity studies conducted on ferrous iron salts demonstrate that iron(II) is not mutagenic except at high concentrations. Dunkel *et al.* (1999) tested the genotoxic potential of both ferrous sulfate and ferrous fumarate. Assays included the Ames reverse mutation assay conducted in seven different *Salmonella typhimurium* histidine auxotroph tester strains (TA97a, TA98, TA100, TA102, TA1535, TA1537, and TA1538) and the L5178Y $TK^{+/-}$ mouse lymphoma forward mutation assay both with and without S9 metabolic activation. Ferrous sulfate did not produce a mutagenic response in the Ames assay at doses up to 10,000 µg of iron per plate either with or without metabolic activation. Ferrous fumarate produced a mutagenic response in the Ames assay, but only in the TA98 strain with metabolic activation. The induction in the number of revertants was 3.3-fold at 987 µg iron/plate and 2.7-fold at 1,647 µg iron/plate compared to vehicle control in the presence of rat and hamster liver S9 mix, respectively. However, at higher concentrations of iron, ferrous fumarate did not induce a mutagenic response in the TA98 strain with or without metabolic activation. In the mouse lymphoma assay, ferrous sulfate produced a weakly positive mutagenic response in the absence of metabolic activation at the highest concentration tested (201 µg iron/mL) and a dose-dependent (1,005 to 1,508 µg iron/mL) response in the presence of S9 metabolic activation. Ferrous fumarate also produced a dose-dependent (296 to 316 µg iron/mL) mutagenic response in the presence of S9 metabolic activation. Doses up to 4,606 µg iron/mL as ferrous fumarate were not mutagenic in the absence of S9 metabolic activation. Ferrous sulfate has also been shown to be weakly mutagenic by Casto *et al.* (1979), but only at high concentrations. Ferrous sulfate was able to enhance the viral transformation of Syrian hamster embryo cells by a simian

adenovirus, SA7. However, the mutagenic activity of ferrous sulfate was deemed low as a concentration of at least 0.9 mM was required to increase transformation by only 2.2-fold.

Ammonium/Ammonia

In the U.S., the daily intake of ammonia from food and drink is estimated to be approximately 18 mg (EHC, 1986). In addition, approximately 4 g of ammonia is produced endogenously within the human intestinal tract per day through the biological degradation of nitrogenous matter (EHC, 1986). Ferrous ammonium phosphate is composed of approximately 7% ammonia. The proposed use-levels of ferrous ammonium phosphate would thus provide an ammonia content (present as the ammonium ion) ranging from 0.17 to 1.26 mg/serving. Therefore, intakes of ammonium, and consequently ammonia, from ferrous ammonium phosphate-fortified foods are negligible compared to those already consumed in the normal human diet or produced endogenously. Nonetheless, toxicity data on ammonium salts and ammonia are presented below to provide a complete toxicological profile on ferrous ammonium phosphate, but are presented in brief.

Acute Toxicity Studies

Ammonium salts have been reported as having very low acute oral toxicity in experimental animals with oral LD_{50} values of 4,400 and 3,100 mg/kg body weight reported for ammonium sulfamate in rats and mice, respectively (Vinokurova and Mal'Kova, 1963). Other investigators have reported similar oral LD_{50} values for ammonium sulfate in the rat, ranging from 3,000 to 5,020 mg/kg body weight (Frank, 1948; Bukhovskaya *et al.*, 1966). Oral LD_{50} values for ammonium salts also have been reported by the WHO as being in the range of 350 to 750 mg/kg body weight (animal species and compounds not specified) (WHO, 2003). Furthermore, lung edema, nervous system dysfunction, acidosis, and kidney damage were reported to occur after the administration of single doses of unspecified ammonium salts ranging from 200 to 500 mg/kg body weight. Adverse effects in rodents following acute oral exposure to ammonium salts, primarily ammonium chloride, include acute pulmonary edema, nervous system dysfunction, metabolic acidosis, and renal hypertrophy and other kidney effects. These effects were observed at ammonium salt levels ranging from 200 to 1000 mg/kg body weight administered for periods of 1 to 8 days. These data have been previously evaluated and summarized in detail by the IPCS in the Environmental Health Criteria (EHC) report compiled for ammonia in 1986 (EHC, 1986).

Subchronic Toxicity Studies

Gupta *et al.* (1979) have reported no adverse effects on the general health, body weights, hematological values, or relative organ weights of adult female and weanling male and female albino rats (20/sex/age/group) orally administered 100, 250, or 5,000 mg ammonium sulfamate/kg body weight/day (equivalent to approximately 16, 40, and 790 mg ammonium/kg body weight/day) 6 days per week for 90 days. Additionally, no histopathological abnormalities

of the kidney, liver, lung, stomach, heart, spleen, thyroid, adrenal, gonads, intestine, or lymph nodes were observed in these animals. Only slight changes were reported, including a slight decrease in food consumption and a slight increase in water consumption in the high-dose weanlings of both sexes. However, no adverse effects on weight gain and food utilization efficiency were observed in male weanling Sprague-Dawley rats (6 rats/group) provided a diet supplemented with approximately 8,600 mg ammonium acetate/kg body weight/day (equivalent to approximately 2,013 mg/kg body weight/day of ammonium) for 21 days compared to rats fed a diet without ammonium acetate (Birnbaum *et al.*, 1957). Similar results were observed in 3 pigs fed a diet supplemented with approximately 1,820 mg diammonium citrate/kg body weight/day (equivalent to approximately 290 mg ammonium/kg body weight/day) for 28 days (Kagota *et al.*, 1979). In addition, the consumption of diammonium citrate did not result in any significant differences in packed cell volume or ammonia-nitrogen levels in the blood or total protein concentration in the plasma of pigs compared to pigs fed the basal diet. No lesions or abnormalities were revealed upon autopsy. Average daily weight gain, average daily food consumption, and food utilization efficiency were similarly not affected in rats or pigs fed a diet supplemented with approximately 1,030 mg/kg body weight/day of ammonium chloride (equivalent to approximately 347 mg ammonium/kg body weight/day) for 4 weeks (Clawson and Armstrong, 1981). Enlargement of the parathyroids was reported after the administration of 83 to 200 mg/kg body weight/day of ammonium hydroxide or 100 to 200 mg/kg body weight/day of ammonium carbonate to rabbits for periods of 3 to 16 months and 5 to 26 months, respectively (Fazekas, 1954a,b). Enlargement of the adrenal glands also have been reported in rabbits following oral administration of 1,000 mg ammonium hydroxide/kg body weight/day by gavage (initially every other day) for 17 months (Fazekas, 1939).

Chronic Toxicity and Carcinogenicity Studies

No carcinogenic effects were observed in Swiss and C3H mice provided ammonium hydroxide in the drinking water at levels between 0.1 to 0.3% for their entire life-span (Toth, 1972). The provision of ammonium chloride did not affect the spontaneous development of breast adenocarcinomas in C3H females. In addition, no evidence for an association of increased tumor incidence with ammonia intake due to increased protein intake was observed in male Sprague-Dawley rats (Topping and Visek, 1976).

Developmental Toxicity Studies

One study was identified in which ammonium chloride was administered to pregnant Sprague-Dawley rats. Ammonium chloride was administered to rats at a concentration of 0.9% in the drinking water (equivalent to approximately 290 mg ammonia/kg body weight/day) from GD 7 to GD 20 (Goldman and Yakovac, 1964). Although the administration of ammonium chloride resulted in inhibition of fetal growth, no teratogenic effects were observed in this study.

Mutagenicity and Genotoxicity Studies

Ammonium sulfate was shown to be non-mutagenic in *Salmonella* and *Saccharomyces* test systems (Litton Bionetics, Inc., 1975d). Ammonia produced mutagenic effects in a reverse mutation test in *S. typhimurium* strains, but only at cytotoxic concentrations resulting in less than 2% survivors (Iwaoka *et al.*, 1981a,b). Ammonia also produced positive results in the BALBe/3T3-transformation test, in a sex-linked dominant/lethal test, and in chromosome aberration assays conducted in fibroblasts of Chinese hamsters; however, these effects were only observed at high concentrations as reported by the U.S. Environmental Protection Agency (EPA) (U.S. EPA, 1989). The U.S. EPA reported that other genotoxicity tests gave negative results (U.S. EPA, 1989). Furthermore, the IPCS has stated that there is no evidence to indicate that ammonia would be mutagenic in mammals (EHC, 1986).

Phosphates

Dietary intakes of phosphorus from foods in European countries have been estimated by the European Food Safety Authority (EFSA) to be on average 1,000 to 1,500 mg/day by adults and ranges up to approximately 2,600 mg/day (EFSA, 2005). The EVM also reports a mean daily intake of phosphorus from foods in the British population as being 1,260 mg/day with higher intakes of 2,110 mg/day (EVM, 2003b). Intakes from drinking water is estimated to be 4.4 mg/day assuming consumption of 2 L of water per day at a maximum allowed content of 2.2 mg/L (EFSA, 2005; EVM, 2003b). Ferrous ammonium phosphate is composed of approximately 53% phosphate. The proposed use-levels of ferrous ammonium phosphate would thus provide a phosphate content ranging from 1.27 to 9.54 mg/serving. Therefore, intakes of phosphate ions, and consequently of phosphorus, from ferrous ammonium phosphate-fortified foods are negligible compared to those already consumed in the normal human diet. Nonetheless, toxicity data on phosphate salts are presented below to provide a complete toxicological profile on ferrous ammonium phosphate, but are presented in brief.

Acute Toxicity Studies

Several acute oral toxicity studies have been conducted using sodium and potassium phosphate salts, including studies conducted by the FDRLI as reported by JECFA in their 1982 evaluation of phosphoric acid and phosphate salts (JECFA, 1982). The results of these studies indicate that inorganic phosphates and the resulting doses of phosphorus are not overtly toxic when administered orally to experimental rodents. Oral LD_{50} values for monosodium phosphate were 3,700, 4,100, and 2,000 mg/kg body weight in mice, rats, and guinea pigs, respectively Eichler, 1950; FDRLI, 1975a). Oral LD_{50} values for monopotassium phosphate were 3,200 and 2,820 mg/kg body weight in mice and rats, respectively (FDRLI, 1975b). For tetrasodium diphosphate, oral LD_{50} values reported were 1,300 and 1,380 mg/kg body weight in mice and rats, respectively (FDRLI, 1975c). For sodium triphosphate and sodium hexametaphosphate, oral LD_{50} values reported in mice were 2,300 and 3,700 mg/kg body weight, respectively

(FDRLI, 1975b) and 1,700 and 2,170 to 2,400 mg/kg body weight in rats, respectively (FDRLI, 1975d). In rabbits, the oral LD_{50} of sodium triphosphate was reported as 2,500 mg/kg body weight (FDRLI, 1975b). Following exposure to a large dose of disodium acid phosphate *via* the diet for periods of 24 to 72 hours, enlargement of the kidneys and lesions in the inner and outer cortex and outer medulla were observed in young Sprague-Dawley rats. The diet was supplemented with 10% disodium acid phosphate, equivalent to approximately 10,000 mg disodium acid phosphate or 2,200 mg phosphorus/kg body weight/day (Craig, 1957).

Subchronic Toxicity Studies

The results of animal studies have demonstrated that excessive dietary intake of phosphorus results in increased plasma phosphorus and decreased serum calcium levels. These imbalances may ultimately result in bone loss and calcification of soft tissues through homeostatic controls (JECFA, 1982). Calcification of soft tissues, especially of the kidneys, stomach, and aorta is a common observation in animal studies conducted with high dietary levels of phosphates. Specifically, increased calcification of the kidneys and nephrocalcinosis were reported to occur in rats provided phosphorus (from tetrasodium diphosphate, sodium monophosphate, sodium tripolyphosphate, or orthophosphate) at levels of approximately 419 and 669 mg/kg body weight/day or greater, respectively, in the diet for 24 weeks (Hahn *et al.*, 1956, 1958; Hahn and Seifen, 1959). In addition, Haut *et al.* (1980) provided uninephrectomized, partially nephrectomized, or intact Sprague-Dawley rats with diets supplemented with 0.5, 1.0, or 2.0% phosphorus (equivalent to approximately 250, 500, and 1,000 mg phosphorus/kg body weight/day) for 18 weeks. No adverse effects were observed at the low-dose level (0.5% phosphorus); however, histological alterations were observed in animals of the mid- and high-dose groups and were more pronounced in the uni- and partially nephrectomized animals. In contrast, no histological alterations of the kidneys were observed in male Wistar rats provided with approximately 1,300 mg phosphorus/kg body weight/day (from orthophosphate) in the diet for 21½ weeks in comparison to rats fed diets containing approximately 420 mg phosphorus/kg body weight/day (Dymsza *et al.*, 1959). Therefore, it is difficult to determine the level at which dietary phosphorus induces kidney damage in rats as kidney alterations were observed in some studies where phosphorus was provided at levels higher than in other studies in which no kidney alterations were reported. Furthermore, it is reported that isolated areas of renal calcification may occur in apparently healthy rats that are provided standard diets with no added phosphate (JECFA, 1982).

Significant increases in relative or absolute kidney weights have been reported in studies in which rats were fed diets supplemented with dibasic potassium phosphate, tetrasodium phosphate, or sodium hexametaphosphate, providing phosphorus at levels ranging from 419 to 3,010 mg phosphorus/kg body weight/day for periods of 1 to 6 months (Hodge, 1956; Hahn *et al.*, 1958; Dymsza *et al.*, 1959; Hahn and Seifen, 1959; Datta *et al.*, 1962). Growth retardation also has been observed in rats at high dietary levels of phosphate salts. These included rats provided with diets containing 10% sodium hexametaphosphate or sodium tripolyphosphate

(providing approximately 3,010 and 2,490 mg phosphorus/kg body weight/day, respectively) for 1 month (Hodge, 1956) as well as rats provided with diets containing a mixture of Kurrol's salt and disodium and tetrasodium diphosphate at a level of 5% for 3 months (van Esch *et al.*, 1957; van Genderen, 1958) and rats provided with diets containing 3 and 5% sodium tripolyphosphate or Graham's salt for 6 months (Hahn *et al.*, 1956, 1958; Hahn and Seifen, 1959).

The addition of 10.94 g/day dicalcium phosphate or 6,250 mg/day ammonium polyphosphate to the diets of Wether sheep resulted in increased appetite, weight gain, and apparent nitrogen retention, regardless of the phosphorus source (Fishwick, 1974). No tissue changes were reported when dogs were provided with diets containing 100 mg/kg body weight/day of sodium tripolyphosphate (approximately 25 mg phosphorus/kg body weight/day) or sodium hexametaphosphate (approximately 30 mg phosphorus/kg body weight/day) for 1 month (Hodge, 1956). In this study, dogs also were provided with sodium tripolyphosphate or sodium hexametaphosphate in the diet in increasing doses ranging from 1,000 to 4,000 mg/kg body weight/day (providing up to 1,204 mg phosphorus/kg body weight/day) for 5 months. The dog fed sodium hexametaphosphate began to lose weight at a dose-level of 2,500 mg/kg body weight/day, whereas the dog fed sodium tripolyphosphate only lost weight at a dose-level of 4,000 mg/kg body weight/day. Urinalysis, hematology, and organ weights were normal in both groups, except for the sodium tripolyphosphate-treated dog, which had an enlarged heart (hypertrophy of the left ventricle). Tubular damage was noted in the kidneys of both dogs at the higher doses.

In a study conducted to examine the effect of the calcium to phosphorus ratio, it was observed that pigs fed diets containing 0.65% calcium plus phosphorus at either 1.3 or 1.95% for 6 months experienced decreased weight gain, slight hypocalcaemia, significant hyperphosphatemia, increased serum parathyroid hormone (PTH) levels, and an increased rate of bone turnover in comparison to pigs fed diets containing equal levels of calcium and phosphorus (DeLuca *et al.*, 1976). Minor (microscopic) bone changes that were not apparent upon gross examination were observed in Cinnamon ringtail monkeys provided diets containing phosphorus at up to 4 times the level of calcium for up to 88 months (Anderson *et al.*, 1977).

Chronic Toxicity Studies

In chronic toxicity studies, no adverse effects on growth, reproduction, pathology, blood chemistry, mineral balance, nitrogen retention, or acidic conditions of the digestive tract were observed in rats fed diets containing 0.4 or 0.75% phosphoric acid (providing approximately 63 and 118 mg phosphorus/kg body weight/day) for 3 successive generations (Lang, 1959; Ellinger, 1972). Furthermore, no effects on growth, fertility, or average lifespan were observed in rats fed diets containing 0.5, 1, or 5% of a mixture of two-thirds disodium diphosphate and one-third Kurrol's salt over 3 generations, although nephrocalcinosis was reported to occur in the 1% and 5% dietary groups (van Esch *et al.*, 1957). In contrast, first and second generation rats provided with a diet containing 5% of a mixture of one-third Kurrol's salt and two-thirds

diphosphate from weaning to the end of their life-spans were reported to display significant growth inhibition and decreased fertility (van Esch *et al.*, 1957). Growth inhibition also was reported in rats provided a diet containing 5% sodium tripolyphosphate (providing approximately 630 mg phosphorus/kg body weight/day) or sodium hexametaphosphate (providing approximately 752 mg phosphorus/kg body weight/day) for 2 years (Hodge, 1960a,b). In addition, rats consuming the 5% sodium tripolyphosphate diet had a decreased survival rate in comparison to the other dietary groups (0.05 and 0.5%), although no differences in mortality rates were reported between the 0.05, 0.5, and 5% sodium hexametaphosphate dietary groups. Similar to observations in subchronic studies, increased kidney weights and calcification of the kidneys were observed in rats consuming diets containing high-levels of phosphates (Hodge, 1960a,b).

Reproductive and Developmental Toxicity Studies

No maternal toxicity or teratogenic effects were observed in albino CD-1 mice, Wistar-derived albino rats, golden hamsters, or Dutch-belted rabbits following exposure to phosphate salts in a series of teratology studies conducted by the FDRLI (FDRLI, 1973a,b, 1975a,b,c,d). Phosphate salts were administered daily by oral intubation from GD 6 through to GD 10 to 18, depending on the species, and included monosodium phosphate, monopotassium phosphate, sodium acid pyrophosphate, tetrasodium diphosphate, sodium hexametaphosphate, and sodium tripolyphosphate. The highest dose levels investigated in mice, rats, hamsters, and rabbits were 370 mg/kg body weight/day (monosodium phosphate and sodium hexametaphosphate), 410 mg/kg body weight/day (monosodium phosphate), 166 mg/kg body weight/day (sodium acid pyrophosphate), and 250 mg/kg body weight/day (sodium tripolyphosphate), respectively. In a study by Bonting and Jansen (1956), no adverse effects on growth or reproduction or significant differences in hematological parameters were observed in rats fed diets containing 0.75 and 1.4% phosphoric acid (equivalent to approximately 200 and 375 mg phosphorus/kg body weight/day) for 90 weeks over 3 generations in comparison to control rats.

Mutagenicity and Genotoxicity Studies

No mutagenic effects of monopotassium phosphate, monosodium phosphate, or tetrasodium pyrophosphate were observed in *in vitro* assays using *Saccharomyces cerevisiae* strain D4 or *S. typhimurium* strains TA1535, TA1537, and TA1538, either with or without metabolic activation (Litton Bionetics, Inc., 1975a,b,c; FDRLI, 1975d). Sodium hexametaphosphate also was not mutagenic either with or without metabolic activation in an *in vitro* assay using *S. typhimurium* strains TA1535, TA1536, and TA1537 (Litton Bionetics, Inc., 1975c). The non-mutagenicity of sodium acid pyrophosphate was demonstrated in a number of tests, including a host-mediated assay in mice using *S. typhimurium* strain TA1530, a mitotic recombination frequency assay in *S. cerevisiae* strain D3, an *in vitro* assay using *S. typhimurium* strains TA1535, TA1536, TA1537, and TA1538 with and without the metabolic activation, a dominant lethal test in rats, and a translocation test in rats (Newell *et al.*, 1974). In addition, the results of several tests,

including a host-mediated assay in mice using *S. typhimurium* strains TA1530 and G46, a mitotic recombination frequency assay in *C. cerevisiae* strain D3, an *in vitro* assay using *S. typhimurium* strains TA1530 and G46 and *S. cerevisiae* strain D3 with and without metabolic activation, an *in vivo* cytogenetic study of rat bone marrow cells, an *in vitro* cytogenetic study of human lung cells in tissue culture, and a dominant lethal study in rats have all demonstrated that sodium tripolyphospate is not mutagenic (Litton Bionetics, Inc., 1974).

E. Studies in Humans

Ferrous Ammonium Phosphate

The general safety and tolerance of ferrous ammonium phosphate was examined as part of the bioavailability study conducted in human volunteers (Nestlé Research Center, 2007b). No serious adverse events occurred during the course of this study.

Ferrous Iron Supplementation

The safety of oral ferrous iron formulations has been investigated in a number of studies involving healthy adult men and women or children, unless otherwise stated. In these studies, high-dose iron supplementation at doses equal to or greater than 50 mg/person/day resulted in GI effects, including nausea, diarrhea, vomiting, and constipation. Low-dose iron supplementation, however, was not associated with adverse effects. A summary of the identified studies on iron in the form of ferrous salts is presented below.

Frykman *et al.* (1994) conducted a study with the objective of assessing the tolerance to daily iron intake from a mixed heme/non-heme iron supplement to that from a purely non-heme supplement (ferrous fumarate) for a period of 1 month. The study was conducted in 97 Swedish adult men and women aged 34 to 52 years who were regular blood donors. Daily intakes of supplemental iron were either 18 mg from the supplement containing both heme and non-heme iron (2 mg from porcine blood and 16 mg from ferrous fumarate) or 60 mg from ferrous fumarate. The subjects served as their own placebo controls. To assess tolerance, subjects completed daily evaluations of GI symptoms. Although no significant differences were detected in basic iron status between the 2 groups, the number of subjects experiencing obstipation (35%) as well as total GI side effects (nausea, epigastric pain, obstipation, and diarrhea) (25%) was significantly greater in the group receiving iron in the form of ferrous fumarate compared to that in the heme/non-heme iron (14% and 14%, respectively) and placebo groups (20% and 14%, respectively). No significant differences in side effects were detected between the heme/non-heme iron and placebo groups.

