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Novel methods for identification and quantification of iron fortificants in cereal flours

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Novel methods for identification and quantification of iron fortificants in cereal flours

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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NOMENCLATURE

ASC	Ascorbic Acid
AACC	American Association of Cereal Chemists
DMT1	Divalent metal transporter 1
EDTA	Ethylenediaminetetraacetate
EFe	Electrolytic iron
FAO	Food and Agriculture Organization of the United Nations
Fe	Iron
Fe^0	Elemental iron
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
Fe-S	Iron-sulfur
FePP	Ferric pyrophosphate
FeCit	Ferrous citrate
FeFum	Ferrous fumarate
$FeSO_4$	Ferrous sulfate
FPN	Ferroportin
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HFE	Human homeostatic iron regulator (**H**igh **FE**)

HJV	Hemojuvelin
IDA	Iron deficiency anemia
IL	Interleukin
IRE	Iron response element
IRE-BP	Iron response element-binding protein
KSCN	Potassium thiocyanate
MCV	Mean corpuscular volume
MFP	Meat, fish, poultry
MNP	Micronutrient powders
N	Normal (concentration)
NaFeEDTA	Sodium ferric ethylenediaminetetraacetate
NH_4SCN	Ammonium thiocyanate
RBC	Red blood cell
RLS	Restless leg syndrome
sTfR	Soluble transferrin receptor or serum transferrin receptor
TCA	Trichloroacetic acid
TfR	Transferrin receptor
TfR1	Transferrin receptor 1
TfR2	Transferrin receptor 2
TGA	Thioglycolic acid

UTR	Untranslated region
WHO	World Health Organization

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ABSTRACT

Iron deficiency is the most common nutrient deficiency globally. Fortification of cereal grains is a main strategy used to ameliorate global iron deficiency due to its safety and efficacy. For fortification to be effective, fortification programs must use appropriate iron compounds at appropriate levels. However, existing laboratory methods to identify and quantify fortificants are time consuming and costly. Our objective was to develop a quick and simple method to identify and quantify iron compounds commonly used for flour fortification. Unfortified whole wheat, refined wheat, and yellow corn flours were fortified with 20–60 mg Fe/kg flour using ferric pyrophosphate (FePP), ferrous sulfate ($FeSO_4$), ferrous citrate (FeCit), ferrous fumarate (FeFum), sodium ferric EDTA (NaFeEDTA), and electrolytic iron (EFe). Using potassium thiocyanate (KSCN) with HCl with and without hydrogen peroxide (H_2O_2), we identified EFe, ferric, and ferrous fortificants. NaFeEDTA, FePP, $FeSO_4$, FeCit, and FeFum were identified based on their solubility in water using ferrozine with and without ascorbic acid (ASC). An alternative method for identification that uses only KSCN as a chromogen was also developed but was inferior to the ferrozine method. Four blinded samples were prepared with randomly selected fortificants (EFe, NaFeEDTA, FePP, FeFum) and all were correctly identified by four personnel. For quantification, those four samples plus an additional $FeSO_4$ sample were tested blindly. The average of each person's reported iron levels for each sample were within 10 mg Fe/kg of actual iron levels 85% of the time. Estimated iron levels from the visual method were not significantly different than iron levels from two standard quantitative methods ($p > 0.05$) for all the fortificants tested suggesting reliability of simple visual testing. These quick, inexpensive, and reliable methods will be useful for agencies to identify the type and amount of iron added to flour to monitor the quality of iron fortification strategies.

CHAPTER 1. GENERAL INTRODUCTION

Background

As of 2017, 2.0 billion people are affected by anemia globally and 1.1 billion people have iron deficiency anemia [1]. The prevalence of anemia is highest among females of reproductive age and children under 5 years old [2]. Iron deficiency is caused by many factors including low iron intake, low intake of bioavailable iron, inflammation, and infections. Strategies to ameliorate global iron deficiency include fortification, supplementation, improving dietary diversity, and biofortification [3]. Fortification is important because of its safety and efficacy, but this can only be insured if there is adequate oversight of fortification programs. However, there are currently no low cost, rapid, and reliable methods available for the identification and quantification of iron fortificants used in flour fortification. Here, we developed a rapid, reliable, and inexpensive method for use by government agencies to identify and quantify iron fortificants in cereal flours for oversight of fortification programs.

Objectives

1. Develop a rapid, reliable, and inexpensive method for the identification of six of the most commonly used iron fortificants (electrolytic iron (EFe), ferric pyrophosphate (FePP), sodium ferric EDTA (NaFeEDTA), ferrous sulfate ($FeSO_4$), ferrous fumarate (FeFum), ferrous citrate(FeCit)) added to cereal flours (refined wheat flour, whole wheat flour, yellow corn flour).
2. Develop a rapid, reliable, and inexpensive method for the quantification of five of the most commonly used iron fortificants (EFe, FePP, NaFeEDTA, $FeSO_4$, FeFum) added to cereal flours.