Regular blood donors (289 male and 237 female) were randomized to receive 0, (placebo), 20, or 40 mg/day of iron for 6 months in a double-blind, placebo-controlled study conducted by Radtke *et al.* (2004). Iron supplementation was provided in the form of capsules as ferrous gluconate. A total of 141 male volunteers dropped out of the study; 44, 44, and 58% from the

40 mg, 20 mg, and placebo groups, respectively. A total of 96 female volunteers dropped out of the study; 28, 44, and 49% from the 40 mg, 20 mg, and placebo group, respectively. Volunteers withdrew from the study due to GI complaints, poor compliance, and other reasons not provided. Dropout rates were marginally (male volunteers) or significantly (female volunteers) greater in the placebo group than in either iron group. Approximately 60% of the subjects did not report any side effects and there were no significant differences in the incidence of adverse effects (such as GI complaints) among groups. Specifically, the frequency of GI complaints was low occurring at 11, 13, and 11% in the 40 mg, 20 mg, and placebo groups, respectively.

In a randomized, single blind study conducted by Brock *et al.* (1985), 543 male and female subjects aged 18 to 39 years (n = 272) were given 50 mg of iron daily either in the form of ferrous sulfate or a wax-matrix ferrous sulfate preparation for 56 consecutive days and were asked to record the occurrence of side effects. Fifty percent (50%) of subjects taking the conventional ferrous sulfate preparation (n=272) reported moderate to severe adverse effects. Gastric effects, including abdominal discomfort, nausea, and vomiting were experience by 27.2% of these subjects and bowel-related effects, including constipation, diarrhea, and dark stool were experienced by 28.3% of subjects. The percentage of subjects that experienced these GI effects amounted to 45.6% (some subjects experience both gastric and bowel-related effects). The most common side effects were abdominal discomfort (19.5%) and constipation (17.3%). Other adverse side effects included moderate and severe headaches reported in 9.2% of subjects. Furthermore, of the 272 subjects taking the conventional ferrous sulfate preparation, 44 did not complete the study due to intolerable adverse effects. The limitation of this single blind study is that although results were compared to those from a group consuming ferrous sulfate in a wax-matrix preparation, there was not an appropriate placebo control group. However, the number of subjects experiencing the various GI effects and headaches was significantly greater in the conventional ferrous sulfate group compared to the wax-matrix iron preparation group.

Other studies that reported on the safety and tolerability of ferrous sulfate, confirm the findings of Brock *et al.* (1985). For example, in their randomized, double-bind, cross-over study, Coplin *et al.* (1991) reported that out of 38 pre-menopausal women (aged 18 to 40 years with normal iron status) that ingested 50 mg/day of iron from ferrous sulfate for 14 consecutive days, 34% experienced GI symptoms that were mild to moderate in nature. Such symptoms included abdominal pain, bloating, constipation, diarrhea, and nausea and did not differ in incidence between Week 1 and 2 of the study. In comparison, 24% of the women that ingested iron in the form of bis-glycino iron(II) chelate experienced similar GI symptoms. Other symptoms during the ferrous sulfate ingestion period included headache and fatigue, which were experienced by 13 and 16% of the women, respectively. There were no reports of severe symptoms. This study did not include an appropriate placebo control group.

Female blood donors (mean age of 29 years) were recruited in a study by Maghsudlu *et al.* (2008). Each volunteer received 150 mg of ferrous sulfate 3 times daily (corresponding to 90 mg of iron daily) or a placebo daily for 1 week after each blood donation (every 4 months). Four hundred and twelve (412) healthy volunteers were scheduled for an initial visit and 3 subsequent visits at 4-month intervals for possible repeat donations. Side effects observed in both the iron-supplemented group and placebo group included nausea and vomiting, abdominal cramps, headaches, and constipation. Side effects were recorded in approximately 15, 18, and 18% of the iron-supplemented group versus approximately 7, 6, and 8% of the placebo group at Visits 1, 2, and 3, respectively. A significant difference was observed in the overall incidence of side effects and in the occurrence of nausea and vomiting in the iron-supplemented group compared to the placebo group.

In a randomized, controlled, double-blind, double dummy study conducted by Liguori (1993), involving confirmed iron-deficient or iron-deficient anemic patients, 1095 men and women aged 15 to 88 (mean age of 40 years) received 105 mg/day of iron as ferrous sulfate or 120 mg/day of iron as ironproteinsuccinylate for a period of 60 consecutive days. Subjects were evaluated on Days 30 and 60 post-treatment. There were no compound-related adverse effects on hematology or serum chemistry and general tolerability to both iron supplements was favorable. Of the patients receiving ferrous sulfate, 26.3% experienced adverse reactions including heartburn, epigastric pain, constipation, abdominal pain, nausea, and skin rash. The number of reported incidences of each adverse reaction type was 33, 31, 23, 32, 14, and 8, respectively, with 141 incidences in total. The general tolerability also was favorable with ironproteinsuccinylate. In this group, 11.5% of patients also experienced heartburn, epigastric pain, constipation, and abdominal pain. The number of reported incidences of these events was 25, 19, and 25, respectively, and was significantly less in frequency and duration than with ferrous sulfate. All events with both iron formulations were transient and none could be classified as severe or potentially harmful.

In 3 series of 3 randomized, double-blind, placebo-controlled studies conducted by Hallberg *et al.* (1966), blood donors ingested a ferrous sulfate supplement or placebo after a blood donation for 14 consecutive days and completed an evaluation following the treatment period. In series I and II, the dose of iron provided as ferrous sulfate was 222 mg/day (two 37-mg tablets 3 times daily), whereas in series III, iron was provided at a dose of 180 mg/day (one 60-mg tablet 2 times daily). Series I, II, and II consisted of 195 (162 males and 33 females), 119 (108 males and 11 females), and 200 (170 males and 30 females) subjects within the placebo group, respectively, and 198 (163 males and 35 females), 120 (107 males and 13 females), and 195 (152 males and 43 females) subjects within the ferrous sulfate group. Within each series, the number of subjects experiencing GI side effects was consistently and significantly greater in the ferrous sulfate group than in the placebo group. Side effects included diarrhea, nausea, and minor side effects, such as slight abdominal distension, constipation, or a mild degree of loose stools. The percentage of subjects that reported these side effects were 13.6% placebo *vs.*

22.9% ferrous sulfate for series I, 13.6% placebo *vs.* 22.9% ferrous sulfate for series II, and 12.4% placebo *vs.* 26.5% ferrous sulfate for series III. The number of subjects that discontinued iron supplementation as a result of side effects was significantly greater in the ferrous sulfate group than in the placebo group for series I (0.9% placebo *vs.* 9.9 ferrous sulfate) and series II (1.1% placebo *vs.* 8.8% ferrous sulfate).

Fourteen healthy subjects (13 females and 1 male, aged 24 to 48 years), serving as their own controls, ingested 650 to 975 mg/day (two to three 325 mg tablets/day) of ferrous sulfate for 2 weeks (Laine *et al.*, 1988). All subjects developed dark brown-black stools during the study. Following 1 and 2 weeks of ferrous sulfate supplementation, symptoms of nausea and vomiting were significantly increased in severity compared to 2 weeks prior to initiation of the study. However, severity scores of abdominal pain were not significantly altered by iron supplementation. Endoscopic examination revealed a significant increase in severity of abnormalities in the stomach after 2 weeks of iron supplementation. No abnormalities were found in the duodenum either before or after iron supplementation. Abnormalities found in the stomach were considered mild and included erythema in 9 subjects, small areas of subepithelial hemorrhage in 6 subjects, and solitary antral erosions in 2 subjects. Three subjects presenting with erythema also had subepithelial hemorrhage and 1 subject presented with all 3 abnormalities. In addition, 2 subjects presenting with abnormalities also had elevated levels of fecal blood loss (determined by quantifying fecal hemoglobin concentrations), although, the overall mean fecal blood loss from all subjects after iron supplementation did not differ significantly from prior to iron supplementation. Endoscopy scores also positively and significantly correlated with those of abdominal pain. Although abnormalities were found in the stomach following iron supplementation, gastric biopsies of the abnormalities and of random areas did not reveal any histologic changes. Biopsies of the antrum and duodenum also were unremarkable.

Effects of Ferrous Iron Supplementation on Human Intra-Uterine Development

Iron supplementation during pregnancy is commonly recommended in order to prevent and treat iron deficiency anemia. Several randomized, placebo-controlled, double-blind human studies have evaluated the safety of iron supplementation during pregnancy on birth outcome measures as summarized below. Additional studies summarized include studies conducted in infants and children. The results of the human studies conducted with ferrous iron supplements or fortified foods during gestation or in infants or children indicate that iron is not teratogenic and does not result in adverse birth outcomes or adverse growth and development, and may be considered safe to the mother developing embryo/fetus and/or infant.

In a study conducted by Cogswell *et al.* (2003), 275 iron replete women (serum hemoglobin concentration ≥110 g/L and serum ferritin concentration ≥20 μg/L) were randomly assigned to receive either 30 mg/day of iron as ferrous sulfate (n = 117) or placebo control (n = 96) from enrolment (<20 weeks of gestation, average 11 weeks) to 28 weeks of gestation. At 28 weeks,

iron status was re-evaluated and women with ferritin concentrations of 12 to <20 µg/L were given 30 mg/day of iron and those with ferritin concentrations <2 µg/L were given 60 mg/day of iron regardless of initial assignment. This protocol was repeated at 38 weeks of gestation. Iron supplementation from the time of enrolment to 28 weeks of gestation resulted in infants with significantly increased birth weight (3,277 g) compared to infants of the placebo group (3,072 g). Infants from the iron supplementation group also displayed a significant decrease in the incidences of pre-term low birth weight (2.6%) and small-for-gestational age (6.8%) compared to placebo infants (10.4 and 17.7%, respectively). In addition, the gestational age at birth was significantly higher in women of the iron supplementation group compared to those receiving placebo. Iron supplementation did not significantly affect the incidences of pre-term delivery or body length at birth. Iron supplementation during pregnancy also did not result in adverse effects on birth outcome measures.

In a similar study, iron-replete, non-anemic pregnant women at <20 weeks of gestation (average 11 weeks) were randomly assigned to receive either 30 mg/day of iron as ferrous sulfate within a multivitamin (n = 166) or a multivitamin without iron (n = 168) until 26 to 29 weeks of gestation (Siega-Riz *et al.*, 2006). At birth, infants from the iron supplement group weighed, on average, 108 g heavier than infants from the control group, a significant increase. In addition, the incidence of pre-term delivery was approximately 50% less likely in the iron group compared to the control group (7.5% *vs.* 13.9%). Iron supplementation did not significantly affect the incidences of low birth weight or small-for-gestational age or gestational age at delivery.

Similar findings were reported in a study in which pregnant women received 100 mg/day of iron from 28 weeks of gestation until the end of gestation (Preziosi *et al.*, 1997). Mean body length at birth was significantly greater in infants from mothers treated with iron (n = 99) compared to infants of the placebo group (n = 98). However, birth weight was unaffected by iron supplementation. Apgar scores were also significantly higher in infants from the iron supplementation group compared with control infants. Apgar scores are based on the evaluation of signs pertaining to the condition of the infant at birth (Finster and Wood, 2005). These signs are heart rate, respiratory effort, reflex irritability, muscle tone, and color, which are evaluated 60 seconds after the complete birth of the infant. Scores of 0 to 2 indicate infants in poor condition, scores of 3 to 7 indicate infants in fair condition, and scores of 8 to 10 indicate infants in good condition. Infants from both treated and control groups scored high (9.2 and 9.6, respectively), and thus, were in good condition.

A study conducted in infants also failed to find adverse effects associated with iron supplementation on growth (Abdelrazik *et al.*, 2007). In this study, breast-fed infants 4 to 6 months of age received either an oral multivitamin containing 1 mg/kg body weight/day of iron in the form of ferrous gluconate or placebo control (the same multivitamin without iron). For analytical purposes, the ferrous gluconate group (n = 198) was further subdivided into well-nourished (n = 86) or malnourished (n = 112) categories, which were in turn stratified into

anemic (n = 58 well-nourished and 72 malnourished) and non-anemic (n = 30 well-nourished and 40 malnourished) subgroups. The placebo group (n = 50), however, consisted only of well-nourished, non-anemic infants. After 6 and 12 months of treatment, weight gain (g/mo) and length gain (mm/mo) were significantly higher in iron-supplemented infants compared to infants receiving placebo. Iron therapy did not affect head circumference gain. Within the ferrous gluconate group, weight and length gain was significantly higher in malnourished infants compared to well-nourished infants as assessed at 6 and 12 months. Within the malnourished group, anemic infants displayed significantly higher weight and length gain compared to non-anemic infants, but only after 6 months of iron therapy. Morbidity risk was measured by incidences of diarrhea and fever and found to be significantly higher in malnourished infants compared to well-nourished infants, both receiving ferrous gluconate. Because the ferrous gluconate group consisted of a large portion of malnourished infants compared to the placebo group, which did not consist of any malnourished infants, iron supplementation appeared to significantly increase the risk of morbidity. The anemic status of the iron-supplemented infants did not affect the risk of morbidity. The authors concluded that the apparent effect of iron therapy on morbidity was linked to the immunologic background of the infants rather than on iron status or iron supplementation *per se*.

To determine the safety of iron fortification of a powdered human milk fortifier, Berseth *et al.* (2004) conducted a 28-day study in which pre-term infants were randomized to receive either 5.76 mg/day of iron from the iron-fortified human milk fortifier or a control human milk fortifier. Results showed that infant characteristics did not differ significantly between the fortifier groups throughout the course of the study. Such characteristics included daily weight gain, weekly weight gain, weekly body length gain, and weekly head circumference gain. Iron fortification of a human milk fortifier product did not adversely affect the growth of infants. Both fortifiers were well-tolerated by infants and did not differ in morbidity or adverse events. Specifically, hematology and clinical chemistry parameters were not significantly affected by iron fortification on Days 14 and 28 of the study. The incidence of septic infections and necrotizing enterocolitis were also similar between the groups and were very low.

In addition to the general growth of the developing fetus or infant, iron supplementation does not adversely affect neurocognitive development as a result of early infant exposure. In a study conducted by Steinmacher *et al.* (2007), infants were randomly assigned to early or late iron supplementation groups. In the early-iron group, iron supplementation was initiated at a median age of 14 days in 52 girls and 38 boys, while in the late-iron group, iron supplementation was initiated at a median age of 61 days in 54 girls and 20 boys. Iron was administered *via* infant formula at a dose of 2 mg/kg body weight/day and increased to 4 mg/kg body weight/day if iron deficiency was detected during the study. Children completed a standardized follow-up assessment at a median age of 5.3 years. Mildly or severely abnormal results on the standardized neurology examination were significantly higher in the late-iron group (26%, n = 74) compared to the early-iron group (17%, n = 90). Standardized assessments of mobility,

motor coordination, cognitive function, visual impairment, and childhood behavior were similar between the 2 groups. There were, however, suggestive trends toward improved cognitive development in infants receiving early iron supplementation compared to infants in the late-iron group. Furthermore, in a study conducted by Zhou *et al.* (2006), *in utero* exposure to iron supplements did not adversely affect the intelligence quotient (IQ) at childhood age. In this study, pregnant women were randomly assigned to receive either 20 mg/day of iron or placebo beginning at 20 weeks of gestation to delivery and the children followed up at 4 years of age. A standardized IQ assessment revealed that children from the iron (n = 153) and placebo (n = 49) groups were similar in their mean composite IQ scores. Both groups also did not differ significantly in any of the IQ subscales, such as verbal reasoning, visual reasoning, quantitative reasoning, and short-term memory. Furthermore, the proportion of children that fell 1 or 2 standard deviations below the mean was similar between the iron and placebo groups. The abnormal total behavior score was significantly elevated in children of the iron group compared to children of the placebo group. However, the mean scores for behavior difficulties, categorized as emotion, conduct, hyperactivity, and peer relations, did not differ significantly between children of the iron and placebo groups.

Ammonium

Most of the adverse effects resulting from the consumption of ammonium ions in humans result from accidental ingestion of solutions of ammonium hydroxide (EHC, 1986). In such cases of accidental exposure, tissue-destructive effects such as esophageal burns have been reported. In one case, the ingestion of a solution containing 24 g ammonia/L resulted in death. Upon autopsy, it was revealed that the solution induced hemorrhagic inflammatory changes in the esophagus, stomach, and small intestine (Klendshoj and Rejent, 1966). In the EHC report compiled for ammonia by the IPCS in 1986, it was reported that no data on the acute oral toxicity of ammonium compounds in humans, other than ammonium chloride, is in existence (EHC, 1986). There is little evidence for ammonium ions to induce toxicity in humans, except in cases of acute accidental exposure (EHC, 1986).

Phosphates

In contrast to effects observed in experimental rats, there have been no reports of ectopic calcification resulting from high dietary intakes of phosphorus in humans with adequate renal function (EFSA, 2005). In addition, the provision of phosphorus to healthy subjects (n = 5 to 16), resulting in phosphorus intakes ranging from 1,000 to 3,000 mg/day for periods of 1 day to 4 months did not result in any adverse effects (Reiss *et al.*, 1970; Portale *et al.*, 1986; Heaney and Recker, 1987; Calvo and Heath, 1988, 1990). As well, the consumption of 6,000 mg $NaH_2PO_42(H_20)$ (approximately 1,194 mg phosphorus) per day for 15 days and the consumption of 5,000 to 7,000 mg NaH_2PO_4 (1,500 mg phosphorus) per day for an unspecified length of time resulted in no adverse effects (Lauersen, 1953; Lang, 1959). Nausea, vomiting, and diarrhea have been reported in studies where phosphorus intakes in healthy subjects (n = 8 to 79)

ranged from 750 to 3,008 mg/day for periods of 1 to 6 weeks (Bell *et al.*, 1977; Brixen *et al.*, 1992; Whybro *et al.*, 1998; Grimm *et al.*, 2001). In one study, in which 8 adult volunteers were provided a diet containing 700 mg calcium and 2,100 mg phosphorus/day for 4 weeks following a 1-month control period, all volunteers reported intestinal distress, soft stools, or mild diarrhea; however, these symptoms subsided in 6 of the volunteers and only persisted intermittently throughout the 4 weeks in the remaining 2 (Bell *et al.*, 1977).

In the study by Bell *et al.* (1977), consumption of the high-phosphorus diet (2,100 mg phosphorus/day for 4 weeks) resulted in increased serum and urinary phosphorus levels and decreased serum and urinary calcium levels. In contrast, the provision of 2,000 to 4,000 mg phosphoric acid/day (equivalent to approximately 632 to 1,264 mg phosphorus/day) to 15 students for 10 days or 3,900 mg phosphoric acid/day (equivalent to approximately 1,232 mg phosphorus/day) to 2 males for 14 days did not result in changes in urinary composition (Lauersen, 1953). In neonates provided infant formulae based on cows' milk, hypocalcaemia has been reported to occur and has been ascribed to the high phosphorus content of the formulae (Venkataraman *et al.*, 1985; Specker *et al.*, 1991). The lower renal capacity of neonates and young infants results in higher serum phosphate values than would be expected to be observed in older infants and adults at comparable relative intakes. If serum phosphate levels are too high, bone accretion may be adversely affected, and rickets and hypocalcemic tetany may result (EFSA, 2005).

Significant increases in serum levels of PTH, which induces demineralization of bone tissue, were observed in some studies. However, this effect is inconsistently observed among studies, which is supported by reports demonstrating no change in the levels of bone resorption markers and serum osteocalcin following supplemental intakes of phosphorus ranging from 750 to 3,008 mg/ day for periods of 7 days to 4 months (Heaney and Recker, 1987; Calvo and Heath, 1990; Brixen *et al.*, 1992; Whybro *et al.*, 1998; Grimm *et al.*, 2001). It has been hypothesized that the variability in serum PTH response may be partly explained by the circadian rhythm of PTH secretion and the variation in the time of blood sampling between the studies (EFSA, 2005). Conflicting effects on serum 1,25-dihydroxyvitamin D, the active form of vitamin D and regulator of serum calcium and phosphorus concentrations, also have been reported. Significant decreases in serum 1,25-dihydroxyvitamin D were reported after the consumption of 3,000 mg phosphorus for 10 days (Portale *et al.*, 1986); however, serum 1,25-dihydroxyvitamin D was reported to increase significantly after consumption of 1,660 mg phosphorus/day for 8 days and was reported not to change significantly after consumption of 1,700 mg phosphorus/day for 28 days (Calvo and Heath, 1988, 1990). The EFSA Scientific Panel on Dietetic Products, Nutrition, and Allergies has reported that no human studies have linked high phosphorus intake, regardless of calcium intake, to lower bone mass or increased rates of bone loss (EFSA, 2005). In addition, the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) was asked to evaluate whether high phosphorus intakes could adversely affect bone health (COT, 2004). After reviewing the available data, the COT concluded that "the

elevated PTH levels associated with supplemental phosphorus intakes reflect a short term adjustment to maintain plasma calcium levels and do not necessarily represent an adverse effect of phosphate on bone health".

F. Evaluations Conducted by Scientific Bodies

The IOM (2001) has established a Tolerable Upper Intake Level (UL) of 45 mg/person/day (equivalent to 0.75 mg/kg body weight/day for a 60 kg adult) for the total intake of iron from all sources by adults and adolescents based on GI effects observed in individuals consuming 60 mg of iron from ferrous fumarate as supplemental iron to the diet in the study by Frykman *et al.* (1994). In infants, doses of supplemental non-heme iron within the range of 5 to 30 mg/day for several months are reported to not cause adverse GI effects (IOM, 2001). Therefore, for infants and young children, the IOM determined the UL of supplemental non-heme iron to be 40 mg/person/day. The IOM concluded that based on the UL, the risk of adverse effects from dietary sources appears to be low and that GI distress does not occur from consuming a diet containing naturally occurring or fortified iron. Importantly, the UL applies only to the general population *i.e.,* excluding those with hereditary hemochromatosis, or to those being treated with iron under close medical supervision.

Iron compounds also have been reviewed by a number of other scientific bodies, including the EVM (2003a), the EFSA Scientific Panel on Dietetic Products, Nutrition, and Allergies (EFSA, 2004), and JECFA (1983). The EVM concluded that there are insufficient appropriate data to establish a safe UL for iron, especially considering that GI effects are associated with iron in supplements rather than in foods (EVM, 2003a). However, the EVM has concluded that for guidance purposes, a supplemental intake of approximately 17 mg/day (equivalent to 0.28 mg/kg body weight/day for a 60 kg adult) would not be expected to produce adverse effects in the majority of people. This value does not apply to those with an increased susceptibility to iron overload. The EFSA Panel also was unable to establish a UL for iron, stating that the adverse GI effects reported after short-term oral supplementation with non-heme iron preparations at doses of 50 to 60 mg daily were not a suitable basis to establish a UL for iron from all sources (EFSA, 2004). At its twenty-seventh meeting, JECFA evaluated iron (JECFA, 1983) and assigned a group provisional maximum tolerable daily intake (PMTDI) of 0.8 mg/kg body weight, which applies to iron from all sources except for iron oxides used as coloring agents, supplemental iron taken during pregnancy and lactation, and supplemental iron for specific clinical requirements. The PMTDI set by JECFA was based on studies showing that normal individuals have taken supplements of 50 mg of iron (ferrous iron) for long periods of time without any adverse effects, and is consistent with the UL set by the IOM.

For phosphorus, the IOM has established a recommended dietary allowance (RDA) in the range of 460 to 1250 mg/person/day and a UL of 3,000 to 4,000 mg/day, depending on the population group (IOM, 1997). In their latest evaluation of phosphoric acid and phosphate salts at the twenty-sixth meeting, JECFA established a Maximum Tolerable Daily Intake (MTDI) of 70 mg

phosphorus/kg body weight/day, which applies to the sum of phosphates present naturally in food and those present as additives (JECFA, 1983). In contrast, the EFSA Panel determined that the critical endpoint of phosphorus-induced nephrocalcinosis observed in rats that was used to set the MTDI was not relevant to humans (EFSA, 2005). The Panel also concluded that although mild GI effects, such as nausea, vomiting, and diarrhea, have been observed with supplemental intakes of 750 mg phosphorus/day and greater, normal healthy individuals can tolerate phosphorus intakes of at least 3,000 mg/day without adverse systemic effects. Therefore, the Panel considered that the GI effects observed were not a suitable basis to establish a UL for phosphorus from all sources. The Panel also determined that a UL could not be established based on the effect of a high phosphorus intake on the activity of calcium regulating hormones, which the Panel considered not to be adverse in themselves, and to have no demonstrable effects on bone mineral density and skeletal mass. The Panel concluded that there was not sufficient data available to establish a UL for phosphorus. The EVM also concluded that insufficient data exist to establish a safe UL for inorganic phosphate (EVM, 2003b). However, taking into consideration the osmotic diarrhea and mild GI effects observed in some individuals with supplemental intakes of 750 mg phosphorus/day and the vulnerability of persons with hypovitaminosis D to hyperparathyroidism, the EVM concluded that, for guidance purposes, a supplemental intake of 250 mg phosphorus/day (equivalent to 4.2 mg/kg body weight/day for a 60 kg adult) would not be expected to result in adverse effects, including mild GI upset. When combined with the maximum intake from food and water (2,100 mg/day), this corresponds to an estimated total intake of 2,400 mg/day (equivalent to 40 mg/kg body weight/day for a 60 kg adult) that would not be expected to result in any adverse effects (EVM, 2003b).

JECFA allocated an acceptable daily intake (ADI) of "not specified" for ammonium salts in the form of ammonium carbonate and ammonium hydrogen carbonate at its twenty-sixth meeting (JECFA, 1982). It was noted in the evaluation that although toxicological data for these ammonium salts were limited, extrapolation of results from studies with ammonium compounds (primarily ammonium chloride) and with sodium or potassium carbonate provided a basis for evaluation. At its twenty-ninth meeting, JECFA prepared a table providing ADIs for a large number of combinations of cations and anions, including ammonium salts (JECFA, 1986). No restriction was placed on the intake of ammonium from ammonium salts, provided that the contribution made to food is assessed and considered acceptable. Ammonia also has been evaluated by the IPCS (EHC, 1986). In their report, the IPCS concluded that ammonia does not present a direct threat to man except as a result of accidental exposure, particularly in industry. As part of their evaluation of drinking-water quality, WHO also has previously assessed the safety of ammonia (WHO, 2003). In their evaluation, WHO concluded that ammonia is not of direct importance for health in the concentrations expected in drinking-water, and therefore, a health-based guideline was not derived.

G. Summary and Basis for GRAS Conclusion

Ferrous ammonium phosphate is an inorganic salt of iron(II), ammonium, and phosphate ions that is produced consistent with cGMP to meet appropriate food grade specifications. Ferrous ammonium phosphate is a source of iron, the bioavailability of which is comparable to iron from other iron compounds already approved for use in fortified foods in the U.S. When subject to the low pH conditions of the stomach, ferrous ammonium phosphate dissociates to iron, ammonium and phosphate ions. Therefore, the safety of ferrous ammonium phosphate was evaluated based on the considerable published database available on iron, phosphate and ammonium salts. A critical evaluation of this scientific data that is generally available in the public domain have been determined by Nestlé not to indicate any potential for adverse effects in humans following consumption of ferrous ammonium phosphate under the intended conditions of use. In addition, iron, ammonium, and phosphate salts have been historically consumed as added or natural components of the normal human diet. Estimated intakes of iron under the intended conditions of use of ferrous ammonium phosphate (*i.e.*, replacement of current iron fortificants) are well below the UL of 45 mg/person/day (0.75 mg/kg body weight/day) set by the IOM for adults and adolescents and the PMTDI of 0.8 mg/kg body weight/day established by JECFA. Furthermore, food fortification with ferrous ammonium phosphate will result in negligible intakes of ammonium and phosphate when compared to levels currently consumed in the diet or produced naturally in the body. Finally, a comprehensive package of data were critically evaluated by a panel of experts, qualified by scientific training and experience to evaluate the safety of ferrous ammonium phosphate as a component of food, who unanimously concluded that "the intended uses in traditional foods of ferrous ammonium phosphate, produced consistent with cGMP and meeting appropriate food-grade specifications presented in the supporting dossier, are safe and suitable". The Expert Panel further concluded that "the intended uses in traditional foods of ferrous ammonium phosphate, produced consistent with cGMP and meeting appropriate food-grade specifications presented in the supporting dossier, are Generally Recognized as Safe (GRAS) based on scientific procedures". Therefore, Nestlé has concluded that ferrous ammonium phosphate is GRAS under the intended conditions of use on the basis of scientific procedures.

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Table of CFR Sections Referenced (Title 21—Food and Drugs)		
Part	**Section §**	**Section Title**
104 – Nutritional quality guidelines for foods	104.20	Fortification policy
182—Substances generally recognized as safe	182.1073	Phosphoric acid
184—Direct food substances affirmed as generally recognized as safe	184.1139	Ammonium hydroxide
	184.1375	Iron, elemental

Appendix A

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APPENDIX A

EXPERT PANEL CONSENSUS STATEMENT REGARDING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF FERROUS AMMONIUM PHOSPHATE FOR USE AS AN INGREDIENT IN FOOD

EXPERT PANEL CONSENSUS STATEMENT REGARDING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF FERROUS AMMONIUM PHOSPHATE FOR USE IN FOODS

26 September 2008

INTRODUCTION

At the request of Nestec Limited (Nestec), an Expert Panel (the "Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information on ferric ammonium phosphate and determine whether the intended use in foods of ferric ammonium phosphate is safe and suitable, and Generally Recognized as Safe (GRAS) based on scientific procedures. The Panel consisted of the following qualified scientific experts: Prof. Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine), Prof. Robert Nicolosi, Ph.D. (University of Massachusetts Lowell), and Prof. John Thomas, Ph.D. (Indiana University School of Medicine).

The Panel, independently and collectively, critically examined a comprehensive package of scientific information and data compiled from the literature and other published sources (the dossier) through February 2008 by Cantox Health Sciences International (Cantox). In addition, the Panel evaluated other information deemed appropriate or relevant, including data provided by Nestec as well as by Dr. Paul Lohmann, the supplier and manufacturer of the ferrous ammonium phosphate. The information evaluated by the Panel included details pertaining to the method of manufacture and product specifications, supporting analytical data, intended use-levels in specified food products, and a comprehensive assessment of the available scientific literature pertaining to the safety of the ionic components of ferrous ammonium phosphate, specifically iron(II), ammonium, and phosphate.

Following independent, critical evaluation of such data and information, the Panel convened on 5 August 2008 and unanimously concluded that the intended use in traditional foods of ferrous ammonium phosphate, manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications is safe and suitable and is GRAS based on scientific procedures. A summary of the basis for the Panel's conclusions provided below.

SUMMARY AND BASIS FOR GRAS

Background

Iron is an essential element required nutritionally in order to maintain a variety of physiological functions. Since iron-deficiency is a common condition, the fortification of foods with iron and various iron salts is commonplace. The development of iron sources that are both bioavailable and stable in food continues to challenge nutritionists, food technologists, and health professionals. Nestec Ltd. intends to use ferrous ammonium phosphate for fortification purposes in the United States (U.S.) as a direct replacement of other iron fortificants currently in use to help meet the Recommended Dietary Allowances (RDAs) established by the Institute of Medicine (IOM, 2001) and summarized in Table 1. Ferrous ammonium phosphate is specifically developed for use in foods in which interactions between other iron salts and components of the food (*e.g.*, polyphenols or fat) can have detrimental effects on the organoleptic properties of the finished product.

Table 1 RDAs for Iron for the General U.S. Population

Life Stage Group		RDA for Iron (mg/person/day)
Children	1-3 y	7
	4-8 y	10
Males	9-13 y and 19->70 y	8
	14-18 y	11
Females	9-13 y and 51->70 y	8
	14-18 y	15
	19-50 y	18
Pregnancy	14-50 y	27
Lactation	14-18 y	10
	19-50 y	9

Technical Information

Ferrous ammonium phosphate is well characterized and defined by a product specification consistent with a 1:1:1 molar ratio of iron(II), phosphate and ammonium and an iron loading of approximately 30%. Ferrous ammonium phosphate is a synthetic compound and is manufactured by the reaction of ammonium hydroxide, iron powder, and phosphoric acid consistent with cGMP. The only significant impurity associated with ferrous ammonium phosphate arises by oxidation of iron(II) to iron(III), however, under the appropriate conditions of production and storage conditions these levels are relatively low (*ca.* 5%) and are not a safety concern. The product specification includes limits for iron(II) (22 to 30%), iron(III) (max. 7%) and the total iron content (min. 28%) to ensure the composition and purity of the material.

Ferrous ammonium phosphate is stable under both the recommended storage conditions and conditions of intended use where stability is measured by monitoring any detrimental effects on the organoleptic properties (*e.g.*, taste, color, odor) of the final fortified foods. In particular, stability experiments demonstrate that in products high in polyphenols or fat and representative of the intended use, the organoleptic properties are comparable or superior to other common fortification salts (Sher *et al.*, 2001; Rekhif *et al.*, 2002). The key property of ferrous ammonium phosphate responsible for the observed stability in final products is its poor solubility at higher pH (*e.g.*, neutral conditions) but high solubility at low pH (acidic conditions). As a consequence, the material exhibits little reactivity in finished products so limiting its effect on the organoleptic properties of the formulation until subject to the low pH conditions of the stomach where dissociation to iron, ammonium, and phosphate occurs.

Bioavailability

Solubilization of ferrous ammonium phosphate at low pH involves hydration of each of the individual ions thereby facilitating bioavailability. In human subjects, iron absorption from a milk product fortified with 5 mg of iron as ferrous ammonium phosphate was shown to be approximately 7%, compared with iron absorption of approximately 10 and 3% obtained when the milk product was fortified with ferrous sulfate and ferric pyrophosphate, respectively (Nestlé Research Center, 2007a,b). The absorption of iron from ferrous sulfate-fortified cereal-based foods and milk-based products ranges from 2 to 11%. These iron absorption values are consistent across all age groups (infants, children, adolescents, and adults). Based on the consistency of this data, it can be expected that the absorption of iron from ferrous ammonium phosphate fortified cereal-based foods and milk-based products will be slightly less than that of ferrous sulfate (*i.e.*, approximately 7%). The solubility and bioavailability of ferric ammonium phosphate is within the range of other forms of iron approved for use as direct food substances by the U.S. Food and Drug Administration (FDA) demonstrating suitability for the intended use.

The bioavailability of iron from iron salts is not only dependent on the formulation of iron, but also on the requirement of the body for iron, the type of food that is fortified (*i.e.*, the matrix) , and the presence of enhancers and inhibitors of iron absorption in the food. Enhancers of iron absorption include meats, vitamins A and C, and various organic acids; inhibitors include phytic acid, polyphenols, and calcium (IOM, 2001; EVM, 2002; Lopez and Cámara Martos, 2004; Teucher *et al.*, 2004). Although ingestion of large amounts of iron (25 mg per person or greater) in the inorganic form from supplementation may inhibit the absorption of zinc (1:1 or greater molar ratio of iron to zinc) (Meadows *et al*, 1983; Solomons *et al.*, 1983; EVM, 2002), the ingestion of iron from iron-fortified foods typically fortified with lower amounts of iron, does not appear to inhibit zinc absorption (Valberg *et al.*, 1984; Sandström *et al.*, 1985; Davidsson *et al.*, 1995; Fairweather-Tait *et al.*, 1995). Once solubilized in the upper gastrointestinal (GI) tract, iron is absorbed by the intestinal mucosa, released into the blood stream, and distributed throughout the body by the transport protein transferrin (INACG, 1993; IOM, 2001; EVM, 2002).

Iron is incorporated into several different types of proteins for utilization in a variety of biological functions. Proteins that require iron for functionality include hemoglobin, myoglobin, and cytochromes. Excess iron that is not utilized is stored in the liver, bone marrow, and in reticuloendothelial cells in the form of ferritin and hemosiderin. The excretion of iron is largely dependent on the production and degradation of erythrocytes as most iron in the body is contained within erythrocytes. Virtually all the iron from erythrocytes is recycled for incorporation into hemoglobin. Thus, only a small amount of iron is excreted daily, predominantly *via* feces.

Proposed Use and Use-Levels

Nestec intends to use ferrous ammonium phosphate as a source of iron in a limited range of food and beverage products as a direct replacement of other iron fortificants. Fortification will be in accordance with the Fortification Policy (21CFR §104.20) outlined in the FDA's Nutritional Quality Guidelines for Foods (21CFR §104.20) (U.S. FDA, 2008) and will comply with the general nutritional labeling guidelines that a "good source" contains between 10 to 19% of the Daily Value (DV) in the Reference Amounts Customarily Consumed (RACC) and an "excellent source" more than 20% of the DV in the RACC (21CFR §101.13) (U.S. FDA, 2008). Fortification at levels between 10 and 30% of the Daily Value in the RACC for iron, currently 18 mg/person/day, is the equivalent of 1.8 to 3.6 mg of iron for children greater than 4 years of age, adults and pregnant and lactating women (see Table 2).

Table 2 Summary of the Use-Levels for Ferrous Ammonium Phosphate and Iron in the U.S. by Population Group Based on Food-Use Levels of Ferrous Ammonium Phosphate that Provide 10 and 30% of the DV of Iron Per Serving (RACC)[a]

Population Group	DV (mg/day)	Use-Level that Provides 10% of DV		Use-Level that Provides 30% of DV	
		Iron (mg/serving)	Ferrous Ammonium Phosphate (mg/serving)	Iron (mg/serving)	Ferrous Ammonium Phosphate (mg/serving)
Children Less Than 4 Years	10	1	3.3	3.0	10.0
Children 4 or more years of age and Adults	18	1.8	6	5.4	18.0
Pregnant or Lactating Women	18	1.8	6	5.4	18.0

DV = Daily Value as reported in Appendices F and G of the *Food Labeling Guide* (U.S. FDA, 2008); [a] serving size or Reference Amounts Customarily Consumed (RACC) (U.S. FDA, 2008)

The IOM (2001) has established a Tolerable Upper Intake Level (UL) of 45 mg/person/day (equivalent to 0.75 mg/kg body weight/day for a 60 kg adult) for the total intake of iron from all

sources by adults and adolescents based on GI effects observed in individuals consuming 60 mg of iron from ferrous fumarate as a supplement to the diet. For infants and young children, the IOM determined the UL of supplemental non-heme iron to be 40 mg/person/day. Representative examples of iron intakes from typical product servings fortified with ferrous ammonium phosphate show that the level of iron consumed is well below the respective ULs set by the IOM for different population groups. A worse case scenario based on fortification at the use level of 20% of the DV per RACC, the level considered an "excellent source" of the mineral, and intake of only larger typical beverage serving size of 14 oz, 7 servings would have to be consumed by adolescents and adults and 6 servings by young children in order to exceed the relevant ULs. Although Nestec does not intend to market or formulate products for consumption by children less than 4 years of age in the event that this population group does consume products fortified at a level of 20% of the DV for other population groups, 11 servings of the RACC/person/day would be required to reach the UL of 40 mg/person/day established for young children (IOM, 2001). Since it is highly unlikely that children of this age would consume 11 servings, there are no safety concerns.

Since the levels of fortification are low and well below the ULs established by the IOM, it is unlikely that adverse GI effects would occur since adverse GI effects have only been associated with high intakes from iron supplements. Neither the Expert Group on Vitamins and Minerals (EVM, 2002) or the European Food Safety Authority (EFSA, 2004) has set a safe or tolerable upper limit for total iron, although the latter noted "the risk of adverse effects from iron overload in the general population, including those heterozygous for hereditary hemochromatosis, is considered to be low". The Joint FAO/WHO Expert Committee on Food Additives (JECFA) (1983) has presented a provisional maximum tolerable intake of 0.8 mg/kg body weight/day based on the safe long-term intake of ferrous supplements of 50 mg/person/day (for a 60 kg individual) which is consistent with the IOM.

The IOM has established an RDA for phosphorus in the range of 460 to 1250 mg/person/day and a UL of 3,000 to 4,000 mg/day, depending on population group (IOM, 1997). Typical use-levels of ferrous ammonium phosphate that provide 20% of the DV of iron will result in a phosphate content of 3.6 to 6.4 mg/serving (equivalent to 1.2 to 2.1 mg phosphorus/serving). Exposure to phosphorus resulting from the consumption of ferrous ammonium phosphate is well below both the RDA for phosphorus and the ULs and is not expected to have an adverse effect on human health.

In the U.S., the daily intake of ammonia from food and drink is estimated to be approximately 18 mg, whereas the estimated amount of ammonia produced per day in the human intestine is approximately 4,000 mg (EHC, 1986). Typical use-levels of ferrous ammonium phosphate that provide 20% of the DV of iron will result in consumption of ammonium at levels of between 0.5 to 0.8 mg/serving which is far lower than either daily dietary intake or endogenous production in the human intestine. As a result, exposure to ammonium (or the deprotonated product,

ammonia) as a consequence of ferrous ammonium phosphate consumption is not expected to induce adverse effects on human health.

Toxicity

Toxicity to ferrous ammonium phosphate has not been specifically evaluated in experimental animals. However, the salt readily dissociates into iron(II), ammonium and phosphate; therefore, it is appropriate to assess the safety of ferrous ammonium phosphate by critically evaluating the available information on iron, ammonium, and phosphorus.

Iron

Animal Studies

The oral LD_{50} of ferrous sulfate is approximately 670, 1,720, and 1,028 mg/kg body weight, equivalent to 134, 344, and 206 mg iron/kg body weight, in mice, rats, and rabbits, respectively (Keith, 1957; Boccio *et al.*, 1998). Adverse effects following acute exposure to ferrous sulfate is consistent among laboratory animals and consists of GI disturbances such as vomiting (observed in dogs) and diarrhea and lesions of the stomach and proximal small intestine (in rodents) (Keith, 1957; Nayfield *et al.*, 1976; Bianchini *et al.*, 1988; Benoni *et al.*, 1993). Adverse effects of subchronic oral dietary administration of iron, mainly in the form of ferrous sulfate or carbonyl iron, are predominantly hepatic in nature in rodents. Such effects include increases in serum indicators of liver toxicity and increased hepatic microsomal lipid peroxidation (Wu *et al.*, 1990; Omara and Blakley, 1993; Olynyk *et al.*, 1995; Stål *et al.*, 1996). These hepatic effects were typically observed following administration of large concentrations of iron as ferrous sulphate or carbonyl iron in the diet resulting in doses of approximately 750 mg/kg body weight/day or greater. These findings were not typically accompanied by macro- or microscopic histopathological abnormalities, including necrosis or fibrosis. Chronic oral exposure to carbonyl iron as a dietary admixture for up to 1 year in mice and rats did not result in cirrhosis or the development of tumors (Iancu *et al.*, 1987; Plummer *et al.*, 1997; Pigeon *et al.*, 1999). In addition, based on the evaluation conducted by JECFA on iron (JECFA, 1983) and the study by Fairweather-Tait *et al.* (1984), iron(II) does not cause maternal toxicity and is not teratogenic in mice and rats.

From the above studies, short-term exposure to high doses of iron in the form of either ferrous sulphate or carbonyl iron produces gastrointestinal disturbances in experimental animals. Repeated exposure to high doses of iron produces hepatic effects, but generally without macro- or microscopic abnormalities or carcinogenic effects.

Human Studies

In humans, acute iron toxicity occurs mostly in children as a result of accidental poisoning from medications and/or dietary supplements intended for adults, but not from iron-fortified foods

(McGuigan, 1996). Repeated oral iron supplementation may lead to adverse GI effects at dosages above 50 mg/person/day (Hallberg *et al.*, 1966; Brock *et al.*, 1985; Laine *et al.*, 1988; Coplin *et al.*, 1991; Liguori *et al.*, 1993; Frykman *et al.*, 1994). The association of iron overload with hepatocellular carcinoma and coronary heart disease is restricted to cases of severe iron overload in individuals with genetic disorders, notably those with hereditary hemochromatosis (IOM, 2001; EVM, 2002). This sub-population and individuals receiving repeated blood transfusions and those with existing hepatic or GI disorders are at an increased risk of chronic toxicity caused by increased iron ingestion by supplementation. The amount of iron provided by iron-fortified foods is substantially less than that provided by oral iron supplements; iron at levels present in fortified foods does not pose a health risk to humans. Findings from human studies indicate that supplementation with iron is not teratogenic and does not result in adverse birth outcomes or adverse effects on growth and development, supporting its safety to the mother, developing embryo/fetus, and/or infant (Preziosi *et al.*, 1997; Cogswell *et al.*, 2003; Berseth *et al.*, 2004; Siega-Riz *et al.*, 2006; Zhou *et al.*, 2006; Abdelrazik *et al.*, 2007; Steinmacher *et al.*, 2007)

Mutagenicity Studies

Results of *in vitro* mutagenicity assays demonstrated that iron(II) has no or very low genotoxic potential at high concentrations (Casto *et al.*, 1979; Dunkel *et al.*, 1999). Therefore, iron(II) intake at the low levels provided in fortified foods will not be genotoxic.