3. Develop a rapid, reliable, and inexpensive method using only potassium thiocyanate as a chromogen for the identification and quantification of five of the most commonly used iron fortificants (EFe, FePP, NaFeEDTA, $FeSO_4$, FeFum) added to cereal flours.

Thesis Organization

This thesis contains four chapters including a general introduction, literature review, a manuscript, and an overall summary, with additional methods not included in the manuscript and detailed instructions of the methods developed in the appendices. The manuscript is titled "Rapid and reliable method for qualitative and quantitative assessment of iron fortificants used for flour fortification" and was written for the submission to the journal *Nutrients*. References throughout this thesis are at the end of each chapter and are formatted using the *Nutrients* citation format. The research described in this thesis focuses on the development of methods for identification and quantification of iron fortificants in cereal flour.

Author's Roles

In the course of my master's degree program, I worked primarily on the development of these methods. The majority of my work is presented in Chapter 3 of this thesis, which includes the development, description, and results of these methods including the results of laboratory assistants testing of blinded samples. For the manuscript, I wrote the draft with the assistance of Isaac Agbemafle, and Dr. Manju Reddy edited the manuscript as a lead investigator on the project.

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CHAPTER 2. LITERATURE REVIEW

Prevalence, Causes, Risk Factors, and Consequences of Iron Deficiency and Anemia

Prevalence of iron deficiency and anemia

As of 2017, 2.0 billion people are affected by anemia globally. While the prevalence of iron deficiency is decreasing, in 2017 1.1 billion people had iron deficiency anemia [1]. Iron deficiency is the leading cause of years lived with disability (YLD) in many countries. Globally, iron deficiency anemia is the fourth leading cause of YLD after low back pain, headache disorders, and depressive disorders [1]. Prevalence of anemia is highest among females of reproductive age and children under 5 years old [2]. Globally, in 2016 42% of children under 5 years of age, 33% of females of reproductive age, and 40% of pregnant females had anemia [3, 4].

Causes and risk factors of iron deficiency and anemia

Iron deficiency can be caused by several factors, including low iron intake, low iron absorption, low consumption of highly bioavailable iron, and high iron loss. In females of reproductive age, there is a wide range in the amount of iron lost during menstruation, and iron deficiency is often caused by high blood loss during menstruation. Since the 1960s, estimated iron losses from menstruation have fallen from 0.7 mg iron lost per day to 0.4 mg lost per day [5, 6]. This is likely due to wider use of oral contraceptives, but there is still a wide range in amount of blood lost [6, 7].

Premature birth is related to iron deficiency in infants. Iron is largely transferred to the fetus in the third trimester. Premature infants are more prone to iron deficiency because they are

born before transfer of iron to the fetus is complete and because they grow at a more rapid rate than full term infants [8].

Iron deficiency anemia (IDA) can result from inflammation associated with chronic diseases, obesity, and acute illness. Infections can also result in secondary nutrient deficiencies and anemia if they result in diarrhea and malabsorption [9]. *Helicobacter pylori*, for example, can cause IDA. Around half of the world's population carry *H. pylori*, with the highest prevalence in Africa, Latin America, and Asia [10]. *H. pylori* may cause IDA by multiple mechanisms including sequestering iron, decreasing gastric acid secretion, altering ascorbic acid (ASC) metabolism in the GI tract, and blood loss from inflammation of gut mucosa [11]. Additionally, decreased stomach acid leads to increased enteropathogenesis and diarrhea, leading to IDA [12]. Anemia is a common feature of HIV and is a predictor of morbidity and mortality. Even with treatment, some remain anemic, likely due to underlying inflammation [13]. Soil-transmitted helminths are a very large and often ignored problem, with 1.5 billion people infected globally, though symptoms are not always present [14]. Helminths feed on the blood and tissues of the host, leading to iron and protein deficits and anemia as well as loss of appetite and diarrhea.

Chronic inflammation from obesity, autoimmune conditions, and chronic kidney disease can result in changes in iron homeostasis or IDA [15, 16]. Systemic inflammation increases levels of the hormone hepcidin, which decreases iron absorption [17]. Additionally, chronic inflammatory conditions of the GI tract, like Crohn's disease and ulcerative colitis, can cause IDA through multiple mechanisms, including inflammatory signaling, GI bleeding, and diarrhea [17].