Phosphate

Free inorganic phosphate is abundant in the diet, and it is the major form in which dietary phosphorus is absorbed (JECFA, 1982; Takeda *et al.*, 2004). Foods that contain high levels of protein generally contain high levels of phosphorus (EFSA, 2005). Data from 5 European countries indicate mean and 97.5th percentile intakes of phosphorus from foods ranging from 1,112 to 1,570 and 1,763 to 2,601 mg/person/day, respectively (EFSA, 2005). In addition to its endogenous presence in food, phosphorus in the form of phosphate salts also may be added to processed foods, soft drinks, and drugs to function as sequestrants, emulsifiers, and buffers (Merck, 2001).

Bioavailability (Absorption)

The net absorption of phosphorus from the diet is reported to range from 55 to 70% in adults and from 65 to 90% in infants and children and to be independent of dietary intakes. The phosphorus content of the diet does not affect calcium absorption. Diets containing calcium to phosphorus ratios ranging from 0.18 to 1.88 do not affect calcium absorption efficiency (Spencer *et al.*, 1965; Heaney, 2000). Inorganic phosphate plays a critical role in skeletal development, mineral metabolism, and diverse cellular functions involving intermediary metabolism and energy-transfer mechanisms (JECFA, 1982; Nordin, 1988; IOM, 1997; Takeda *et al.*, 2000, 2004; EVM, 2003). Phosphate is filtered by the glomeruli in the kidney, the majority

(80 to 90%) is reabsorbed in the proximal renal tubules, and the remainder excreted in the urine. Only in adult patients where the glomerular filtration rate has decreased below 20% of the adult value has hyperphosphatemia been reported (IOM, 1997).

Animal Studies

The reported oral LD_{50} values in rats, mice, and rabbits for various phosphate salts range from 1,300 to 4,100 mg/kg body weight, equivalent to approximate oral LD_{50} values for phosphorus ranging from 120 to 1,060 mg/kg body weight. Calcification of soft tissues, especially of the kidneys, stomach, and aorta is a common observation in sub-chronic studies conducted with rats and high dietary levels of phosphates. Specifically, increased calcification of the kidneys and nephrocalcinosis was reported to occur in rats provided phosphorus (from tetrasodium diphosphate, sodium monophosphate, sodium tripolyphosphate, or orthophosphate) at levels of approximately 419 and 669 mg/kg body weight/day or greater, respectively, in the diet for 24 weeks (Hahn *et al.*, 1956, 1958; Hahn and Seifen, 1959). In contrast, no histological alterations of the kidneys were reported to occur in male Wistar rats provided with approximately 1,300 mg phosphorus/kg body weight/day (from orthophosphate) in the diet for 21½ weeks in comparison to rats provided diets containing approximately 420 mg phosphorus/kg body weight/day (Dymsza *et al.*, 1959). Therefore, it is difficult to determine the level at which dietary phosphorus induces kidney damage in rats as kidney alterations were observed in some studies where phosphorus was provided at levels higher than in other studies in which no kidney alterations were reported. Furthermore, it is reported that isolated areas of renal calcification may occur in apparently healthy rats that are provided standard diets with no added phosphate (JECFA, 1982).

Feeding a diet containing 0.75% phosphoric acid, providing approximately 118 mg phosphorus/kg body weight/day, to rats over 3 generations did not result in any adverse effects on growth, reproduction, pathology, blood chemistry, mineral balance, nitrogen retention, or the acidic conditions of the digestive tract (Lang, 1959; Ellinger, 1972). First and second generation rats provided a diet containing 5% of a mixture of one-third Kurrol's salt (potassium phosphate) and two-thirds diphosphate (level of phosphorus unable to be determined) from weaning to the end of their life-spans were reported to display significant growth inhibition and decreased fertility (van Esch *et al.*, 1957). However, no maternal toxicity or teratogenic effects were reported to occur in albino CD-1 mice, Wistar-derived albino rats, golden hamsters, or Dutch-belted rabbits administered various phosphate salts by oral gavage in a series of teratology studies (FDRLI; 1973a,b, 1975a,b,c,d). Hodge (1960a,b) reported increased kidney weights and calcification of the kidneys in rats consuming diets containing 5% sodium tripolyphosphates or hexametaphosphates (equivalent to approximately 630 to 750 mg phosphorus/kg body weight/day), but in not in rats consuming diets containing levels of 0.05 or 0.5% of these sodium phosphates (Hodge, 1960a,b). Phosphates tested included various sodium, potassium, and calcium phosphate salts with *in utero* exposures occurring from day 6 of gestation through to day 16, 15, 10, and 18 in mice, rats, hamsters, and rabbits, respectively. Highest dose levels

tested were in the 100 to 500 mg/kg body weight/day range and did not result in maternal toxicity or teratogenicity.

Animal studies indicate that excessive dietary intake of phosphorus results in increased plasma phosphorus and decreased serum calcium levels. These imbalances may ultimately result in bone loss and calcification of soft tissues through homeostatic controls (JECFA, 1982).

Human Studies

The possibility of the above mentioned effects in animals occurring in humans was evaluated by the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) and the COT concluded that "the elevated PTH levels associated with supplemental phosphorus intakes reflect a short term adjustment to maintain plasma calcium levels and do not necessarily represent an adverse effect of phosphate on bone health" (COT, 2004). In addition, it has been reported that no clinical studies have linked high phosphorus intake, regardless of calcium intake, to lower bone mass or increased rates of bone loss (EFSA, 2005). There have been no reports of ectopic calcification resulting from high dietary intakes of phosphorus in humans with adequate renal function; however, this effect may be observed in patients with end-stage renal disease (EFSA, 2005). Hyperphosphatemia under these conditions is a secondary effect of renal disease as opposed to a direct effect of dietary intake.

JECFA evaluated phosphoric acid and phosphate salts and concluded that the Maximum Tolerable Daily Intake (MTDI) for man would be 70 mg phosphorus/kg body weight/day and applies to the sum of phosphates present naturally in food and those present as additives. In contrast, the EFSA Panel determined that the critical endpoint of phosphorus-induced nephrocalcinonsis observed in rats that was used to set the MTDI was not relevant to humans, and concluded that there was not sufficient data available to establish an upper limit for phosphorus. It was noted that mild GI effects, such as nausea, vomiting, and diarrhea, have been observed with supplemental intakes of 750 mg phosphorus per day and greater; however, it was determined that normal healthy individuals can tolerate phosphorus intakes of at least 3,000 mg per day without adverse systemic effects. The EVM also concluded that insufficient data exists to establish a safe upper level for inorganic phosphates; however, taking into consideration the osmotic diarrhea and mild GI effects observed in some individuals with supplemental intakes of 750 mg phosphorus/day and the vulnerability of persons with hypovitaminosis D to hyperparathyroidism, a safety factor of 3 was applied to the no-observed-adverse-effect level (NOAEL). With regards to phosphorus, the EVM concluded that "for guidance purposes only, a supplemental intake of 250 mg/day would be expected to not produce adverse effects, including mild gastrointestinal upset. This is equivalent to 4.2 mg/kg body weight in a 60 kg adult. Assuming a maximum intake of 2,100 mg/day from food and water, an estimated total intake of 2,400 mg/day (equivalent to 40 mg/kg body weight/day in a 60 kg adult) would not be expected to result in any adverse effects". Furthermore, UL values

established by the IOM range from 3,000 to 4,000 mg/person/day, depending on population group (IOM, 1997).

Mutagenicity

No mutagenic effects were reported to occur when different phosphates (monopotassium phosphate, monosodium phosphate, tetrasodium pyrophosphate, sodium hexametaphosphate, sodium acid pyrophosphate, and sodium tripolyphospate) were evaluated using a variety of assays, including *in vitro* assays using *Saccharomyces cerevisiae* strain D4 or *Salmonella typhimurium* strains TA1530, TA1535, TA1536, TA1537, TA1538, and G46 either with or without the addition of a mammalian metabolic activation preparation, a host-mediated assay in mice using *S. typhimurium* strain TA1530, a mitotic recombination frequency assay in *S. cerevisiae* strain D3, an *in vivo* cytogenetic study of rat bone marrow cells, an *in vitro* cytogenetic study of human lung cells in tissue culture, and a dominant lethal study in rats (Litton Bionetics, Inc., 1974, 1975a,b,c; Newell *et al.*, 1974; FDRLI, 1975d).

Ammonium

Ammonium salts are added to processed foods for various technological purposes. In the U.S., ammonium phosphate is GRAS as a general purpose food additive when used in accordance with cGMP (U.S. FDA, 2008). Ammonium phosphate is used as a buffering agent, a dough conditioner, a leavening agent, and in yeast (EHC, 1986). Unprocessed foods also may contain ammonium salts; however, intake from this source is reported to be negligible in comparison to the amount of ammonia produced in the intestine by the deamination of the amino groups of food proteins (EHC, 1986).

Following absorption from the gastrointestinal tract, ammonium is processed by the liver and excreted as urea (EHC, 1986; Health Canada, 1987). As a result, very little ammonium reaches the systemic circulation. It is reported that the consumption of ammonium ions poses little risk to both animals and humans as these species have fast and efficient enzyme systems to process ammonium, thus maintaining low tissue levels (Health Canada, 1987). Furthermore, toxicity will result only in cases where excessive amounts of ammonium have been consumed, thus overwhelming the conjugation and excretion systems, or if these mechanisms are defective (EHC, 1986).

Safety of Ferrous Ammonium Phosphate

In summary, the safety assessment of the intended use of ferrous ammonium phosphate is based on the following:

1. The bioavailability of iron from foods fortified with ferrous ammonium phosphate is comparable to foods incorporating ferrous sulfate which is approved for use in fortified foods in the U.S.

2. Ferrous ammonium phosphate dissociates to iron, ammonium, and phosphate ions in the stomach. Therefore, it was appropriate to assess the toxicity of ferrous ammonium phosphate based on the considerable published database available for iron, phosphorus, and ammonium.

3. Exposure estimates from representative examples of products fortified with ferrous ammonium phosphate in accordance with the FDA's Nutritional Quality Guidelines and for Foods show that the level of iron consumed is well below the respective Upper Limits established by the Institute of Medicine for different population groups.

4. Food fortification with ferrous ammonium phosphate will result in negligible intakes of ammonium and phosphate when compared to levels currently consumed in the diet or produced naturally in the body.

5. Ferrous ammonium phosphate was well tolerated (no adverse effects reported) by human subjects following consumption of two servings within one day of a milk product fortified with ferrous ammonium phosphate at a level of 2.5 mg of iron per serving.

CONCLUSION

We, the Expert Panel, have independently and collectively, critically evaluated the data and information summarized above and conclude that the intended uses in traditional foods of ferrous ammonium phosphate, produced consistent with cGMP and meeting appropriate food-grade specifications presented in the supporting dossier, are safe and suitable.

We further conclude that the intended uses in traditional foods of ferrous ammonium phosphate, produced consistent with cGMP and meeting appropriate food-grade specifications presented in the supporting dossier, are Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

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Appendix B

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APPENDIX B

SUMMARY OF ANALYTICAL DATA AND OTHER COMPOSITIONAL INFORMATION

APPENDIX B SUMMARY OF ANALYTICAL DATA AND OTHER COMPOSITIONAL INFORMATION

Composition of Ferrous Ammonium Phosphate

Three manufactured batches were analyzed to show that the manufacturing process produces a product consistent with the specification parameters on the physical appearance, identification, and purity of the final ingredient. For all 3 batches, the relative percentages (w/w/w) of iron, ammonia, and phosphate are consistent with a 1:1:1 inorganic salt associated with a small amount of water ($FeNH_4PO_4.xH_2O$ where x = 1 or less typically). Furthermore, the analytical data confirm the total amount of iron(III) in the final product to be no more than 7%. The analytical data for these 3 batches are summarized below in Table B-1.

Table B-1 Analytical Data Relating to the Composition of Three Commercial Batches of Ferrous Ammonium Phosphate

Parameter	Assay Results (% by weight)			Specification
Batch Number	**140503**	**140505**	**140506**	
Date of Manufacture	**10/12/2007**	**11/12/2007**	**11/12/2007**	
Iron(II) Test	Positive	Positive	Positive	Positive
Ammonium (NH_4^+) Test	Positive	Positive	Positive	Positive
Phosphate (PO_4^{3-}) Test	Positive	Positive	Positive	Positive
Iron(II) Assay	28.3	25.5	24.5	22-30%
Iron(III) Assay	4.0	5 5	6 5	Max. 7%
Total Iron [sum Fe(II) and Fe(III)]	32.3	31.1	31.0	Min 28%
Ammonia (NH_3) Assay	7.0	6 9	6.6	5-9%
Phosphate (PO_4^{3-}) Assay	52.7	53 6	53 6	49-55%
Water (H_2O)	0 9%	1 5%	0.7%	Max. 3 0%

Batch Analysis of Ferrous Ammonium Phosphate

Impurities in the final product will typically be derived from either the starting materials or as a consequence of the manufacturing process. The quality of the raw materials is controlled by well defined specifications and internal inspection prior to use. The impurity profiles for three commercial batches of ferrous ammonium phosphate are presented in Table B-2 and confirm the quality and consistency of the material produced by the manufacturing process. The analytical data show compliance to the product specifications for chemical and microbiological parameters and demonstrate the absence of chemical and microbiological contamination.

Table B-2 Analytical Data Relating to the Impurities in Three Commercial Batches of Ferrous Ammonium Phosphate

Parameter	Assay Results			Specification
Batch Number	**140503**	**140505**	**140506**	
Date of Manufacture	**10/12/2007**	**11/12/2007**	**11/12/2007**	
Sulfate (SO_4^{2-})	<0.2%	<0.2%	0.2%	Max. 0.2%
Aluminum (Al)	<10 mg/kg	<10 mg/kg	<10 mg/kg	Max 30 mg/kg
Arsenic (As)	<3 mg/kg	<3 mg/kg	<3 mg/kg	Max 3 mg/kg
Lead (Pb)	<1 mg/kg	<1 mg/kg	<1 mg/kg	Max. 10 mg/kg
Cadmium (Cd)	<1 mg/kg	<1 mg/kg	<1 mg/kg	Max. 3 mg/kg
Chromium (Cr)	<10 mg/kg	<10 mg/kg	<10 mg/kg	-
Copper (Cu)	<1 mg/kg	<1 mg/kg	<1 mg/kg	-
Manganese (Mn)	30 mg/kg	82 mg/kg	120 mg/kg	-
Nickel (Ni)	<10 mg/kg	<10 mg/kg	<10 mg/kg	Max. 50 mg/kg
Selenium (Se)	<1 mg/kg	<1 mg/kg	<1 mg/kg	-
Mercury (Hg)	<1 mg/kg	<1 mg/kg	<1 mg/kg	-
Zinc (Zn)	12 mg/kg	17 mg/kg	22 mg/kg	-
Fluoride (F^-)	<10 mg/kg	<10 mg/kg	<10 mg/kg	Max. 50 mg/kg
Iodine (I)	<10 mg/kg	<10 mg/kg	<10 mg/kg	-
Total aerobic count	<1000 0 K/g	<1000.0 K/g	<1000 0 K/g	-
Aerobic mesophilic count	<10 cfu/g	<10 cfu/g	<10 cfu/g	Max. 500 cfu/g
Aerobic mesophilic spores	150 cfu/g	<10 cfu/g	<10 cfu/g	-
Aerobic thermophilic spores	<10 cfu/g	<10 cfu/g	<10 cfu/g	-
Anaerobic mesophilic spores	<10 cfu/g	<10 cfu/g	<10 cfu/g	-
Anaerobic thermophilic spores	<10 cfu/g	<10 cfu/g	<10 cfu/g	-
Enterobacteriaceae	Absent	Absent	Absent	-
Enterobacter sakazakii	Absent	Absent	Absent	-
Salmonella	Absent	Absent	Absent	-

SUBMISSION END

000081

2-19-09 Correspondence

AM

West-Barnette, Shayla

From: Nelson,Mark,GLENDALE,Regulatory Affairs/Label/Nutrition [Mark.Nelson@us.nestle.com]
Sent: Thursday, February 19, 2009 8:22 PM
To: West-Barnette, Shayla
Subject: RE: Update About Tomorrow's 2pm ET Meeting (GRN 271)

Shayla -

We had a brief but productive call with Dr. DiNovi and his colleagues last Friday. We addressed all of the questions, however we are still waiting for the formal release of the results of the bioavailability study by my Nestle colleagues in Switzerland.

Unfortunately the people who are authorized to do this are traveling. I hope to have an answer next week when I will send all of the requested information.

I have a process question. Is an email with attachments acceptable or do you need hard copies as with the original submission?

Thank you.

Mark

Mark F Nelson, PhD
Director Regulatory Affairs US and Canada
Nestlé USA, Inc
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mark.nelson@us.nestle.com

From: West-Barnette, Shayla [mailto:Shayla.WestBarnette@fda.hhs.gov]
Sent: Thursday, February 12, 2009 8:12 AM
To: Nelson,Mark,GLENDALE,Regulatory Affairs/Label/Nutrition
Subject: Update About Tomorrow's 2pm ET Meeting (GRN 271)

Mr. Nelson,

I will be out of the office tomorrow due to unforeseen circumstances. In my absence, Dr. Michael DiNovi will speak with you and the representatives from Cantox during tomorrow's telephone conference about GRN 271. The meeting time and call-in instructions remain the same. If you have any difficulties calling in, Dr. DiNovi can be reached at (301) 436-1320. My apologies for the last-minute change. I can be reached by phone or email today if you have any questions. Thanks,

Shayla

AM

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SCIENTIFIC & REGULATORY AFFAIRS

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APR 10 2009
BY: (b)(6)

April 8, 2009

Shayla West-Barnette, Ph.D.
Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

RE: GRAS Notice No. GRN 00271

Dear Dr. West-Barnette:

I am writing to provide responses to the comments from the Office of Food Additive Safety review team included in your note of February 5, 2009.

Toxicology Issues

Comment 1. Page 9: Why the average all-user body weight is 65 kg, while that of the heavy consumer is 48 kg? Based on iron intake, the all-user body weight is 51 kg, and that of the heavy consumer is 49 kg?

Response: Estimates for the daily intake of ferrous ammonium phosphate represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES 2003-2004 data; these average amounts comprised the distribution from which mean and percentile intake estimates were produced. Mean and percentile estimates were generated incorporating survey weights in order to provide representative intakes for the entire U.S. population. Thus, intakes on a per kg body weight basis are calculated for each individual based on their own measured body weight. From the distribution of individual intakes, the mean and 90th percentile are obtained, as opposed to assuming a standard 60 kg body weight and calculating the per kg body weight intakes. In addition, the number of respondents that reported body weight data were fewer than the total number of respondents, thus the distribution of intakes on a per kg body weight basis is based on a smaller sample size than the intakes estimated on a mg per day basis. Furthermore, the value that lies at the 90th percentile in the mg per day estimates is most likely not obtained from the same individual as the value that lies at the 90th percentile in the mg per kilogram body weight day estimates.

Comment 2. Page 26: The results of bioavailability study in human volunteers (Nestle Research Center, 2007b) are requested according to the section "Availability of Information" on page 2.

Response: Two documents are attached. The first is RDLS-RD080105, Fe Absorption from Full Cream Milk Powder fortified with Ferrous Ammonium Phosphate (FAP) in Healthy Young Woman, August 21, 2008. The second is TRIAL N° 05.09.NRC,.Evaluation of Iron Absorption from Ferric Pyrophosphate and Ferric Ammonium Phosphate from an Instant Milk Drink, March 6, 2007.

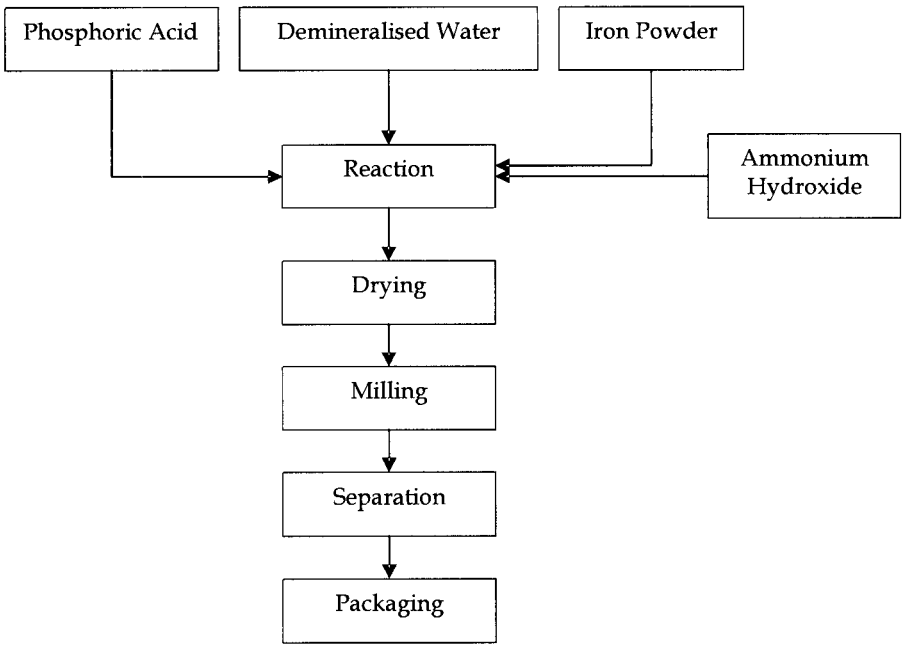
Comment 3. Appendix A, page 1, lanes 9 and 10: probably "ferrous" instead of "ferric".

Response: Appendix A, page1, lanes 9 and 10 are to read "ferrous" and not "ferric". This was a typographical error.

Chemistry Issues

Comment 1. Regarding the manufacturing of ferrous ammonium phosphate, on page 3 of the notice it is stated that excess starting materials or insoluble salts (e.g. un-reacted iron powder and ammonia) are removed during the purification and spray drying processes. The schematic overview in Figure 2 includes a drying step, but no purification step that could account for the removal of insoluble materials is indicated. Please explain in more detail when and how insoluble salts are removed during the manufacturing.

Response: Following spray drying, the material is passed through a sieve and a magnetic separation unit. This stage of the manufacturing process allows for the removal of any non-ferrous salt and ensures that the product meets the well-defined ranges for iron (II), phosphate and ammonia consistent with a 1:1:1 inorganic salt and minimal impurities. For completeness, the flow chart has now been revised to include this purification stage of the process (below).



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Comment 2. The notice gives the molecular formula of ferrous ammonium phosphate as FeNH4PO4.xH2O, where x is stated to be "1 or less typically". For one molecule of water, the expected ionic percentages would be Fe, 30%; NH4, 9.6%; PO4, 51% and the remaining 9.6% being water. We note that in Appendix B, in 3 batches of ferrous ammonium phosphate, the percentage of water is only about 1%, the percentage of Fe is close to the expected value of 30%, while the percentages of NH4 and PO4 are in slight disagreement with the expected values. Please provide us with a more accurate estimate of the amount of water of crystallization in ferrous ammonium phosphate that might account for the measured percentages of the ionic components.

Response: The specification states that the water content of ferrous ammonium phosphate will not exceed 3% where batch data has demonstrated that typically levels are measured to be in the region of 1%. A more accurate description of the water of crystallization that that provided in the notification (x <1) is therefore, x is typically around 0.1 and always less than 0.3. With this very low water content, ferrous ammonium phosphate can almost be considered anhydrous.

Comment 3. We have noticed that when back-calculations are performed using the given per person and per kilogram body weight intake estimates, unrealistic body weights are obtained. For example, to obtain the given 0.6 mg/kg/day 90th percentile estimate, the given 29 mg/person/day estimate would have to be divided by a body weight of 48 kg, while to obtain the given 0.074 mg/kg/day 90th percentile estimate of iron intake, the given 3.8 mg/person/day estimate would have to be divided by a body weight of 51 kg. Normally, adult body weights are assumed to be 60 or 70 kg. Please explain the inconsistencies and the unusually low body weights resulting from your intake estimates.

Response: The Response to Comment 1 under "Toxicological Issues" applies here as well.

Please let me know if you or the members of the review team need anything further.

Sincerely,

(b)(6)

Mark F. Nelson, Ph.D.
Director, Scientific & Regulatory Affairs

Nestlé Research Center
Lausanne

R&D REPORT

RDLS-RD080105

Fe Absorption from Full Cream Milk Powder fortified with Ferrous Ammonium Phosphate (FAP) in Healthy Young Woman

By **Peter Kastenmayer (NRC, Lausanne)**
M. Vigo, D. Grathwohl, A. Besson (NRC, Lausanne)

Project Number: RDLS-600032

Keywords: Fe, fortification, absorption, stable isotopes, ferrous ammonium phosphate, ferrous pyrophosphate, full cream milk

Date of Issue: August 21, 2008 Secretary Ref: bba

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P. Zahner, Vevey, NN/R&D
B.K. Low, Vevey, B-C&B
O. Chmiel, PTC Orbe/Beverages

SUMMARY

BUSINESS AND TECHNICAL IMPACT:

A variety of Nestlé products are fortified with iron, in order to help prevent iron deficiency. Fe compounds used for fortification have to be carefully selected considering bioavailability and organoleptic characteristics. Most frequently used compounds in Nestlé products include ferrous sulfate, ferrous fumarate and ferric pyrophosphate. In products, which are, sensitive to taste, colour changes and lipid oxidation such as milk powders, chocolate drinks and infant cereals, ferric pyrophosphate is used because of its low reactivity. Ferric pyrophosphate, however, is known to have a very low bioavailability.

Recently, a new inorganic iron compound, ferrous ammonium phosphate (FAP) was developed by PTC New Milford and patented for food fortification. FAP has excellent organoleptic properties and showed promising results in in-vitro bioavailability assays. To confirm this in-vivo we have measured Fe absorption in young women in a double blind, two-periods, cross-over trial from full cream milk powder (FCM) fortified with ferrous sulphate, ferrous ammonium phosphate (FAP) or ferric pyrophosphate. Fractional Fe absorption (geometric mean) was 10.4 %, 7.4 % and 3.3 % respectively. Fe from FAP was highly significantly better absorbed (2.3 x) than Fe from ferric pyrophosphate ($p<0.0001$). Compared to ferric pyrophosphate, FAP has double the advantage of being proprietary to Nestlé and having a much better bioavailability at approximately comparable fortification cost.

OBJECTIVE:

Objective of the Project:
Establish knowledge on Fe bioavailability from FAP

Objective of the Work:

The primary objective of the clinical trial is to compare fractional iron absorption from FAP against ferrous sulfate and ferric pyrophosphate as established iron fortificants.

METHODOLOGY AND TRIALS:

The clinical trial was done in collaboration with the group of Prof. Richard Hurrell at ETH Zurich. Fe absorption from FAP, ferrous sulfate and ferric pyrophosphate was determined in 38 healthy young women using the erythrocyte incorporation dual stable isotope technique (^{57}Fe, ^{58}Fe) and a randomized, double blind, two period cross-over trials in 2 parallel groups: AB or BA and AC or CA.

FAP and ferric pyrophosphate intrinsically labelled with ^{57}Fe and ferrous sulfate intrinsically labeled with ^{58}Fe were produced by Dr. P. Lohmann GmbH (Emmerthal, Germany) to be comparable to the commercially available compounds. Fe fortification compounds were given to the subjects in NIDO Full Cream Milk without added Fe produced by PTC Konolfingen. Isotopically labelled compounds were added to reconstituted milk at a level of 2.5 mg Fe/250 ml (10 mg/L). Absorption was calculated based on the enrichment of Fe tracers incorporated in erythrocytes 14 days after test meal administration. Enrichments were determined by negative thermal ionization mass spectrometry (NTIMS)

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RESULTS AND CONCLUSIONS:

For the first time data in humans have been established for fractional Fe absorption of FAP in comparison with ferrous sulphate and ferric pyrophosphate. When added, NIDO Full Cream Milk at a level of 2.5 mg Fe/250 ml (10 mg/L) geometric mean, Fe absorption from Fe sulphate, FAP and ferric pyrophosphate was 10.4 %, 7.4 % and 3.3 %, respectively. Fe from FAP was highly significantly better absorbed from milk (2.3 x) than Fe from ferric pyrophosphate ($p<0.0001$). Fe absorption of FAP was significantly lower than Fe absorption from ferrous sulphate, which was used as water-soluble reference compound ($p=0.0002$). Absorption ratios of FAP/ferrous sulphate and ferric pyrophosphate/$FeSO_4$ as a measure of relative bioavailability were 0.71 and 0.32 respectively.

We have demonstrated superior bioavailability of FAP compared to ferric pyrophosphate in this clinical trial. Replacement of ferric pyrophosphate by FAP in Fe sensitive products were soluble Fe compounds such as ferrous sulfate cannot be used would allow improving significantly the benefit of Fe fortification for the consumer without compromising product quality or significantly increasing cost. This could be protected, as FAP is proprietary to Nestlé.

ACTIONS:

Support process for obtaining regulatory approval in collaboration with CT-R and Cantox Health Sciences. Establish scientific dossier on FAP in collaboration with SNS.

SIGNATURE/S OF ALL AUTHORS:

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DETAILED REPORT

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1 Introduction

Iron deficiency is a major nutritional problem in the world today. It affects hundreds of millions of people and is especially prevalent in infants, young children and women of childbearing age. In children, it can retard psychomotoric development and mental performance. In pregnant women, it can result in premature delivery as well as fetal and maternal mortality. In addition, iron deficiency can reduce work capacity, impair immune response and perhaps lead to higher incidence of infection (Hurrell & Cook, 1990).

Food fortification is generally regarded as the best long-term approach to reducing the incidence of Fe deficiency. Fe compounds used for fortification have to be carefully selected considering bioavailability and organoleptic characteristics. In general, for a given food product, the iron compound chosen is that which has the highest bioavailability, but without provoking unacceptable organoleptic alteration during production, storage or after reconstitution.

Iron fortification of a number of products is problematic (milk powders, chocolate drinks and infant cereals) due to accelerated fat oxidation or color changes. Therefore, often water-insoluble Fe compounds, such as ferric pyrophosphate, elemental iron or Ferric orthophosphate are used, but has been shown that Fe absorption from these compounds is low (Davidsson et al., 2000).

Recently, a new inorganic iron compound, ferrous ammonium phosphate (FAP) was developed by PTC New Milford and patented for food fortification. It is prepared by the interaction of ferrous sulphate, phosphoric acid and ammonium hydroxide and contains about 30 % Fe. The compound is almost insoluble in water, does not cause taste or color problems in a variety of Fe sensitive products as shown by stability trials done at PTC Konolfingen (RTD Milo, Nesquik, FCM), PTC Orbe (Nesquik powder), PTC Singen (Maggi cube) and PTC New Milford (infant formula).

In-vitro tests carried out at PTC New Milford and NRC assessing the solubility of the FAP showed that Fe was released in dilute HCl under physiological conditions. For Fe compounds solubility is usually a good predictor for in-vivo bioavailability but his needed confirmation through a clinical trial in humans. Therefore Fe absorption from full cream milk powder (FCM) fortified from FAP was determined in healthy young women.

The primary objective of the clinical trial was to compare fractional iron absorption from FAP to Fe absorption from ferrous sulphate and ferric pyrophosphate as established iron fortificants. Ferrous sulphate was chosen as freely water-soluble reference compound because it has a proven high bioavailability. Ferric pyrophosphate (Fepyr) was selected as Fe compound currently used in Fe sensitive products, which could be replaced by FAP. The study was done in collaboration with Prof. Richard Hurrell at the Laboratory of Human Nutrition, ETH Zurich.

2 Methodology and Trials

2.1 SUBJECTS

The study was performed on 38 healthy women aged 18 to 30 years recruited from the student and staff population at ETH Zurich and the University Zurich by advertisement and by word of mouth. The following inclusion criteria were used:

- maximum body weight approx. 60 kg (to ensure sufficient enrichment of Fe tracer in blood at low absorption)
- apparently healthy
- no intake of mineral/vitamin supplements before and during the study
- willing to comply with the study procedure
- no metabolic or gastrointestinal disorders
- having received oral and written information about the aims and procedures of the study
- having provided oral and written informed consent

Exclusion criteria for recruitment were:

- Pregnancy or lactation
- Regular intake of medication (except oral contraceptives)
- Blood donation or significant blood loss (accident, surgery) over the past 6 months
- Currently participating in another clinical trial or having participated in another clinical trial during the last 3 months prior to the beginning of this study
- Former participation in a study involving administration of iron stable isotope labels
- Patient who cannot be expected to comply with treatment

Iron status and infection were used to describe the study population but were not considered as an inclusion/exclusion criteria. A possible effect of iron status, infection or other possible factors influencing iron absorption would cancel out as each subject was used as his/her own control.

2.2 ETHICS

Written consent was obtained from each volunteer after detailed explanation of the study goals and methods. The protocol was approved by the ethics committee of "Gesundheits Direktion Kanton Zürich", Switzerland June 27, 2006. Coding of all samples and information (blood and anthropometrical data) ensured the anonymity of the subjects.

2.3 EXPERIMENTAL DESIGN

The study was set up as two randomized, double blind, two period crossover trials in parallel:
AB or BA and AC or CA.

Subjects were assigned to one of 2 groups and within each study to one of the 2 sequences:

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Table 1: Treatment allocation.

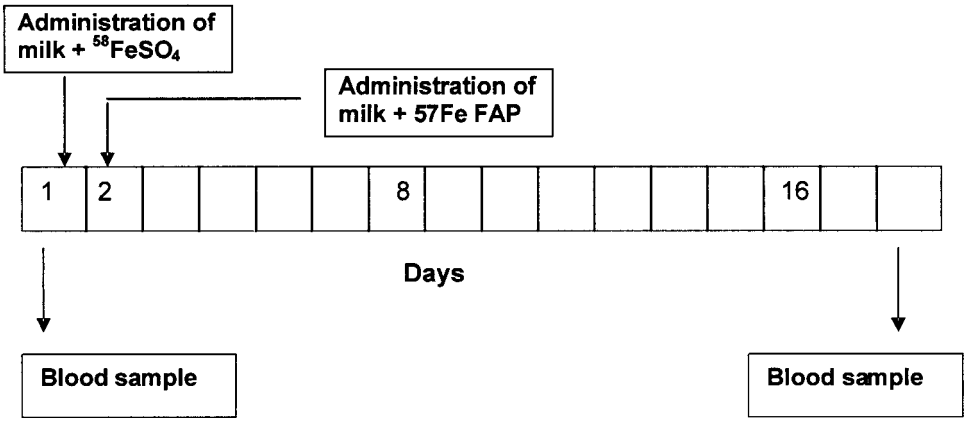
	Sequence 1	Sequence 2
Group 1:	$FeSO_4$ – FAP	FAP – $FeSO_4$
Group 2:	$FeSO_4$ – Fepyr	Fepyr – $FeSO_4$

Iron absorption was determined in fasting subjects by a double stable isotope technique assessing erythrocyte incorporation of isotopic labels (^{57}Fe, ^{58}Fe) 14 days after test meal intake (Kastenmayer et al., 1994; Walczyk, 1996). FAP and ferric pyrophosphate (^{57}Fe) and ferrous sulphate (^{58}Fe) were intrinsically labelled with stable isotopes with the support of Dr. Paul Lohmann GmbH, Emmerthal, Germany. Fractional Fe absorption was calculated based on the shift of the iron isotope ratios in the blood samples and the amount of iron circulating in the body 14 days after test meal administrations assuming 80% incorporation of the absorbed Fe into red blood cells.

The detailed schema for the two test periods is given in Figure 1.

Fig 1: Design of Fe absorption study

Group 1 (20 Subjects):



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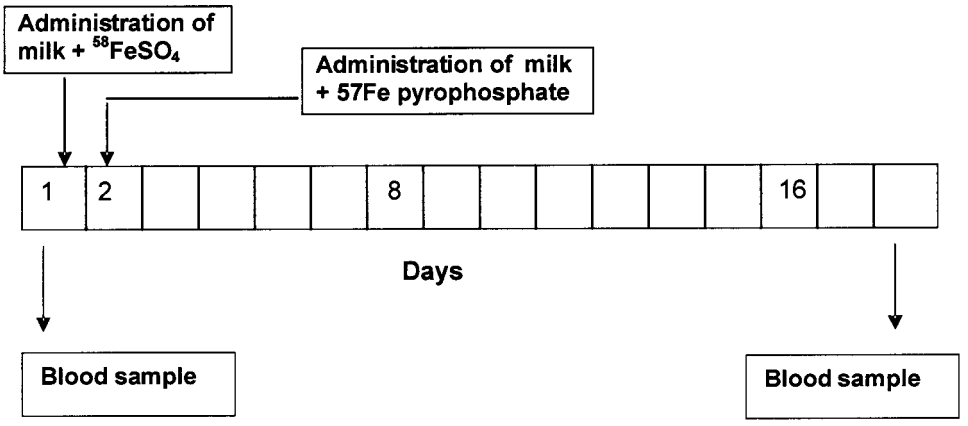
Group 2 (20 Subjects):



2.4 FRACTIONAL FE ABSORPTION

2.4.1 Preparation of stable isotope labelled Fe compound

Fe sulphate labelled with ^{58}Fe

^{58}Fe labelled Fe sulphate was supplied by ETH Zurich. It had been previously prepared by Dr. P. Lohmann GmbH at lab scale according to the procedure used for the commercially available compounds. High purity Fe metal enriched in ^{58}Fe (92.99 %) was dissolved in 16 % H_2SO_4 under Argon. Iron sulfate solution was evaporated to dryness at 80 C° under vacuum. The Fe sulfate was then dried in a vacuum oven at 120 C°. The production batch number was Lot No.: 129258. The powder was analyzed at NRC for microbiological safety. Heavy metal impurities were controlled by inductively coupled plasma mass spectrometry (ICP-MS model ELAN 6000, Perkin Elmer; Rotkreuz, Switzerland). 58-Fe concentration determined by ICP-AES (Model Varian Vista MPX, Varian AG, Zug; Switzerland after dissolution in 1 M HNO3 was 0.3106±0.0036 g/g Fe sulphate. ^{58}Fe isotopic enrichment determined by ICP-MS was 92.9897 ± 0.0098 % (n=3). Isotopic composition (atom %) was: ^{54}Fe: 0.02015 %, ^{56}Fe: 0.3654 %, 57Fe 6.625 %, ^{58}Fe: 92.9897 %.

Ferrous ammonium phosphate (FAP) labelled with ^{57}Fe

^{57}Fe labelled FAP was prepared by Dr. P. Lohmann GmbH at lab scale according to the procedure used for the commercially available compounds. High purity Fe metal powder enriched in ^{57}Fe (95.1 %) obtained from AMT (Israel, Lot No. 11-1-05) was suspended in water and reacted with phosphoric acid to Fe(II)-mono-hydrogen-phosphate. Ferrous ammonium phosphate (FAP) was obtained by adding ammonium hydroxide to the solution and dried in a vacuum oven. Amount produced was: 3.06 g. The production batch number was Lot No.: 132012

The powder was analyzed at NRC for microbiological safety. Heavy metal impurities were controlled by ICP-MS. 57-Fe concentration determined by ICP-AES after dissolution in 1 M HNO3 was 0.2887±0.0066 g/g Fe sulphate. ^{57}Fe isotopic enrichment determined by ICP-MS was 94.495 ± 0.0023 % (n=3). Isotopic composition (atom %) was: ^{54}Fe: 0.0667 %, ^{56}Fe: 3.279 %, 57Fe: 94.495 %, ^{58}Fe 2.159 %.

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Ferric pyrophosphate labeled with 57Fe

^{57}Fe labelled ferric pyrophosphate was prepared by Dr. P. Lohmann GmbH at lab scale according to the procedure used for the commercially available compounds. High purity Fe metal powder enriched in ^{57}Fe (95.1 %) obtained from AMT (Israel, Lot No. 11-1-05) was suspended in water and reacted with sulphuric acid to Fe(II) sulfate. Fe(II) sulfate was oxidized to $Fe(III)_2(SO_4)_3$ using H_2O_2.

$Fe(III)_2(SO_4)_3$ was precipitated using di-sodium-di-hydrogen-pyrophosphate after pH adjustment with Na_2CO_3. The precipitate was then washed to be free from sulfate and dried under vacuum.
The production batch number was Lot No.: 132013. Amount produced was: 3.87 g
The powder was analyzed at NRC for microbiological safety. Heavy metal impurities were controlled by ICP-MS. 57-Fe concentration determined by ICP-AES after dissolution in 1 M HNO3 was 0.24286±0.00055 g/g Fe sulphate. ^{57}Fe isotopic enrichment determined by ICP-MS was 94.573 ± 0.0012 % (n=3). Isotopic composition (atom %) was: ^{54}Fe: 0.06233 %, ^{56}Fe 3.208 %, 57Fe 94.573 %, ^{58}Fe 2.1568 %.

2.4.2 Solubility test of stable isotope labeled compounds

Solubility of ^{57}Fe labeled FAP and ferric pyrophosphate was measured in vitro at two different pH (1 and 1.7) simulating the conditions of the human stomach. For comparison unlabeled micronised FAP (Lohman batch: 130090) and Fe sulphate (Merck, No.: 103965)

The first test at pH=1.7 was based on the method proposed by Forbes et al., 1989. Aliquots of Fe compounds calculated to contain 20 mg Fe were weighed into a 500-mL conical flask and 250 mL 0.02 mol HCl/L (pH 1.7), preheated to 37°C, was added. The flasks were placed in a shaking water bath at 37°C and gently shaken at a rate of 1 Hz for 180 min. Two mL aliquots were taken at regular time intervals and centrifuged for 2 min at 1000 g. An aliquot of 0.375 ml of supernatant was transferred to a 15-mL tube and diluted to volume with 1 M HNO_3 after addition of Sc and Cs as internal standards. Fe content was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Results are given in Figure 2.

The second, more rapid was based on a recently established in vitro dissolution method proposed by Lynch et al., 2007. For this method the authors have tested dissolution of different Fe powders at two pH levels (pH=1 and pH=1.7) and different time points (15 to 150 min) and found that the best prediction of RBV for the rat AOC method was found after a dissolution time of 30 min at a pH=1. Aliquots of Fe compounds calculated to contain 20 mg Fe were weighed into 500-mL conical flask and 250 mL 0.1 mol HCl/L (pH 1), preheated to 37°C, was added. The flasks were placed in a shaking water bath at 37°C and gently shaken at a rate of 1 Hz. Two 2 mL aliquots were taken after 30 min and analyzed as described above. Results are shown in Figure 3.

At pH=1.7 solubility of ^{57}Fe labeled ferric pyrophosphate, 57Fe FAP, unlabeled micronised FAP and Fe sulphate after 180 min was 11.6%, 33.8 %, 57.8 % and 99.2 %, respectively. The ^{57}Fe labeled FAP had a lower solubility than the unlabeled, commercial micronised FAP, which most likely can be explained by the production scale. Due to the cost of the isotope enriched Fe the ^{57}Fe labeled compound is produced on the lab scale at the g level whereas the unlabeled FAP is produced at the ton level. For the ^{57}Fe enriched ferric pyrophosphate the solubility of 11.1 % is very similar to the solubility of the unlabeled, commercial ferric pyrophosphate, which is approx. 8%.