Iron deficiency anemia constitutes 50% of anemia cases globally [9]. Deficiencies of other nutrients, including vitamins A, B_2, B_6, B_{12}, C, D, E, folate, and copper, can also result in nutritional anemias. These nutrients are needed for hemoglobin synthesis, the production or maintenance of red blood cells (RBC), or are involved in iron metabolism. Additionally, anemia can be the result of genetic abnormalities like sickle cell disease, alpha and beta thalassemia, and glucose-6-phosphate dehydrogenase (G6PD) deficiency, all of which predominantly affect those in developing countries [18]. In general, sickle cell disease predominately affects those in Sub-Saharan Africa, thalassemia affects Southeast Asia and Africa, and G6PD deficiency affects Africa and the Mediterranean [18]. Eating disorders like anorexia nervosa can also result in anemia, though this may not be due to iron deficiency [19]. Iron deficiency can, however, exacerbate the hormone dysregulation and bone loss associated with female athlete triad [20].

Consequences of iron deficiency and anemia

General symptoms of IDA include fatigue, weakness, headache, hair loss, brittle nails, decreased work capacity, inability to maintain body temperature in a cold environment, behavior changes, decreased resistance to infection, and adverse pregnancy outcomes [21, 22].

During pregnancy, both mother and fetus can face short- and long-term consequences due to iron deficiency [23]. Anemia causes cardiovascular strain and reduced physical and mental performance for the mother [24]. Anemia increases the risk of maternal mortality from other factors, like blood loss, and increases the chance that blood transfusions will be required. Postpartum, anemia is associated with insufficient milk production [24]. IDA is associated with increased risk of low birth weight and preterm delivery [7]. Three mechanisms have been proposed to explain why IDA in pregnancy could lead to preterm delivery [25]. First, hypoxia and increased norepinephrine induce fetal and maternal stress, which leads to increased

production of corticotropin-releasing hormone (CRH). CRH is a risk factor for preterm labor, pregnancy-induced hypertension, eclampsia, and premature rupture of membranes, and leads to increased fetal cortisol production, which may reduce growth. Second, iron deficiency increases oxidative damage. Third, iron deficiency increases maternal risk of infection. Babies born at term usually have adequate iron stores regardless of maternal iron status [26].

Iron deficiency during infancy and childhood has significant negative effects, particularly on immune function and brain development. Signs and symptoms of IDA in children include pale skin, fatigue, slowed development, poor appetite, behavioral problems, and frequent infection [27]. Anemia in childhood is associated with an increased risk of seeking medical attention for a lower respiratory tract infection [28]. IDA is also correlated with asthma [29]. Iron deficiency and anemia in childhood may have lifelong effects, and are associated with impaired performance in school, cognitive problems, and behavior problems. Specifically, iron deficiency can negatively impact neural development early in life. In follow up longitudinal studies, iron deficiency in infancy was linked with poorer cognitive function in childhood, and worse executive function in adolescence [30]. In rats, iron deficiency has been shown to have long lasting negative effects on myelin formation. Early research showed that even short-term iron supplementation in infants with IDA could improve development and motor control, even without improving hemoglobin levels [31]. This suggests that iron deficiency could have negative effects, even if anemia is not present. The behavioral effects of IDA in infancy appear to be mitigated if anemia is corrected by two years of age [32]. Four-year-old children who were anemic in infancy and at 2-years-old fared worse on measures of social emotional development compared to children who never had IDA. However, children whose anemia was corrected by 2-years-old fared similarly to those who never had IDA. Iron deficiency is thought to contribute to

pica in children [22]. This increases the risk that children will consume toxic non-foods like chips of lead paint, which can further damage the brain.

In adulthood, iron deficiency is associated with decreased productivity and decreased immune function. New evidence has also found an association with iron and restless leg syndrome (RLS) [33]. RLS is associated with iron deficiency or low iron levels in the brain, and individuals with both RLS and IDA have worse RLS symptoms.

Iron Absorption

Iron in the body

The adult human body contains 3–5 g iron [34]. Iron is found primarily in red blood cells, liver, macrophages, and myoglobin. Red blood cells contain 65–75% of the body's iron. Another 10–20% of iron is stored as ferritin in macrophages or hepatocytes, and 3–4% of the body's iron is found in muscle as myoglobin [34, 35]. There is no dedicated method for iron excretion and iron is lost only by sloughing off the epithelium of the gastrointestinal tract, blood loss, and sweating. To account for these losses, 1–2 mg of iron needs to be absorbed per day.

Iron in foods

Dietary iron is present in foods as nonheme iron or heme iron. Nonheme can be ferric or ferrous, or elemental if from fortified foods. Ferric and elemental iron are the least bioavailable, and heme iron is the most bioavailable. Heme and nonheme iron are often bound to proteins in foods. Nonheme iron is released from proteins in the acidity of the stomach [36], and heme iron is released from proteins during proteolytic digestion in the stomach and small intestine [37].

Heme iron absorption

Heme and nonheme iron are absorbed in the duodenum [38]. Heme iron consists of an iron molecule within a protoporphyrin ring, and dietary heme iron comes largely from

hemoglobin and myoglobin from animal-based foods. Heme iron is better absorbed than nonheme iron. An early radioisotope study found that in young adult males 37% of heme iron was absorbed, while only 5% of nonheme iron was absorbed [39]. It is currently believed that 15–35% of heme iron is absorbed [40]. The mechanism of heme iron absorption is not well understood. It is believed that heme is absorbed via heme carrier protein 1 (HCP1), also called proton-coupled folate transporter (PCFT) because it also has a role in folate absorption [41, 42]. Once in the enterocyte, the iron molecule is removed from the porphyrin ring by the enzyme heme oxygenase [41]. Intact heme may also exit the enterocyte for entry into the blood via the heme exporter FLVCR (feline leukemia virus subgroup C receptor-related protein).

Nonheme iron absorption

Nonheme iron is found in both plant and animal sources and is the form of iron in most supplements and iron fortified foods. Nonheme iron can be ferric (Fe^{3+}), ferrous (Fe^{2+}), or elemental (Fe^{0}). Nonheme iron may be bound to proteins like ferritin, chelated to compounds like EDTA, or freely dissolved. In an aqueous environment, freely dissolved ferrous iron reacts with dissolved oxygen and hydroxide ions to form less soluble ferric compounds, and this process occurs more rapidly at a neutral pH compared to an acidic pH [43, 44].

To be absorbed, ferric iron must be reduced to ferrous iron. This is done by ferrireductase duodenal cytochrome b (DCYTB), an enzyme on the apical surface of enterocytes that likely uses ASC [36]. Additionally, ASC and amino acids like cysteine can also reduce iron nonenzymatically [45]. Ferrous iron is then absorbed via divalent metal transporter 1 (DMT1) [46]. Other modes of absorption for iron are debated. Nonheme iron, particularly when bound to ferritin, may also be absorbed by endocytosis [47, 48]. There has been speculation that the zinc transporter ZIP4 may have a role in iron absorption, but this has not been confirmed [49].

Factors affecting iron absorption

Many factors can enhance or inhibit iron absorption, with most of these factors affecting only nonheme iron. Dietary factors that enhance iron absorption if present in the GI tract with iron include ASC, cysteine, meat fish poultry (MFP) factor, sugars, organic acids, and possibly alcohol [50–52]. Dietary factors that inhibit iron absorption include polyphenols, phytate, oxalic acid, and other minerals including copper, zinc, and calcium [51].

The effect of ASC on iron absorption is likely two-fold. First, ASC reduces iron from ferric to ferrous allowing it to be absorbed by DMT1. Second, ASC chelates iron and reduces pH to increase iron's solubility [53].

An unknown factor in meat, fish, and poultry, termed MFP factor, has been known to increase nonheme iron absorption. Several potential mechanisms have been proposed. First, a component in MFP, like amino acids, proteins, or other factors, could chelate iron to maintain its solubility. Second, a component in MFP may stimulate secretion of gastrin or other factors that chelate iron. Third, a component in MFP may stimulate gastric acid production, which reduces pH and keeps iron soluble [54].

Polyphenols are a wide class of molecules that bind to iron and prevent its absorption. Foods and beverages rich in polyphenols include coffee and tea primarily, but also wine, fruit, vegetables, legumes, some cereals, and herbs. The type of polyphenol likely affects the degree to which iron absorption is inhibited [55].

Phytate (myo-inositol hexakisphosphate) is the storage form of phosphorus in plants, especially in seeds. Foods high in phytate include grains, where phytate is largely found in the bran, as well as nuts and legumes. Phytate binds to iron preventing its absorption and is the main inhibitor of iron absorption in plant-based diets [55]. Milling to remove bran, as well as soaking,

germinating, and fermenting high phytate food can help to remove phytate. Degrading phytate in soy protein isolates has been shown to increase iron absorption [56]. Adding exogenous phytase to meals can also improve iron absorption [55]. Phytate degrading probiotics that are active in the small intestine could be another potential tool to increase iron absorption in high phytate diets [57]. Phytate can chelate other essential minerals including copper, zinc, cobalt, manganese, calcium, and magnesium, and may lead to their deficiencies [58, 59]. Phytate may also function as an antioxidant in foods due to its ability to chelate iron, preventing it from participating in the Fenton reaction in which iron reacts to produce free radicals [60]. Animal studies suggest that phytate may have anti-colon-cancer properties, and in vitro studies suggest phytate reduces cell proliferation [58].

Other minerals including calcium, zinc, copper, and manganese are known to decrease iron absorption [61]. Zinc, copper, and manganese can be absorbed via the iron transporter DMT1 and these minerals may compete for absorption with iron [46]. Calcium negatively affects both heme and nonheme iron absorption, though the effect is limited, and the mechanism of its inhibition is not totally understood [55].