Figure 2: Solubility of labelled Fe compounds at pH=1.7

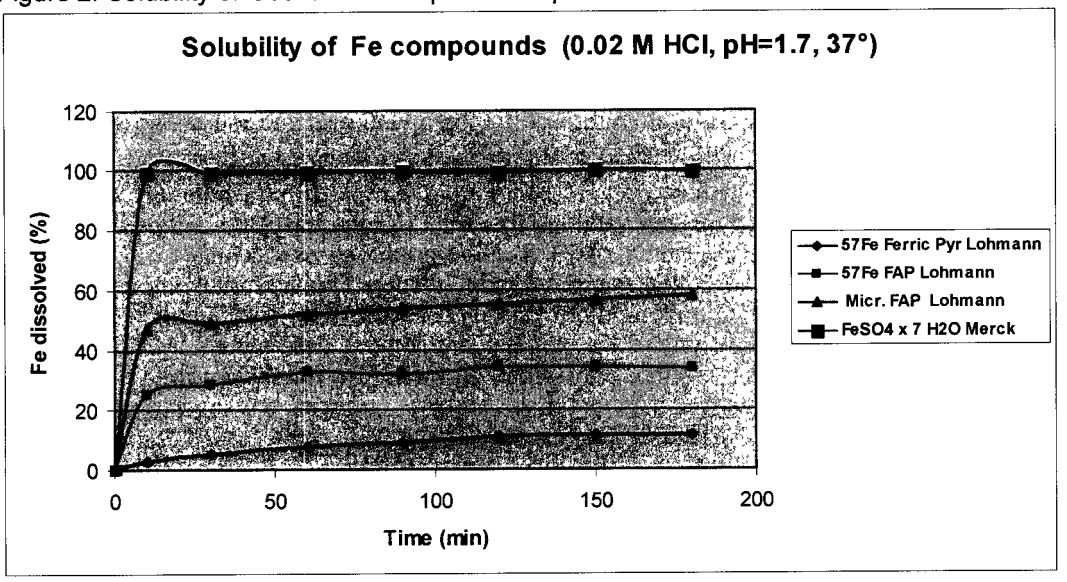
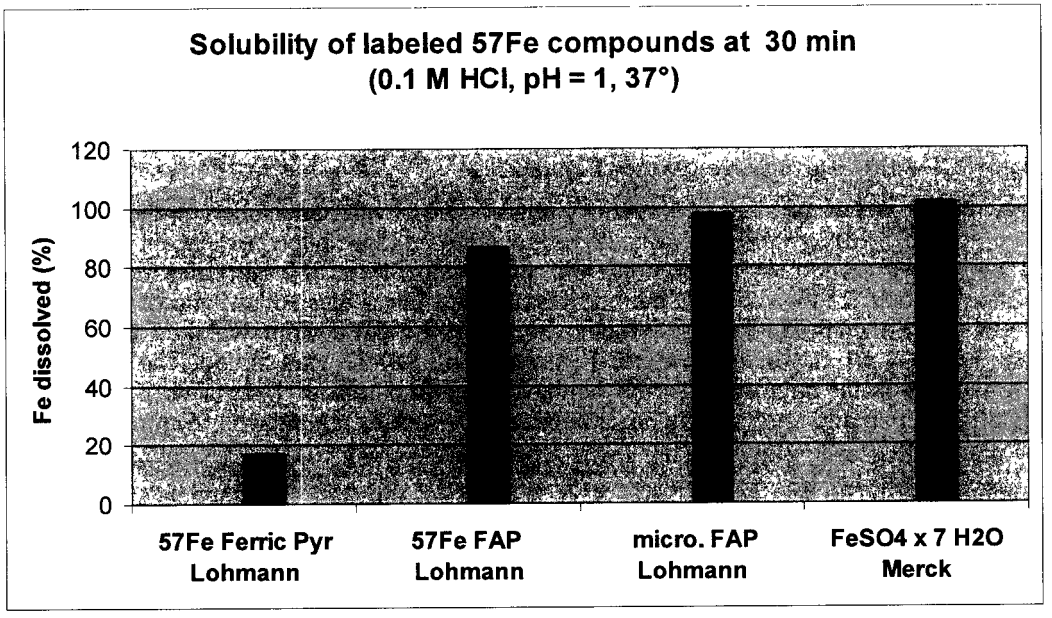


Figure 3: Solubility of labelled Fe compounds at pH=1



The difference in solubility for ^{57}Fe labeled FAP and the unlabeled, commercial micronised FAP is pH dependent and almost disappears at higher pH as I shown by the solubility trial at pH=1 (see Figure 3). Under this conditions after 30 min 86.5 % of Fe is released from ^{57}Fe FAP compared to 98% for the commercial compound.

2.4.3 Stable isotope administration

On day 1, a venous blood sample (7 ml) was drawn after an overnight fast for determination of iron status parameters (hemoglobin, serum ferritin and C-reactive protein as an inflammation marker). Body weight and height was measured and the first labeled test meal (^{57}FAP or ^{57}Fepyr or $^{58}FeSO_4$) was served twice with feedings separated by a 2 hour interval. NIDO Full Cream Milk powder without added Fe produced by PTC Konolfingen served as test meal. Composition of milk was: Fat: 36.7 g/L; Protein: 33.4 g/L; Carbohydrate: 48.6 g/L; Vitamin C: 120.9 mg/L. Isotopically labelled compounds were added to reconstituted milk at a level of 2.5 mg Fe/250 ml (10 mg/L). Vitamin C: Fe molar ratio was 3.8:1.

On day 2 the test meal administration was repeated with the second Fe compound to be tested (^{57}FAP or ^{57}Fepyr or $^{58}FeSO_4$)

Test meals were fed under strictly standardized conditions under close supervision of the investigators. Complete intake of isotopically labeled fortificants was assured by washing the glass after consumption of the milk drink two times with 20 ml of deionized water and letting the subjects consume the washings. No intake of food and fluids was allowed between the two servings and for 3 hours after the second serving of the day. Apart from this, diet was unrestricted during the study. Subjects were asked to complete a short food frequency questionnaire to assess dietary habits regarding iron nutrition either on the first or second feeding day. The following day, the second test meal was administered in fasted condition.

A second venous blood sample (7 ml) was drawn 14 days after intake of the second test meal (day 16) for iron isotopic analysis. Test meal feedings were conducted at the human study facilities of the Human Nutrition Laboratory ETHZ. Venous blood samples were taken by an experienced nurse in EDTA-treated tubes.

2.4.4 Analysis of Fe status parameter

Determination of hemoglobin concentration in whole blood

Hemoglobin concentrations in whole blood were determined using the cyanmethemoglobin method(1). Twenty µl of fresh whole blood were diluted in 5 ml of Drabkin's reagent (D5941, Sigma-Aldrich, Saint-Louis, USA) containing 0.015% Brij 35 solution (B4184, Sigma-Aldrich, Buchs, Switzerland). The absorbance of the formed cyanmethemoglobin was read at 540 nm in a double beam UV/VIS spectrophotometer (Uvikon 940, Kontron, Schlieren, Switzerland). An external calibration curve was established, using pure human hemoglobin (H7379, Sigma-Aldrich, Buchs, Switzerland) as a standard. All determinations were performed in duplicates
A three levels blood control (Eightcheck-3WP, Sysmex, Kobe, Japan) was analyzed with each set of measurements.

Determination of serum ferritin concentrations

Blood samples were stored on ice for 3h and serum was separated after centrifugation for 15 min
Ferritin concentration was determined in plasma samples using an automated solid-phase, two-site chemiluminescent immunometric assay (Immulite One, Diagnostic Products Corporation (DPC), Los Angeles CA, USA). A two levels serum control (EURO/DPC, Llamberis, UK) was analyzed with each set of measurements.

Determination of serum C-reactive protein (CRP) concentration

CRP concentration was determined in plasma samples using an automated solid-phase, two-site chemiluminescent immunometric assay (Immulite One, Diagnostic Products Corporation (DPC), Los Angeles CA, USA). A three levels serum control (DPC, Los Angeles CA, USA) was analyzed with each set of measurements.

2.4.5 Mineral analysis of full cream milk powder used as test meal

Fe content of food samples was determined by ICP-AES (Model Varian Vista MPX, Varian AG, Zug; Switzerland after mineralization in microwave digestion system (Model Mars 5, CEM, USA) Approx. 400 mg sample were weighed in duplicate into Xpress PFA bombs and mineralized using 5 ml HNO_3 65%/ 2 ml H_2O_2. Mineralized sample were diluted to 15 ml with ultra-pure water.
For Ca, Fe and Zn analysis by inductively coupled atomic emission spectrometry ICP-AES (Model Varian Vista-MPX (Varian AG, Mulgrave, Australia) samples aliquots were diluted sample to 15 ml after addition of internal standards Sc and Cs. Ca, Fe and Zn were determined at 422.673 nm, 238.204 nm and 213.857 nm using external calibration.

Accuracy of the method was checked by analyzing the NIST standard reference materials Typical Diet (SRM 11548a) and Bovine Liver (SRM 1577b).

2.4.6 Fe stable isotope analysis

Iron separation

Whole blood samples were mineralized followed by separation of the sample iron from matrix elements by anion-exchange chromatography (Walczyk et al., 1997). An aliquot of 0.5 ml of whole blood was mineralized by microwave digestion (MLS ETHOS, MLS GmbH, Leutkirch, Germany) with 7 ml concentrated HNO_3 and 3 ml H_2O_2 25 %. After evaporation to dryness, the residue was dissolved in 5 ml HCl 6M, and evaporated to dryness once again. The residue was dissolved in 1 ml HCl 6 M, and loaded onto a disposable plastic column (7.5 ml Spectrum PP column, Rockbourne & Co, Stockbridge, UK) filled with 1 ml of AG1-X8 anion exchange resin (100-200 mesh, Bio-Rad Laboratories, Hercules CA, USA). The resin was preconditioned to its chloride form with 10 ml HCl 6 M. After loading of the sample, the column was rinsed with 6 ml HCl 6 M, and iron was finally eluted with 8 ml HNO_3 1M. The eluate was evaporated to dryness and the residue dissolved with 2 ml HCl 6M. The iron-chloride complex was extracted with 3x 0.5 ml diethyl ether. The combined organic phases were evaporated to dryness at 65°C overnight. Each blood sample was prepared in duplicate. The complete separation process was performed under a laminar flow box.

Chemical blank was monitored through the complete process by isotopic dilution, using a known amount of ^{57}Fe solution as indicator.

All acids used in the process were of sub-boiled quality. Only ultra pure water (18 MOhm) was used. Other reagents were of suprapure quality. Only acid-washed material was used through the procedure.

NTIMS measurement

All measurements were performed by negative thermal ionization mass spectrometry (NTIMS) using a magnetic sector field mass spectrometer (MAT 262, Thermo-Finigan, Bremen, Germany) equipped with a multi-collector system for simultaneous ion beam detection. (Walczyk et al., 1997). The samples were measured using a double filament technique. Iron isolated from the blood matrix (30 µg Fe) was loaded onto a BaF_2 coated rhenium filament, together with AgF to promote the formation of negatively charged FeF_4^- ions (evaporation filament). The ionization filament was coated with BaF_2. Measured isotopic ratios were normalized for the natural $^{56}Fe/^{54}Fe$ ratio to correct for mass dependant fractionation effects in the source (Taylor et al., 1992). .

Baseline iron isotope ratios in blood were not determined as natural variations in iron isotope composition caused by mass-dependent biological fractionation effects are corrected for by normalization of measured data for the natural $^{56}Fe/^{54}Fe$ isotope ratio. Normalization will correct for the occurrence of mass-dependent fractionation effects in nature and during analysis

Each isotopically enriched blood sample was analyzed in duplicate for the iron isotopic composition under chemical blank monitoring.

2.4.7 Calculation of fractional Fe absorption

Based on the shift of the iron isotope ratios in the blood samples and the amount of iron circulating in the body, the amounts of ^{57}Fe and ^{58}Fe isotopic label present in the blood 14 days after test meal administrations were calculated based on the principles of isotope dilution and considering that the iron isotopic labels are not monoisotopic (Walczyk et al. 1997). Circulating Fe was calculated based on blood volume and hemoglobin concentration (Kastenmayer et al. 1994). Blood volume calculations were based on height and weight according to Brown et al. (1962). For calculations of fractional absorption, 80% incorporation of the absorbed Fe into red blood cells was assumed.

2.5 Statistical Analysis

Data entry was done at NRC into the Clintrial database with a double entry (Clintrial™ 4.3 from Phase Forward, Waltham, USA).

For sample size calculation relative differences in iron absorption greater than 30% between compounds were considered nutritionally relevant (a fractional iron absorption of 2.1 % from one test meal has to be differentiated from 2.8 % for the alternate test meal in the same subject). Based on literature and in vitro solubility data, the Fe absorption values expected were assumed to be in the range of 10% for $FeSO_4$, 7% for FAP and 3% for Fepyr. Thus the relevant differences expected were approximately $\Delta(FeSO_4\text{-}FAP)=3\%$ and $\Delta(FAP\text{-}Fepyr)=4\%$. From data made available by the ETHZ, the within subject standard deviation after $\log_{10}$ transformation could be estimated with 0.142 and the between subject standard deviation with 0.328. A two-sided superiority test will need a sample size of n = 19 in order to show $\Delta(FeSO_4\text{-}FAP)$ as significant and n=18 in order to show $\Delta(FAP\text{-}Fepyr)$ as significant on an α-level of 5% with a power of 90% (PASS 2000). In order to full fill both hypotheses n = 19 subjects had to be enrolled in study 1 and n = 18 in study 2. To ensure against possible dropouts, 20 subjects were enrolled per study

Primary outcome, fractional iron absorption was $\log_{10}$ transformed to achieve approximately normality. $\log_{10}$ Iron absorption was analyzed by a mixed model. The treatment difference $FeSO_4$ vs. FAP was estimated within subjects, the treatment difference FAP vs. Fepyr was estimated between subjects. The two treatment differences were estimated from the mixed model by appropriate contrasts. Additionally the p-values for the two treatment differences FeSO4 vs. FAP and FAP vs. Fepyr were adjusted for multiple tests. Thus experiment wise false positive rate was controlled on a 5% level. Analysis was performed with R 2.2.1 with library "nlme" and "multcomp".

3 Results

3.1 SUBJECT DESCRIPTION

In this trial, 40 healthy women were enrolled and randomized. Since randomization was before informed consent, subject N° 31 was randomized but has not signed the informed consent, consequently no CRF exists nor were measurements performed. Subject N° 34 did not match the inclusion criteria, 18-30 years, however all measurements were performed. This was done because at this time of the trial, the investigator feared not to reach the required sample size. All remaining subjects were seen as compliant according to the protocol. The PP data set contains n = 38 subjects. According protocol, only a PP analysis is performed (no ITT analysis).
The demographic and anthropometrical characteristics of the subjects are given in Table 2.

Table 2: Summary of and anthropometrical subject characteristics

		n	mean	sd	min	max
Study group 1	**age (years)**	19	22.42	2 06	19.00	26.00
	weight (kg)	19	55.69	4.80	47.10	65.90
	length (cm)	19	166 2	6.37	154.0	174 0
	BMI (kg/m^2)	19	20.16	1.39	17.37	22.35
Study group 2	**age (years)**	19	22.63	3.04	19.00	29.00
	weight (kg)	19	57.73	4.85	47.30	66.40
	length (cm)	19	167.7	4.97	156.0	175.0
	BMI (kg/m^2)	19	20.52	1.36	18.00	22.79
All	**age (years)**	38	22 53	2.57	19.00	29 00
	weight (kg)	38	56 71	4.87	47.10	66 40
	length (cm)	38	166.9	5.69	154.0	175.0
	BMI (kg/m^2)	38	20.34	1 37	17.37	22.79

Iron status of subjects is described in Table 3.

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Table 3: Summary statistics on iron status.

		n	mean	sd	min	max
Study group 1	**CRP (mg/dl)**	19	0.14	0.15	0.01	0.54
	Ferritin (ng/ml)	19	23.75	18.23	5.80	67.20
	Hb (mg/dl)	19	13.07	1.14	10.60	15.39
Study group 2	**CRP (mg/dl)**	19	0.24	0.38	0.01	1.57
	Ferritin (ng/ml)	19	27.20	25.90	1.50	83.70
	Hb (mg/dl)	19	12.95	0 71	11.74	14.32
All	**CRP (mg/dl)**	38	0.19	0 29	0.01	1.57
	Ferritin (ng/ml)	38	25.47	22.16	1.50	83.70
	Hb (mg/dl)	38	13 01	0 94	10.60	15.39

3.2 PRIMARY OUTCOME

Primary study outcome was Fe absorption from FAP, Fepyr and $FeSO_4$. Summary statistics on iron absorption of the compounds are presented in Table 4. Individual values can be found in Annex 1.

Table 4: Summary statistics on iron absorption.

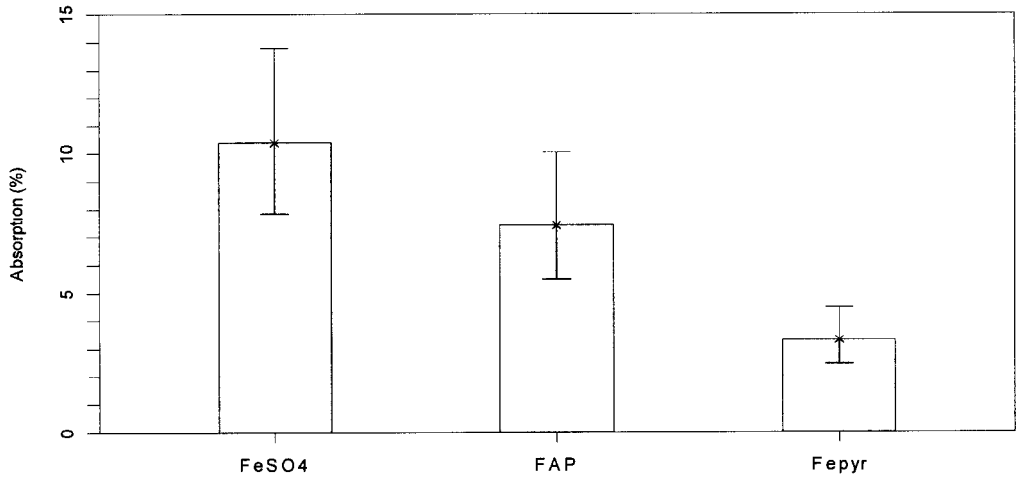
Iron absorption (%)	Period 1			Period 2			All		
	n	mean	sd	n	mean	sd	n	mean	sd
FeSO4	18	13.8	10.1	20	15.1	9.4	38	14.5	9.6
FAP	10	11.1	5.8	9	10 1	9.8	19	10.6	7.7
Fepyr	10	3.9	2.5	9	4.3	2.6	19	4.1	2.5

As it is well known Fe absorption values are not normal distributed. Therefore for statistical analysis results for iron absorption were $\log_{10}$ transformed in order to achieve approximately normal distributed residuals. Geometric means and 95% confidence intervals estimated by the mixed model are presented in Table 5 and Figure 4. Fractional Fe absorption (geometric mean) for $FeSO_4$, FAP and Fepyr was 10.4 %, 7.4 % and 3.3 %, respectively.

Table 5: Geometric means and 95% confidence intervals estimated by the mixed model.

(%)	geo.mean	lower	upper
FeSO4	10.4	7.8	13.8
FAP	7.4	5.5	10.0
Fepyr	3.3	2.4	4.5

Figure 4: Geometric means and 95% confidence intervals estimated by the mixed model



Treatment differences were assessed by a linear mixed effect model correcting for period and are given in Table 6.

Table 6: Treatment differences, 95% confidence intervals and p-values (adjusted for multiple tests) on log 10 transformed iron absorption measurements.

log10(%)	estimate	lower	upper	Adj. p-value
FAP-Fepyr	0.35	0.25	0.46	<0.0001
$FeSO_4$-FAP	0.15	0.07	0.22	0 0002

Fe from FAP was highly significantly better absorbed (2.3 x) than Fe from ferric pyrophosphate ($p<0.0001$) when consumed with the full cream milk powder. Fe absorption of FAP was significantly lower than Fe absorption from ferrous sulphate ($p=0.0002$), which was used as water-soluble reference compound. Absorption ratios of FAP/ferrous sulphate and ferric pyrophosphate/$FeSO_4$ as a measure of relative bioavailability (RBV) were 0.71 and 0.32 respectively.

As can be expected from data published in the literature serum ferritin had an effect on Fe absorption as can be seen for Figure 5. An increase of ferritin by 20 ng/ml will lead to decrease of ≈ 5.3 % iron absorption in geometric means

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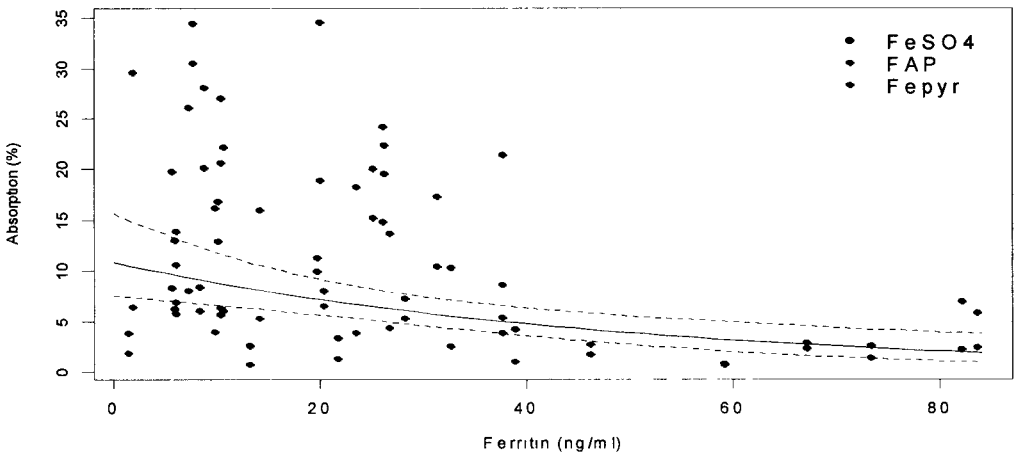
Figure 5: Iron absorption over serum ferritin and geometric mean development and 95% confident bands estimated by a mixed model.



Results for treatment differences remain unchanged when serum ferritin at baseline was included in the statistical analysis as can be seen from Table 7.