Transport and exit of iron from enterocytes

After iron is absorbed into the enterocyte, it is chaperoned around the cell. Mobilferrin is a cytosolic protein that binds to intracellular ferrous iron and transports it to the basolateral membrane [38, 62]. Ferroportin (FPN) is found on the basolateral membrane of enterocytes and it exports ferrous iron out of enterocytes for entry into the blood [63]. FPN is inhibited by hepcidin and is heavily regulated as it is the main determinant of iron into the body. FPN is the only known iron exporter and is present on all cell types. Hephaestin is a copper dependent ferroxidase in the small intestine that converts extracellular ferrous iron to ferric [64].

Iron Metabolism

Iron is essential for many cellular processes, but excess iron in cells can be detrimental. Free iron not bound to proteins may participate in the Fenton reaction where iron reacts with oxygen to produce hydroxyl radicals inducing oxidative stress [65].

Iron transport and storage

Transferrin in blood binds to up to two ferric iron molecules [66]. Transferrin receptor 1 (TfR1) is a glycoprotein on cell membranes that binds to two transferrin molecules and internalizes them into endosomes [67]. Serum (or soluble) transferrin receptor (sTfR) is a cleaved portion of TfR in circulation that reflects TfR levels in the body. High sTfR indicates intracellular iron deficiency, and, unlike some other measures of iron deficiency, sTfR is not affected by inflammation [68]. Within the acidic environment of the endosome, transferrin releases its bound ferric iron molecules [66]. This ferric iron is then reduced to ferrous iron by a metalloreductase and is exported into the cytosol by DMT1 [65].

Ferritin is a cage-like protein composed of 24 light or heavy subunits that reversibly binds up to 4500 iron atoms [69]. It is the iron reservoir within the cytosol of all cells and the buffer against intracellular iron toxicity and deficiency. Ferrous iron enters through ion channels on ferritin and is oxidized to ferric iron and stored. Iron can be reduced and released from ferritin as ferrous iron when needed. When intracellular iron levels become high, iron is thought to be converted into an insoluble form called hemosiderin made of deposits of ferritin [22, 70].

Control of iron metabolism

Transferrin receptor 2 (TfR2) plays a role in iron signaling [71]. Human homeostatic iron regulator (HFE) works with TfR2 to alter hepcidin production [72]. HFE normally interacts with TfR1, but when transferrin binds TfR1, HFE is displaced and interacts with TfR2 [73]. This

leads to a signaling cascade that increases hepcidin production [72]. If genetic abnormalities exist in HFE, hepcidin levels will be low and hereditary hemochromatosis will result. Hemojuvelin (HJV) is another cell surface protein involved in iron metabolism. While its exact mechanism is unclear, it is known that genetic abnormalities in the gene for HJV result in juvenile hemochromatosis [73].

Hepcidin is the main iron regulatory hormone and it downregulates iron levels [74, 75]. In mice, when hepcidin is absent due to knocking out an upstream transcription factor, mice develop iron overload. Many factors can cause a change in hepcidin levels, including iron stores, inflammation, and hypoxia, and high iron levels typically upregulate hepcidin [76]. In addition to HJV, HFE, and TfR2, interleukin-6 (IL-6), IL-1α, and IL-1β also appear to have a role in affecting hepcidin production, with IL-6 being the main method by which inflammation increases hepcidin production [76–78] . Hepcidin downregulates iron's entry into the body from mucosal cells by binding to FPN, resulting in its internalization and degradation [79]. Hepcidin also downregulates iron release from macrophages and hepatocytes via its effect on FPN [78].

Synthesis of proteins involved in iron metabolism is partially regulated post-transcriptionally. Iron response elements (IRE) are short regions of mRNA that form stem-loops in either the 5' or 3' untranslated region [80]. Iron response element binding proteins (IRE-BP) are proteins that bind to IREs. There are two IRE-BPs, the most abundant being iron regulatory protein-1 (IRP-1) [81]. IRP-1 is also cytoplasmic aconitase, which contains a [4Fe-4S] cluster when enzymatically active [82, 83]. This can be converted to a [3Fe-4S] cluster and disassembled allowing for binding to an IRE in mRNA.

Transcripts with an IRE located in the 5' UTR, like those for ferritin, have decreased translation when intracellular iron levels are low [84]. When iron levels are low, binding of an

IRE-BP to the IRE in the 5' region of ferritin's mRNA blocks translation. Transcripts with IREs in the 3' UTR, like those for TfR, have increased translation when intracellular iron levels are low [85]. When iron levels are low, IRE-BPs bind to IREs in the 3' UTR of TfR mRNA, increasing its stability and translation [86].

Measurements of iron deficiency

Many measurements are used to assess iron deficiency and anemia. Hemoglobin is used to diagnose anemia. The World Health Organization defines anemia as hemoglobin <110 g/L for children 6–59 months old and pregnant females, <115 g/L for children 5–11 years, < 120 g/L for children aged 12–14 years and nonpregnant females ≥15 years, and <130 g/L for males ≥15 years [87]. Serum ferritin is used to determine the presence of iron deficiency. A small amount of ferritin from cells is released into serum and can be used as an indicator of iron stores [88]. Serum ferritin is normally between 30 to 300 ng/mL, and serum ferritin < 12 ng/mL is usually the cutoff for diagnosing iron deficiency [89]. Iron depletion can still occur with higher ferritin levels as ferritin is a positive acute phase protein that increases with inflammation. Mean corpuscular volume (MCV) is the average size of red blood cells, and it is calculated by dividing hematocrit by RBC concentration. MCV may be a good way to diagnose IDA during early pregnancy due to normal lowering of hemoglobin in pregnancy making it difficult to identify true low hemoglobin levels [7]. Other measures used to assess iron deficiency and anemia include hematocrit, RBC count, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), transferrin concentration, transferrin saturation, sTfR, zinc protoporphyrin (ZPP), and hepcidin.

Stages of iron deficiency

If the amount of iron absorbed does not match the amount of iron lost, iron depletion will occur. Iron depletion is usually divided into three stages that are measurable with blood analyses [61]. The first stage is iron depletion, where serum ferritin is lowered, which is reflective of lowered iron in body cells. The second stage is iron-deficient erythropoiesis, where transferrin saturation is decreased and sTfR is increased. The third stage is iron-deficiency anemia, where blood hemoglobin, along with hematocrit and MCV, are lowered.

Iron Functions

Heme iron enzymes and proteins

Iron's primary responsibility in the body is as a cofactor for many enzymes and proteins. Both heme and nonheme iron can function as cofactors. Heme proteins have roles in oxygen transport, energy metabolism, preventing oxidative damage, inflammation, the immune system, and thyroid hormone production [90–92]. The majority of iron in the body is in red blood cells as hemoglobin for oxygen transport. Hemoglobin has four subunits, each with one heme, and a single hemoglobin protein can bind up to 4 O_2 molecules. Myoglobin, an oxygen binding protein in muscle, is composed of a single subunit with a single heme molecule and binds to a single O_2 molecule. Heme iron is important for energy metabolism due to its role in complexes III and IV of the electron transport chain [90]. Catalase uses heme as a cofactor to convert H_2O_2 to O_2 to prevent oxidative damage. Another group of enzymes that use heme as a cofactor are halide oxidizers, which are involved in innate immune response and thyroid hormone production [92].

Nonheme iron enzymes and proteins

Nonheme enzymes and proteins can be bound to iron in iron-sulfur (Fe-S) clusters or may be bound to just iron. Non Fe-S iron dependent proteins have functions in the immune system,

and DNA, collagen, carnitine, amino acid, and biogenic amine synthesis [93–96]. Fe-S clusters are molecular ensembles of iron and sulfide involved in many reactions, particularly reactions involving the transfer of electrons. They are involved in energy metabolism and redox reactions. Complexes I, II, and III of the electron transport chain and aconitase in the citric acid cycle contain Fe-S clusters [90, 97–99]. A class of Fe-S enzymes is ferredoxins. Ferredoxins perform redox reactions and are most well known for their roles in photosynthesis and nitrogen fixation. Human ferredoxins are involved in steroid hormone formation and are important in cellular iron metabolism due to their roles in heme and Fe-S cluster formation [100].

Iron plays a role in many biological processes including energy metabolism, immune function, and hormone and neurotransmitter synthesis. This explains the many negative consequences of iron deficiency including fatigue, impaired immune response, and, in children, iron deficiency can negatively affect brain development.

Interventions

There are four approaches to ameliorate dietary iron deficiency and IDA: iron supplementation, increased dietary diversity, biofortification, and iron fortification [101].

Iron supplementation and home fortification

Supplementation allows for a targeted approach to iron deficiency, but the most effective iron supplements, like $FeSO_4$, can have significant GI side effects including diarrhea, constipation, and changes in stool color, which may lessen compliance. The WHO recommends 10–12.5 mg iron be given to children daily in the form of a drop or syrup in areas where iron deficiency is common [102]. Daily iron supplementation during pregnancy is effective at reducing the risk of iron deficiency, but the benefits of widespread supplementation depend on the population [103].