Table 7: Treatment differences, 95% confidence intervals and p-values (adjusted for multiple tests) on log 10 transformed iron absorption measurements corrected for ferritin at baseline.

log10(%)	estimate	lower	upper	Adj p-value
FAP-Fepyr	0.35	0.24	0.45	<0.0001
FeSO4-FAP	0.15	0.07	0.22	0.0001

4 Discussion

Study results

For the first time data in humans have been established for fractional Fe absorption of FAP in comparison with ferrous sulphate and ferric pyrophosphate. Thirty-eight healthy young women participated in the study. Fe absorption was determined using ^{57}Fe and ^{58}Fe labelled Fe compounds and the erythrocyte incorporation method. When added NIDO Full Cream Milk at a level of 2.5 mg Fe/250 ml (10 mg/L) geometric mean Fe absorption from Fe sulphate, FAP and ferric pyrophosphate was 10.4 %, 7.4 % and 3.3 %, respectively. Fe from FAP was highly significantly better absorbed (2.3 x) than Fe from ferric pyrophosphate ($p<0.0001$) when consumed with the full cream milk powder. Fe absorption of FAP was significantly lower than Fe absorption from ferrous sulphate, which was used as water-soluble reference compound ($p=0.0002$). Absorption ratios of FAP/ferrous sulphate and ferric pyrophosphate/$FeSO_4$ as a measure of relative bioavailability were 0.71 and 0.32 respectively.

The study was done in healthy young women although the potential target consumer group for FAP fortified FCMP will be infants and young children. This had two main reasons: Firstly studies in infants and young children are much more time consuming and costly and recruitment of infant subjects might have been difficult for a Fe compound which had been not tested before in a human trial and for which a full safety dossier was not available at the time of the study. On the other hand young menstruating women represent a subject group ranging from moderately iron deficient to iron sufficient but with low iron stores. These subjects represent therefore an adequate model of iron absorption roughly equivalent to infants older than 6 months as shown by Hertrampf et al. (Hertrampf et al., 1998) for absorption of Fe from cow's milk fortified with ferrous sulphate. This is also supported by studies done in our laboratory in collaboration with J. Cook. (Hurrell et al., 1998) where Fe absorption from milk based infant formula was 6.5 % in adults and 6.7 % in infants. Furthermore infant and adult do react in a similar was to inhibitors and enhancers as shown by the Fe absorption from regular and dephytinsed soy formula with 2 levels of VitC. Therefore one could expect the relative difference of Fe absorption for the 3 compounds would be maintained in children.

The absorption study was done using stable isotope labelled FAP, ferrous sulphate and ferric pyrophosphate. These compounds were produced by Dr. P. Lohman GmbH on the lab scale according to the procedures used for the production of the commercial compounds. For cost reasons, it is not possible to produce stable isotope labelled compounds on industrial scale and micronise them. Fe content and solubility of the stable isotope label compounds were compared to the industrially produced compounds. Fe contents were well within the specifications of the commercial compounds. Solubility was tested at two different pH levels using two standard in-vitro dissolution methods proposed by Forbes et al., 1995 and Lynch et. al, 2007. These dissolution tests are screening methods, which can provide estimates on the relative ranking of RBV of Fe compounds but do not allow making precise predictions of in-vivo absorption values. The solubility of ^{57}Fe labelled FAP with regular particle size at lower pH range of the stomach was very close to solubility of the commercial micronised FAP (86.5 vs. 98%). At higher pH (pH=1.7) solubility of 57Fe labelled FAP was lower than for the micronised FAP (33.8 vs. 57.8). For 57Fe labelled ferric pyrophosphate solubility was not much affected by pH (11.6 vs. 17.2 %).

For our study results, this means that if in the human study digestive conditions would be close to lower pH in the worst case, FAP absorption would have been slightly underestimated compared to the commercial compound. In other words, Fe absorption from FAP from the commercial compound would be higher and even closer to the Fe absorption of ferrous sulphate. In our study the observed differences for Fe absorption from the different Fe compounds (RBV FAP and ferric pyrophosphate approx. 0.7 and 0.3) correspond well to the solubility data obtained with the commercial compounds indicating that the ^{57}Fe FAP is absorbed as well as micronised FAP.

The RBV of ferric pyrophosphate of 0.3 is in good agreement with literature data for compounds poorly soluble in dilute acid such as ferric pyrophosphate, ferric orthophosphate or elemental Fe powders which have been shown to have a relative bioavailability of <0.5 in most studies (Hurrell, 2002, Fidler et al., 2004).

Limitations for application of FAP

FAP was specially developed for fortification of products where an addition of iron causes organoleptic problems such as accelerated fat oxidation, taste or color changes (e.g. milk powders, chocolate drinks, certain culinary products and infant cereals). FAP has been shown to have an equivalent or slightly better stability in products compared to ferric pyrophosphate at approx. neutral pH. However, as for ferric pyrophosphate, in highly acidic products such as yogurts, small quantities of iron can be released which can lead to organoleptic changes.

As determined in a human trial, FAP has a significantly better bioavailability (2.3x) compared to ferric pyrophosphate but is slightly less bioavailable than ferrous sulphate (Relative bioavailability RBV=70 %). Therefore for products, which can safely be fortified with ferrous sulfate, the cheapest iron fortification compound, there would be no advantage to use FAP.

Iron fumarate, a compound also used by Nestlé, can be used for fortification of products, which are slightly iron sensitive due to its relatively low solubility. The relative bioavailability (RBV) of iron fumarate has long-time been considered to be equivalent to the bioavailability of ferrous sulfate. New studies, however, show that, in iron deficient subjects, iron absorption from iron fumarate is significantly lower than from iron sulfate. As iron fumarate was not included in the iron absorption study, there are for the moment no data available, which would allow comparing the bioavailability of the two compounds (FAP *vs* iron fumarate).

Cost and supply issues and cost

The exact given data on the cost of different Fe compounds mentioned above is beyond the scope of this report because prices do change depending on supplier, country, exact specifications and amounts acquired. To just give a rough estimate approximate cost for Fe sulphate, ferrous fumarate and ferric pyrophosphate is in the range of (expressed as CHF/kg Fe): 8.3 CHF, 40.- CHF and 45.- CHF. This compares to an approximate price for FAP of 39.- CHF. More information on cost of different Fe compounds can be found in the R&D report from PTC Konofingen (RDKO-RD60021).

The supply issue has been solved by now as a contract was established with Dr. P. Lohmann GmbH who agreed to produce FAP for Nestle.

Safety and regulatory issues

FAP is a new iron compound for food fortification therefore, regulatory approval has to be obtained before it can be used. According to a preliminary review established by Cantox Health Sciences International, a consultant specialized in regulatory issues, the available scientific information and data indicate that it should be feasible to get the approval for the use of FAP as an iron source in foods. A full dossier related to FAP overall safety has been elaborated in collaboration with CT-R Vevey and Cantox Health Sciences. This safety dossier will be shortly submitted to EFSA for approval of use in Europe. An adapted version of the dossier will be submitted for GRAS approval in the US. Finally a request for evaluation of FAP as fortification compound was made to JECFA. For actual information on the status of the different approval procedures CT-R in Vevey should be contacted.

5 Conclusions

We have demonstrated superior bioavailability of FAP compare to ferric pyrophosphate in this clinical trial. Replacement of ferric pyrophosphate by FAP in Fe sensitive products would allow improving significantly the benefit of Fe fortification for the consumer without compromising product quality or increasing cost. This could be protected, as FAP is proprietary to Nestlé.

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7 Appendices

Appendix I: Listing of iron absorption measurements.

ID	Study	TRT	PP	Visit	Iron abs. (%)	Ferritin (ng/ml)	ID	Study	TRT	PP	Visit	Iron abs. (%)	Ferritin (ng/ml)
1	1	FeSO4	1	1	5 39	37.7	21	2	Fepyr	1	1	5.27	14 2
1	1	FAP	1	2	3 83	37.7	21	2	FeSO4	1	2	15.91	14 2
2	2	Fepyr	1	1	4.34	26.8	22	1	FAP	1	1	15.18	25 2
2	2	FeSO4	1	2	13.66	26.8	22	1	FeSO4	1	2	20.08	25.2
3	2	FeSO4	1	1	22.13	10.7	23	2	Fepyr	1	1	8.01	7.3
3	2	Fepyr	1	2	6.04	10 7	23	2	FeSO4	1	2	26.09	7.3
4	2	Fepyr	1	1	6.45	2	24	1	FAP	1	1	10.42	31.4
4	2	FeSO4	1	2	29.58	2	24	1	FeSO4	1	2	17.27	31.4
5	1	FeSO4	1	1	7.22	28 3	25	2	FeSO4	1	1	13 84	6.1
5	1	FAP	1	2	5 24	28.3	25	2	FAP	1	2	5.74	6.1
6	1	FAP	1	1	6.24	6	26	1	Fepyr	1	1	5 68	10.4
6	1	FeSO4	1	2	12 97	6	26	1	FeSO4	1	2	20.66	10.4
7	2	FeSO4	1	1	18 24	23.5	27	2	FeSO4	1	1	3.4	21.8
7	2	Fepyr	1	2	3.87	23.5	27	2	Fepyr	1	2	1.3	21.8
8	1	FAP	1	1	6.07	8.5	28	1	FAP	1	1	2.67	46.2
8	1	FeSO4	1	2	8.37	8.5	28	1	FeSO4	1	2	1.78	46 2
9	1	FeSO4	1	1	22.33	26.3	29	2	Fepyr	1	1	0.64	13.3
9	1	FAP	1	2	19.53	26 3	29	2	FeSO4	1	2	2.59	13.3
10	1	FeSO4	1	1	0 81	59.2	30	1	FeSO4	1	1	34.47	7.8
10	1	FAP	1	2	0.7	59.2	30	1	FAP	1	2	30.51	7.8
11	1	FAP	1	1	18 94	20.1	32	1	FAP	1	1	12.85	10.2
11	1	FeSO4	1	2	34.58	20.1	32	1	FeSO4	1	2	16 77	10.2
12	2	FeSO4	1	1	7.99	20.4	33	2	Fepyr	1	1	1.39	73.4
12	2	Fepyr	1	2	6.46	20.4	33	2	FeSO4	1	2	2.63	73 4
13	2	Fepyr	1	1	3.93	9.9	34	1	FeSO4	0	1	2.14	40.6
13	2	FeSO4	1	2	16.11	9.9	34	1	FAP	0	2	3.78	40.6
14	1	FAP	1	1	6 93	6.1	35	2	Fepyr	1	1	2 51	32.8

14	1	FeSO4	1	2	10.61	6.1	35	2	FeSO4	1	2	10.3	32.8
15	2	Fepyr	1	1	0.97	38.9	36	1	FAP	1	1	20.11	8 9
15	2	FeSO4	1	2	4.26	38.9	36	1	FeSO4	1	2	28.01	8.9
16	2	FeSO4	1	1	7.01	82.2	37	1	FAP	1	1	11.3	19.8
16	2	Fepyr	1	2	2.22	82.2	37	1	FeSO4	1	2	9.95	19.8
17	2	FeSO4	1	1	5.79	83.7	38	2	FeSO4	1	1	27.05	10.5
17	2	Fepyr	1	2	2.36	83.7	38	2	Fepyr	1	2	6.33	10.5
18	1	FeSO4	1	1	2.9	67.2	39	1	FeSO4	1	1	19.72	5.8
18	1	FAP	1	2	2.3	67.2	39	1	FAP	1	2	8.3	5.8
19	1	FeSO4	1	1	24.15	26.1	40	2	FeSO4	1	1	21.4	37.8
19	1	FAP	1	2	14.8	26.1	40	2	Fepyr	1	2	8.57	37.8
20	2	FeSO4	1	1	3.82	1.5							
20	2	Fepyr	1	2	1.83	1.5							

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Appendix 2: Fe status measurements

Subject ID	Hb (g/l)	SF (ng/ml	CRP (mg/dl)
1	123.7	37.7	0.181
2	117.4	26.8	0.084
3	129.8	10.7	0.137
4	139.8	2.0	0.555
5	142.0	28.3	0.022
6	118.5	6.0	0.374
7	133.0	23.5	1.57
8	127.5	8.5	0.058
9	131.9	26.3	0.011
10	140.1	59.2	0.169
11	141.3	20.1	0.037
12	127.4	20.4	0.011
13	123.4	9.9	0.035
14	106.0	6.1	0.129
15	131.1	38.9	0.051
16	139.4	82.2	0.037
17	129.9	83.7	0.011
18	132.2	67.2	0.102
19	127.3	26.1	0.061
20	143.2	< 1.5	0.099
21	132.9	14.2	0.408
22	153.9	25.2	0.543
23	120.1	7.3	0.015
24	148.2	31.4	0.017
25	134.8	6.1	0.223
26	125.4	10.4	0.268
27	128.7	21.8	0.155
28	127.5	46.2	0.026
29	122.4	13.3	0.747
30	124.5	7.8	<0.01
32	120.6	10.2	0.039
33	128.0	73.4	0.023
34	139.3	40.6	0.863
35	120.3	32.8	0.212
36	140.9	8.9	0.119
37	122.9	19.8	0.059
38	125.3	10.5	0.035
39	128.8	5.8	0.386
40	134.2	37.8	0.073

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FINAL VERSION

EVALUATION OF IRON ABSORPTION FROM FERRIC PYROPHOSPHATE AND FERRIC AMMONIUM PHOSPHATE FROM AN INSTANT MILK DRINK

Iron absorption from FAP

Project leader: Dr. Peter Kastenmayer
Principal investigator: Prof. Dr. Richard Hurrell

STATISTICAL REPORT

TRIAL N° 05.09.NRC

Applied Mathematics Group: Biostatistics

Author: **Dominik Grathwohl**
Reviewer: **Dr. Marc Enslen**

March 6, 2007

SUMMARY

Evaluation of iron absorption from ferric pyrophosphate and ferric ammonium phosphate from an instant milk drink

Principal Investigator: Prof. Richard Hurrell

Study center: Laboratory of Human Nutrition, ETH Zurich.

Design: Double blind, randomized, two period, cross-over clinical trial.

Description of subjects: Healthy women 18-30 years.

Product:
- Ferric ammonium phosphate (FAP)
- Ferric pyrophosphate (Fepyr)
- Ferrous sulfate ($FeSO_4$)

Number of patients: (enrolled / PP): 40 / 38.

Primary objective:
Iron absorption of FAP, Fepyr and $FeSO_4$.

Results:

Primary outcome:
Best absorbed is $FeSO_4$ 10.4 [7.8, 13.8] % followed by FAP 7.4 [5.5, 10.0] % and finally Fepyr 3.3 [2.4, 4.5] %, presented are geometric means and 95% confidence intervals. The three absorptions are statistically significantly different.

Additionally analyses:
Impact of cold/flue on iron absorption: the three subjects suffering from cold/flue during measurement period do not show different iron absorption than the other test persons.

Impact of baseline ferritin on iron absorption: baseline ferritin has a significant impact on iron absorption. Test persons with high baseline ferritin have likely a low iron absorption, where as a low ferritin level leads to a high absorption, e.g. an increase of ferritin by 20 ng/ml will lead to decrease of ≈ 5.3 % iron absorption in geometric means.

During the study, no subject suffered from a serious adverse event.

Comment:
Striking is how good Iron absorption in humans can be predicted with solubility experiments in vitro. $FeSO_4$ vs. FAP: solubility: 0.16, this trial: 0.15, and FAP vs. Fepyr: solubility: 0.37, this trial: 0.35 on $\log_{10}(\%)$ scale.

Evaluation of iron absorption from ferric pyrophosphate and ferric ammonium phosphate from an instant milk drink

Prof . Dr. Richard Hurrell

Author: Dominik Grathwohl

March 6, 2007

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INTRODUCTION

This report presents the statistical analysis of Study N° 05.09.NRC. It refers to the protocol dated June 20, 2006. The protocol was approved by the ethics committee of "Gesundheits direktion Kanton Zürich", Switzerland on June 27, 2006. All data can be found in Clintrial database under NRC_0509_IRON.

1 Objectives of the study

The primary objective of the clinical trial is to compare fractional iron absorption from ferric ammonium phosphate against ferrous sulfate and ferric pyrophosphate as established iron fortificants.

MATERIAL AND METHODS

2 Study set-up

The study set up is two randomized, double blind, two period cross-over trials in parallel:

- AB or BA
- AC or CA

3 Study population

The study population is healthy women, 18 to 30 years old.

Subject inclusion criteria:

- 18-30 year old females.
- Maximum body weight = 60 kg.
- Apparently healthy.
- No intake of mineral/vitamin supplements before and during the study.
- Willing to comply with the study procedure.
- No metabolic or gastrointestinal disorders.
- Having received oral and written information about the aims and procedures of the study.
- Having provided oral and written informed consent.

Subject exclusion criteria:

- Pregnancy or lactation.
- Regular intake of medication (except oral contraceptives).
- Blood donation or significant blood loss (accident, surgery) over the past 6 months.
- Currently participating in another clinical trial or having participated in another clinical trial during the last 3 months prior to the beginning of this study.
- Former participation in a study involving administration of iron stable isotope labels.
- Patient who cannot be expected to comply with treatment.

4 Treatment allocation, treatment groups and timing

Subjects were assigned to one of 2 studies and within each study to one of the 2 sequences.

Table 1: Treatment allocation.

	Sequence 1	**Sequence 2**
Study 1:	$FeSO_4$ — FAP	FAP — $FeSO_4$
Study 2:	$FeSO_4$ — Fepyr	Fepyr — $FeSO_4$

The following colors were used, blue/red for study 1 and green/yellow for study 2. The code was broken on February 19, 2007, the colors were:

Table 2: Coding of the product.

	Color	Iron
Study 1	blue	$FeSO_4$
	red	FAP
Study 2	green	$FeSO_4$
	yellow	Fepyr

The timing of the trial is presented in Table 3.

Table 3. Study plan.

	Screening	V1 (baseline)	V2	V3
Timing	**before day 1**	**1 day**	**2 day**	**16 days**
Randomization	X			
Consent	X			
Inclusion/Exclusion criteria	X			
Height and weight		X		
Blood sample		X		
Food frequency questionnaire	X			
Test meal intake		X	X	
Blood sample (Iron absorption)				X

5 Data collection, management and validation

A Excel spread sheet was sent on January 31, 2007 to NRC containing all stable isotopes measurements. In this Excel spread sheet, the assignment of isotopes to the color code was done at random at ETHZ. This piece of information was not given to NRC. On February 19, 2007 the code was broken and the $FeSO_4$ absorption was different in the two studies. This led us to query ETHZ. The assignment could be recovered by using the information that $FeSO_4$ was marked with ^{58}Fe, FAP and Fepyr was marked with ^{57}Fe.

Assignment of randomization code for subject 25 and 26 was confounded, however, well documented and considered in the analysis. The balance of treatments was preserved.

The formulas used for calculating iron absorption from measured isotope ratios, natural isotopic composition, Hb, body weight and height can be found in the publication of Walczyk et al. [1].

6 Statistical methods

6.1 Determination of sample size

Relative differences in iron absorption greater than 30% between compounds are considered nutritionally relevant (a fractional iron absorption of 2.1 % from one test meal has to be differentiated from 2.8 % for the alternate test meal in the same subject). Based on literature and in vitro solubility data, the expected Fe absorption values could be in the range of 10% for $FeSO_4$, 7% for FAP and 3% for Fepyr. Thus the relevant differences would be approximately $\Delta(FeSO_4\text{-}FAP)=3\%$ and $\Delta(FAP\text{-}Fepyr)=4\%$. From data made available by the ETHZ, the within subject standard deviation after $\log_{10}$ transformation could be estimated

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with 0.142, and the between subject standard deviation with 0.328. A two-sided superiority test will need a sample size of n=19 in order to show $\Delta(FeSO_4\text{-}FAP)$ as significant and n=18 in order to show Δ(FAP-Fepyr) as significant on an α-level of 5% with a power of 90% (PASS 2000). In order to fullfill both hypotheses 19 subjects have to be enrolled in study 1 and 18 in study 2. To ensure against possible dropouts, 20 subjects will be enrolled per study.

6.2 Definitions of sub-groups to be analyzed

Intention to treat analysis

Since the objective of this trial is not acceptance of test meal administration or tracer intake, no intention to treat analysis will be performed. We are only interested in the physiological effects as measured by stable isotope techniques.

Per protocol analysis

Per protocol evaluation will exclude data from subjects in the following conditions:

- Incomplete intake of test meal/tracers.
- Non-compliance with fasting after test meal intake.
- Significant blood loss over the study period due to an accident/surgery.

6.3 Statistical analyses planned in the protocol

Outcome, fractional iron absorption will be $\log_{10}$ transformed to achieve approximately normality.

Hypothesis 1: The difference in absorption between FAP and $FeSO_4$ will be assessed by a mixed model.

Hypothesis 2: The difference in absorption between FAP and Fepyr will be assessed by a mixed model corrected by $FeSO_4$ absorption, which can be considered as a baseline.

6.4 Changes in study plan and planned analyses

The two mixed models were fitted jointly together and the treatment differences were derived by appropriate contrasts. Additionally the p-values for the two treatment differences $FeSO_4$ vs. FAP and FAP vs. Fepyr were adjusted for multiple tests.

Analysis was performed with R 2.2.1 with library "nlme" and "multcomp".

RESULTS

7 Description of the treatment groups

7.1 Disposition of subjects

In this trial, 40 healthy women were enrolled and randomized. Since randomization was before informed consent, subject N° 31 was randomized but has not signed the informed consent, consequently no CRF exists nor were measurements performed. Subject N° 34 did not match the inclusion criteria, 18-30 years, however, all measurements were performed. This was done because at this time of the trial, the investigator feared not to reach the required sample size. All remaining subjects were seen as compliant according to the protocol. The PP data set contains n = 38 subjects. According protocol, only a PP analysis is performed (no ITT analysis).

7.2 Demographic and Baseline data

All subjects were women; anthropometric characteristics are presented in Table 4.

Table 4: Summary statistics on anthropometry.

		n	mean	sd	min	max
Study 1	**age (years)**	19	22.42	2.06	19.00	26.00
	weight (kg)	19	55.69	4.80	47.10	65.90
	length (cm)	19	166.2	6 37	154 0	174.0
	BMI (kg/m^2)	19	20.16	1.39	17.37	22.35
Study 2	**age (years)**	19	22.63	3.04	19 00	29.00
	weight (kg)	19	57.73	4.85	47.30	66.40
	length (cm)	19	167.7	4.97	156.0	175.0
	BMI (kg/m^2)	19	20.52	1 36	18.00	22.79
All	**age (years)**	38	22.53	2.57	19.00	29.00
	weight (kg)	38	56 71	4.87	47 10	66.40
	length (cm)	38	166.9	5.69	154.0	175 0
	BMI (kg/m^2)	38	20.34	1.37	17.37	22.79

Iron status is described in Table 5.

Table 5: Summary statistics on iron status.

		n	mean	sd	min	max
Study 1	**CRP (mg/dl)**	19	0.14	0.15	0 01	0.54
	Ferritin (ng/ml)	19	23.75	18.23	5.80	67 20
	Hb (mg/dl)	19	13.07	1.14	10 60	15.39
Study 2	**CRP (mg/dl)**	19	0.24	0.38	0.01	1.57
	Ferritin (ng/ml)	19	27.20	25 90	1.50	83.70
	Hb (mg/dl)	19	12.95	0 71	11.74	14.32
All	**CRP (mg/dl)**	38	0.19	0.29	0.01	1.57
	Ferritin (ng/ml)	38	25.47	22.16	1 50	83.70
	Hb (mg/dl)	38	13.01	0.94	10.60	15.39

7.3 Compliance

Compliance was defined as:

- Complete intake of test meal/tracers.
- Fasting after test meal intake.
- No significant blood loss over the study period due to an accident/surgery.

All subjects of the PP data set were fulfilling compliance criteria.

8 Outcomes

8.1 Primary outcome

Primary outcomes are Iron absorption of FAP, Fepyr and $FeSO_4$. Iron absorption was measured by double stable isotopes technique. Summary statistics on iron absorption is presented in Table 6.

000116

Table 6: Summary statistics on iron absorption.

Iron absorption (%)	Period 1			Period 2			All		
	n	mean	sd	n	mean	sd	n	mean	sd
$FeSO_4$	18	13.8	10.1	20	15 1	9.4	38	14.5	9 6
FAP	10	11.1	5.8	9	10.1	9.8	19	10.6	7 7
Fepyr	10	3.9	2.5	9	4.3	2.6	19	4.1	2 5

Iron absorption was $\log_{10}$ transformed in order to achieve approximately normal distributed residuals. Repeated measurement analysis was done by a mixed model correcting for period. Treatment differences are presented in Table 7. The three forms of iron could be statistical significantly distinguished.

Table 7: Treatment differences, 95% confidence intervals and p-values (adjusted for multiple tests) on $\log_{10}$ transformed iron absorption measurements.

$\log_{10}$(%)	estimate	lower	upper	adj p-value
FAP-Fepyr	0.35	0.25	0.46	<0 0001
$FeSO_4$-FAP	0.15	0.07	0.22	0 0002

Geometric means and 95% confidence intervals estimated by the mixed model are presented in Table 8 and Figure 1.

Table 8: Geometric means and 95% confidence intervals estimated by the mixed model.

(%)	geo.mean	lower	upper
$FeSO_4$	10.4	7.8	13.8
FAP	7.4	5.5	10.0
Fepyr	3.3	2.4	4.5

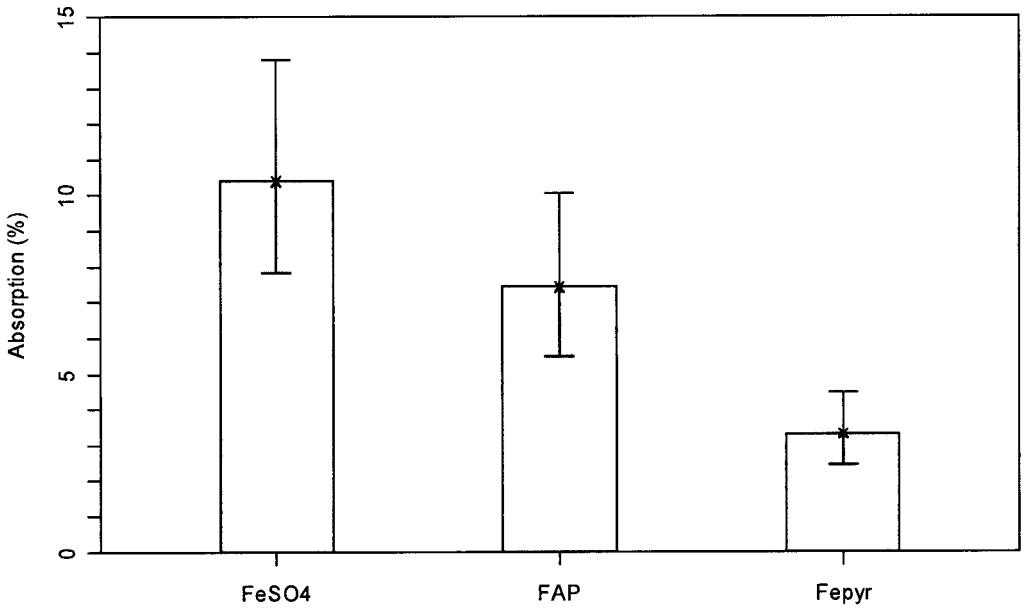

Figure 1: Geometric means and 95% confidence intervals estimated by the mixed model.

A comparison with the assumptions made in the protocol is presented in Table 9. The measured treatment effects and the standard deviations are in good agreement. The treatment differences for the planning were estimated from solubility experiments: $\log_{10}(10) - \log_{10}(7)=0.16$ and $\log_{10}(7) - \log_{10}(3)=0.37$. The standard deviations were estimated from some data provided by ETH Zürich.

Table 9: Treatment differences and between/within subject differences planned according to protocol and estimated from the data.

Treatment difference ($\log_{10}(\%)$)	Planned	Measured
$FeSO_4$-FAP ($\Delta \pm sd_{within}$)	0.16 ± 0.14	0.15 ± 0.10
FAP-Fepyr ($\Delta \pm sd_{between}$)	0.37 ± 0.33	0.35 ± 0.37

8.2 Secondary outcomes

No secondary outcomes were defined.

8.3 Additional Analyses

8.3.1 Impact of cold/flue on iron absorption

Subjects having had a cold during measurement period are identified from Table 12. Individual measurements of iron absorption by forms of iron are presented in Figure 2. Subjects having had a cold are presented by red dots. It seems not that subjects having had a cold have lower iron absorption then other subjects.

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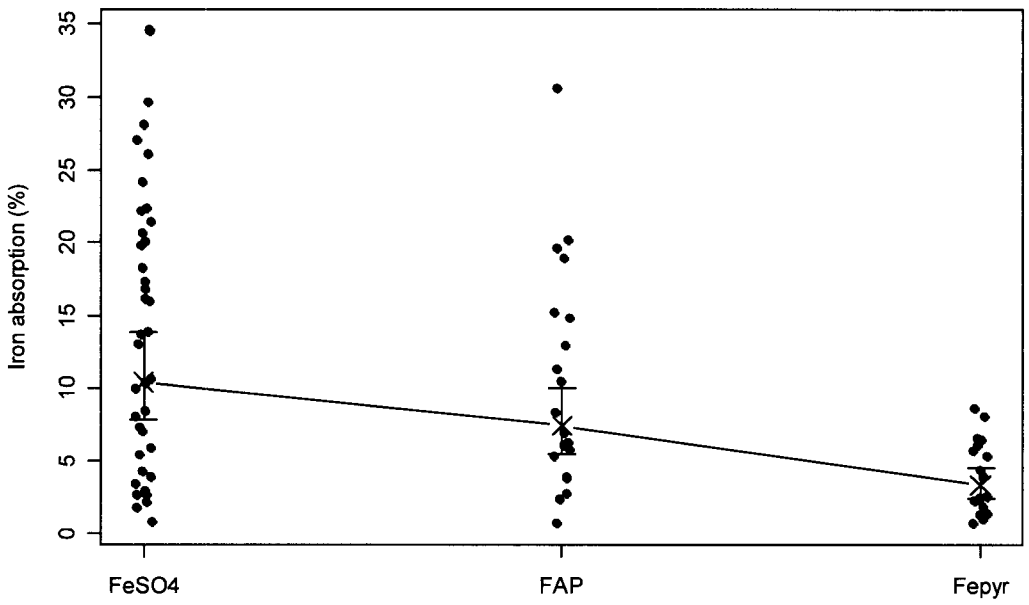

Figure 2: Raw measurements and geometric means and 95% confidence intervals estimated by the mixed model. Subjects having had cold are red dots. There is an artificial noise added to measurements (horizontally) that they are better visual distinguishable.

8.3.2 Impact of serum ferritin on iron absorption

Repeated measurement analyses on Iron absorption (log_{10} transformed) was done by a mixed model correcting for period and ferritin at baseline. Treatment differences are presented in Table 10. Treatment differences are not changed compared to statistical analysis (Table 7). Ferritin has a statistically significant impact on log_{10} iron absorption ($\Delta \pm se = -0.0089 \pm 0.0024$, $p = 0.0007$).

Table 10: Treatment differences, 95% confidence intervals and p-values (adjusted for multiple tests) on log_{10} transformed iron absorption measurements corrected for ferritin at baseline.

log_{10}(%)	estimate	lower	upper	adj p-value
FAP-Fepyr	0.35	0 24	0.45	<0.0001
$FeSO_4$-FAP	0.15	0.07	0.22	0.0001

Iron absorption over serum ferritin with geometric mean development and 95% confident bands estimated by a mixed model are presented in Figure 3, e.g. an increase of ferritin by 20 ng/ml will lead to decrease of ≈ 5.3 % iron absorption in geometric means.

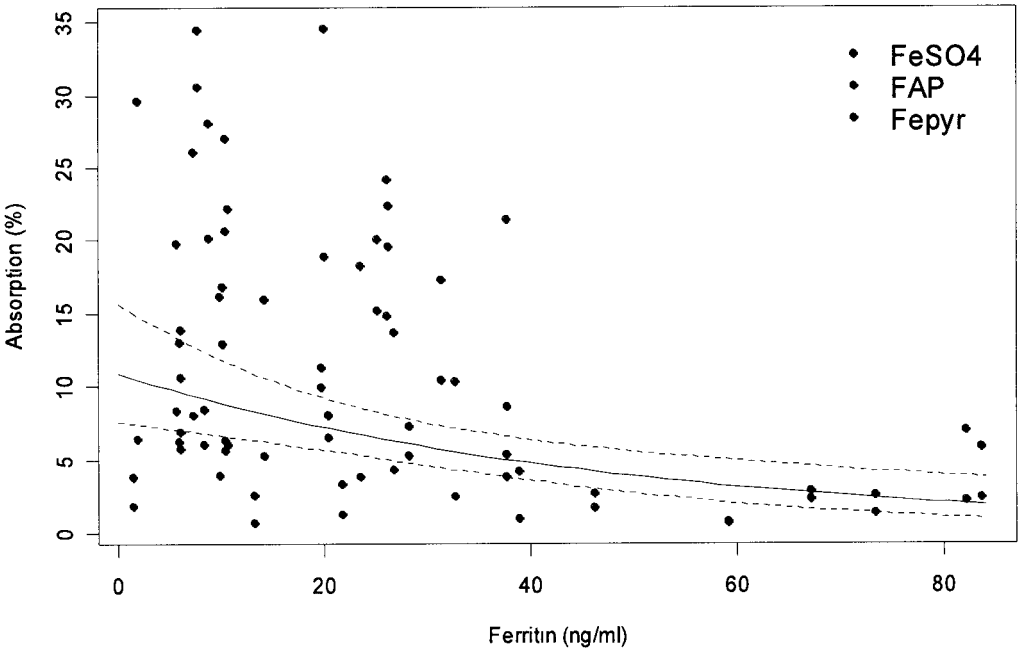

Figure 3: Iron absorption over serum ferritin with geometric mean development and 95% confident bands estimated by a mixed model.

9 Safety and tolerance

9.1 Extent of exposure

All subjects (n=39) have intaken 1 l of test drink (2 servings of 250ml at 2 days). This are 10.4 ± 0.4 mg/l Fe. Isotope and complex intake by treatment group are presented in Table 11.

Table 11: Isotope and compound intake.

Form	n	Isotope	Compound
$FeSO_4$	19	5.2 ± 0.2 mg ^{58}Fe	18.01 ± 0.69 mg
FAP	10	5.2 ± 0.2 mg ^{57}Fe	16.58 ± 0.64 mg
Fepyr	9	5.2 ± 0.2 mg ^{57}Fe	21.41 ± 0.82 mg

9.2 Blood levels and Vital Signs

Measured at baseline, see section 7.2.

9.3 Stool characteristics, flatulence and behavior

Not measured in this trial.

9.4 Adverse Events (AE) and comedication

In this trial happened 9 minor adverse events and zero serious adverse events. The minor adverse events are listed in Table 12. For subject 34, this was excluded from PP analysis, no adverse event aucured. If there is a timely overlap with measurement period, some impact of cold on iron absorption is considerable.

Table 12: Listing of minor adverse events.

ID	Description	Start	End	Intake (1 day)	Blood sample	Date overlap
06	RUNNY NOSE	27/11/06		04/12/06	19/12/06	No
13	COLD	13/10/06	27/10/06	04/12/06	19/12/06	No
15	RUNNY NOSE	20/10/06	26/10/06	04/12/06	19/12/06	No
17	COMMON COLD	01/11/06	05/11/06	28/11/06	13/12/06	No
21	BLOCKED NOSE	27/11/06	01/12/06	30/11/06	15/12/06	Yes
24	COUGH SORE THROAT	07/12/06	15/12/06	28/11/06	13/12/06	Yes
24	RUNNING NOSE	16/12/06	19/12/06	23/11/06	8/12/06	No
32	SORE THROAT	07/11/06	10/11/06	30/11/06	15/12/06	No
33	SORE THROAT	30/11/06	09/12/06	28/11/06	13/12/06	Yes

10 Conclusions and Discussion

The aim of this study is to identify differences in fractional iron absorption from ferrous sulfate ($FeSO_4$), ferric pyrophosphate (Fepyr) and ferric ammonium phosphate (FAP).

The study was designed as a double blind, two-periods, cross-over trial for $FeSO_4$ vs. FAP and the same design was applied for $FeSO_4$ vs. Fepyr. Consequently the difference between FAP and Fepyr has to be contrasted against the between subject standard deviation where as the differences $FeSO_4$ vs. FAP and $FeSO_4$ vs. Fepyr against the within subject standard deviation.

For the PP analysis, 38 subjects were available. Repeated measurement analysis was performed by a linear mixed effect model correcting for period. It could be shown, that the three forms of iron lead to a statistically significant different absorptions. Best absorbed is $FeSO_4$ 10.4 [7.8, 13.8] % followed by FAP 7.4 [5.5, 10.0] % and finally Fepyr 3.3 [2.4, 4.5] %, presented are geometric means and 95% confidence intervals.

Additionally we investigated the impact of cold/flue on iron absorption. The three subjects suffering from cold/flue during measurement period do not show different iron absorption than the other test persons.

We also investigate the impact of baseline plasma ferritin level on iron absorption. Here we could show a significant impact; test persons with high baseline ferritin have likely a low iron absorption, where as a low ferritin level leads to a high absorption, e.g. an increase of ferritin by 20 ng/ml will lead to decrease of ≈ 5.3 % iron absorption in geometric means. However, an analysis of iron absorption by a mixed model correcting for period and baseline ferritin does not change treatment differences and lead approximately to the same confidence intervals.

Striking is how good Iron absorption in humans can be predicted with solubility experiments in vitro. $FeSO_4$ vs. FAP: solubility: 0.16, this trial: 0.15, and FAP vs. Fepyr: solubility: 0.37, this trial: 0.35 on $\log_{10}(\%)$ scale. We could take advantage of this good background knowledge for planning of this trial.

During the study, no subject suffered from a serious adverse event.

References to literature

[1] Walczyk T., Davidsson L., Zavaleta N. and Hurrell R. F. (1997) Stable isotope labels as a tool to determine iron absorption by Peruvian school children from a breakfast meal. Fresenius J. Anal. Chem. 359, 445-449.

LIST OF APPENDIX

Appendix I: Listing of iron absorption measurements

ID	Study	TRT	PP	Visit	Iron abs. (%)	Ferritin (ng/ml)	ID	Study	TRT	PP	Visit	Iron abs. (%)	Ferritin (ng/ml)
1	1	$FeSO_4$	1	1	5 39	37 7	21	2	Fepyr	1	1	5.27	14.2
1	1	FAP	1	2	3 83	37 7	21	2	$FeSO_4$	1	2	15.91	14.2
2	2	Fepyr	1	1	4.34	26.8	22	1	FAP	1	1	15.18	25.2
2	2	$FeSO_4$	1	2	13.66	26.8	22	1	$FeSO_4$	1	2	20.08	25.2
3	2	$FeSO_4$	1	1	22.13	10.7	23	2	Fepyr	1	1	8.01	7.3
3	2	Fepyr	1	2	6.04	10.7	23	2	$FeSO_4$	1	2	26.09	7.3
4	2	Fepyr	1	1	6.45	2	24	1	FAP	1	1	10.42	31.4
4	2	$FeSO_4$	1	2	29.58	2	24	1	$FeSO_4$	1	2	17.27	31.4
5	1	$FeSO_4$	1	1	7.22	28.3	25	2	$FeSO_4$	1	1	13.84	6.1
5	1	FAP	1	2	5.24	28.3	25	2	FAP	1	2	5.74	6.1
6	1	FAP	1	1	6.24	6	26	1	Fepyr	1	1	5.68	10.4
6	1	$FeSO_4$	1	2	12.97	6	26	1	$FeSO_4$	1	2	20 66	10.4
7	2	$FeSO_4$	1	1	18.24	23.5	27	2	$FeSO_4$	1	1	3.4	21.8
7	2	Fepyr	1	2	3.87	23.5	27	2	Fepyr	1	2	1.3	21.8
8	1	FAP	1	1	6.07	8.5	28	1	FAP	1	1	2.67	46 2
8	1	$FeSO_4$	1	2	8.37	8.5	28	1	$FeSO_4$	1	2	1.78	46.2
9	1	$FeSO_4$	1	1	22.33	26.3	29	2	Fepyr	1	1	0.64	13.3
9	1	FAP	1	2	19.53	26.3	29	2	$FeSO_4$	1	2	2.59	13.3
10	1	$FeSO_4$	1	1	0.81	59.2	30	1	$FeSO_4$	1	1	34.47	7.8
10	1	FAP	1	2	0.7	59.2	30	1	FAP	1	2	30.51	7.8
11	1	FAP	1	1	18.94	20.1	32	1	FAP	1	1	12.85	10.2
11	1	$FeSO_4$	1	2	34.58	20.1	32	1	$FeSO_4$	1	2	16.77	10 2
12	2	$FeSO_4$	1	1	7.99	20.4	33	2	Fepyr	1	1	1.39	73.4
12	2	Fepyr	1	2	6.46	20.4	33	2	$FeSO_4$	1	2	2.63	73.4
13	2	Fepyr	1	1	3.93	9.9	34	1	$FeSO_4$	0	1	2.14	40.6
13	2	$FeSO_4$	1	2	16.11	9.9	34	1	FAP	0	2	3.78	40.6
14	1	FAP	1	1	6.93	6.1	35	2	Fepyr	1	1	2.51	32.8
14	1	$FeSO_4$	1	2	10.61	6.1	35	2	$FeSO_4$	1	2	10.3	32 8
15	2	Fepyr	1	1	0.97	38.9	36	1	FAP	1	1	20 11	8.9
15	2	$FeSO_4$	1	2	4.26	38.9	36	1	$FeSO_4$	1	2	28.01	8.9
16	2	$FeSO_4$	1	1	7.01	82.2	37	1	FAP	1	1	11.3	19.8
16	2	Fepyr	1	2	2.22	82.2	37	1	$FeSO_4$	1	2	9.95	19.8
17	2	$FeSO_4$	1	1	5.79	83.7	38	2	$FeSO_4$	1	1	27 05	10.5
17	2	Fepyr	1	2	2.36	83 7	38	2	Fepyr	1	2	6.33	10.5
18	1	$FeSO_4$	1	1	2.9	67 2	39	1	$FeSO_4$	1	1	19.72	5.8
18	1	FAP	1	2	2.3	67.2	39	1	FAP	1	2	8.3	5.8
19	1	$FeSO_4$	1	1	24.15	26.1	40	2	$FeSO_4$	1	1	21.4	37.8
19	1	FAP	1	2	14.8	26.1	40	2	Fepyr	1	2	8.57	37.8
20	2	$FeSO_4$	1	1	3.82	1.5							
20	2	Fepyr	1	2	1.83	1 5							

4-30-09 Correspondence from Notifier

AM

West-Barnette, Shayla

From: Nelson,Mark,GLENDALE,Regulatory Affairs/Label/Nutrition [Mark.Nelson@us.nestle.com]
Sent: Thursday, April 30, 2009 2:55 PM
To: West-Barnette, Shayla
Subject: RE: Request for Clarification Regarding Intended Use in GRN 271 (Ferrous Ammonium Phosphate)

Dr. West-Barnette -

The text in the amendment is in the attachment to our response to the reviewers questions. This attachment, Nestlé R&D Report RDLS-RD080105, is a description of the clinical study that assessed the bioavailability of FAP.

The text " . . . the potential target consumer group for FAP fortified FCMP will be infants and young children. . ." applies to other markets in which Nestlé does business and for a product, full cream milk powder, that we produce and sell in markets outside the US.

The statement in our original submission on pages 8 and 16 of GRN 271 that FAP "will not be used in products specially formulated or processed for use by infants or children less than 4 years of age" continues to be our intention in the US.

Please feel to contact me if you have further questions.

Mark

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From: West-Barnette, Shayla [mailto:Shayla.WestBarnette@fda.hhs.gov]
Sent: Thursday, April 30, 2009 11:17 AM
To: Nelson,Mark,GLENDALE,Regulatory Affairs/Label/Nutrition
Subject: Request for Clarification Regarding Intended Use in GRN 271 (Ferrous Ammonium Phosphate)

Dr. Nelson,

During the process of reviewing the amendment to GRN 271 (ferrous ammonium phosphate (FAP)) dated April 8, 2009, I found a statement on page 18 which reads "the potential target consumer group for FAP fortified FCMP (Full Cream Milk) will be infants and young children." However, on pages 8 and 16 of GRN 271, Nestle states that FAP "will not be used in products specially formulated or processed for use by infants or children less than 4 years of age."

Could you please clarify whether Nestle intends to add ferrous ammonium phosphate to products that will be consumed by infants and young children? An email response would be sufficient to clarify this point. So that the OFAS review team can stay on schedule, a response no later than a week from today (no later than May 8) would be appreciated. Please feel free to contact me if you have any questions. Thanks, and I look forward to hearing from you soon.

Regards,

Shayla West-Barnette, Ph.D.
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Center for Food Safety and Applied Nutrition
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