Micronutrient powders (MNP) are similar to supplements but are added directly to foods. MNPs allow for the prevention of micronutrient deficiencies in targeted groups, and because they can be added to many foods, are able to be used in traditional diets [104]. MNP products include Sprinkles, which contains 12.5 mg iron as microencapsulated FeFum along with other nutrients including 30 mg vitamin C, and MixMe, which contains 10 mg Fe per 1 g sachet as NaFeEDTA along with vitamin C and phytase. However, MNPs have been shown to produce GI side effects like diarrhea and promote GI inflammation [105]. MNPs may also have a negative effect on the microbiome. Pathogenic bacteria like *Salmonella* and *E. coli*, require iron for virulence, while bacteria thought to be beneficial, like *Bifidobacteria* and *Lactobacilli* require little or no iron [106].

Improved dietary diversity

Increasing availability and consumption of fruits (high in vitamin C), vegetables (high in vitamin C and iron), and meat (high in bioavailable iron) should be the main long-term strategy for decreasing global iron deficiency. However, dietary diversity as an approach is difficult, especially in developing countries, due to high costs and low supply of iron rich foods [101]. In addition, dietary diversity alone will not be enough to prevent iron deficiency in all situations. For example, during pregnancy it is very difficult to meet iron needs without supplementation and fortified foods [107, 108].

Biofortification

Biofortification is increasing the vitamin or mineral content of a food through plant breeding, biotechnology, or agricultural practices [109]. Iron biofortified foods exist but are not currently available for widespread introduction into the food supply. Common beans (*Phaseolus vulgaris*) bred with traditional breeding techniques to be high in iron have been the subject of

much of the current research into iron biofortified foods. In one study, women who were fed iron biofortified beans for 128 days consumed an average of 5.9 mg additional iron per day and had significantly greater increases in hemoglobin compared to women fed non-biofortified beans [110]. Additionally, the women who consumed biofortified beans had greater improvements in tests of cognitive performance compared to women fed non-biofortified beans [111].

Iron fortification

Fortificants vary in cost, bioavailability, and in the organoleptic changes they cause to foods, with more bioavailable fortificants generally producing more unwanted organoleptic changes. Choice of vehicle used in a fortification program is important because of iron's reactivity and potential for toxicity. Common vehicles for iron fortification include refined wheat flour, whole wheat flour, corn flour, rice, breakfast cereals, breads, pasta, and infant cereals and formulas. Legislation in 83 countries mandates fortification of wheat flour alone or in combination with maize flour or rice [112]. Maize flour fortification is required in 16 countries, all of which also require wheat fortification. Fortification of rice is only required in 7 countries. Other vehicles for fortification include salt, beverage powders like Nestle's Milo and Nesquik, spice and curry powders, bouillon, sauces, and sugar.

The amount of iron added to a food also matters to ensure effectiveness without risking toxicity and organoleptic changes. The amount of iron added varies by region, vehicle, and fortificant. In the case of refined wheat flour, the amount of iron added is usually set at the amount needed to restore iron levels to preprocessing levels [113, 114]. Unfortified refined wheat flour contains around 10 mg Fe/kg flour, while whole wheat flour contains 40 mg Fe/kg. In general, fortification of refined wheat flour adds iron to return the iron level back to 40 mg Fe/kg [115, 116]. In more specific guidelines, the amount of iron added to flour depends on the

fortificant being used and the average flour availability per person in that region [117]. Thirty mg Fe/kg as ferrous sulfate ($FeSO_4$) is recommended for both refined and whole wheat flour, and 45 mg Fe/kg is recommended for countries with low wheat flour intake (<200 g per person per day) [114]. For example, Brazil requires the addition of at least 4.2 mg of iron (42 mg Fe/kg) and 150 µg of folic acid to each 100 g industrialized wheat and maize flour [118]. Overall, recommended levels of added iron vary but are generally 15–60 mg Fe/kg [117]. One of the highest levels of iron fortification was 6.5 mg iron per 100 g flour (65 mg Fe/kg), which was in Sweden until its fortification program was withdrawn in 1995 due to improved dietary habits, contraceptive use, and concerns about fortification's effect on those with hemochromatosis [119].

Ferrous fortificants

$FeSO_4$ is water soluble, has a high bioavailability, and is considered the gold standard for bioavailability [120]. It is more commonly used for supplementation than for fortification. Because it is water soluble, $FeSO_4$ is reactive and can cause foods to turn gray, green, or blue [121]. Because $FeSO_4$ promotes fat oxidation and rancidity, it can only be used to fortify foods with a short shelf-life, like baked goods. It also may contribute to a metallic taste. $FeSO_4$ is also used to fortify pasta and infant formulas [122].

FeFum is poorly water soluble and soluble in dilute acid [120]. Despite being less soluble, it is believed to be as bioavailable as $FeSO_4$. However, if gastric acid is insufficient, as in infants, absorption of iron from FeFum may be reduced. Because FeFum is insoluble in water it can cause fewer organoleptic problems [123]. FeFum is used in supplements and infant formulas, and is used to fortify maize flour in Venezuela, and wheat flour in Central America [120].

Other ferrous fortificants include ferrous citrate (FeCit), ferrous bisglycinate, ferrous gluconate, ferrous lactate, ferrous succinate, ferrous ammonium sulfate, and ferrous tartrate. FeCit, is poorly water soluble and soluble in dilute acid [124]. While this is the same classification as FeFum, in our experience, FeCit appears to be more soluble than FeFum in water and dilute acid. Iron from FeCit is believed to be absorbed relatively well (74% that of $FeSO_4$), but FeCit is not commonly used and not as well studied [120, 124]. Ferrous bisglycinate is water soluble and used in some infant formulas and supplements [120]. It is more bioavailable than $FeSO_4$, but it has a high cost, causes color changes, and promotes fat oxidation in cereal flour [120, 124]. Ferrous gluconate is water soluble and has similar bioavailability as $FeSO_4$ but is more expensive [120]. It is used in Mexico because it causes fewer sensory problems, but it still has high potential for organoleptic problems [122, 125].

Ferric fortificants

Ferric pyrophosphate (FePP) is water insoluble, poorly soluble in dilute acid. Iron from FePP has a low bioavailability and is absorbed 25–75% as well as $FeSO_4$ [120]. FePP has a low potential for promoting oxidation and organoleptic changes when added to flour [121]. It is commonly used to fortify rice, and rice fortified with FePP is well accepted by consumers [120, 126]. FePP has also been used in Europe to fortify infant cereals and chocolate drink powder [122]. Micronizing to decrease particle size may increase bioavailability, and this product is used in some dairy products in Japan [120].

Sodium ferric EDTA (NaFeEDTA) is water soluble, but despite that, it does not appear to promote lipid oxidation in foods, though it may still cause color changes [120, 124]. NaFeEDTA is significantly more expensive than other fortificants like $FeSO_4$. The main benefit of NaFeEDTA is that it is able to overcome the challenges of fortifying foods high in phytate. In

low phytate foods, iron from NaFeEDTA is absorbed as well as iron from $FeSO_4$, but in high phytate foods, iron from NaFeEDTA is absorbed 2–3 times better than iron from $FeSO_4$ [122]. This is believed to be because EDTA stays bound to iron in the digestive tract and protects it from binding to phytate and other inhibitors [127]. Research looking at NaFeEDTA fortified curry powders has shown success with significant reductions in anemia and increases in hemoglobin and ferritin in females who received fortified curry powder [128].

Other ferric fortificants include ferric citrate, ferric ammonium citrate, ferric saccharate, ferric choline citrate, ferric orthophosphate, and ferric glycerophosphate. Ferric ammonium citrate is used less commonly, but it has been used in the UK for fortification of wheat flour. Early research showed absorption of iron from ferric ammonium citrate in bread is low, being similar to that of reduced iron [129]. Other early research showed that when baked into chapati (unleavened flat-bread) only around 2% of iron from ferric ammonium citrate was absorbed [130]. Ferric saccharate is poorly water soluble, soluble in dilute acid, is as bioavailable as $FeSO_4$, and has been used to fortify chocolate drink powders [120]. Ferric orthophosphate, like ferric pyrophosphate, is water insoluble and poorly soluble in acid [120]. Iron from ferric orthophosphate has 25–32% the absorption of iron from $FeSO_4$. Ferric glycerophosphate is a newer fortification product. Bioavailability and safety of ferric glycerophosphate is not well understood [131]. It appears to have good bioavailability in rats, with 93% the absorption of $FeSO_4$, however ferric glycerophosphate is 10 times as expensive as $FeSO_4$ [122].

Elemental iron fortificants

There are 5 types of elemental fortificants produced via different manufacturing processes [124]. They have varying particle sizes and densities, which affects their bioavailability. In general, elemental iron fortificants have a low bioavailability. The WHO

recommends doubling the amount of iron added to fortified foods if using an elemental fortificant to compensate for lower bioavailability [120]. Elemental fortificants are usually inexpensive and they are relatively unreactive in foods due to low solubility. The five elemental fortificants are carbonyl iron, hydrogen-reduced iron, atomized iron, carbon monoxide reduced iron, and electrolytic iron. Electrolytic iron has the highest bioavailability, and hydrogen-reduced iron can also have a high bioavailability when it's processed to have a very small particle size. Atomized and carbon monoxide reduced iron have very low bioavailability (12–32% that of $FeSO_4$) and are not generally recommended for fortification. The bioavailability of carbonyl-iron is not well understood but it could be absorbed as well as electrolytic iron. Electrolytic iron is used in infant cereals in the U.S. and used in Nesquik chocolate beverage powder and in Milo powder.

Encapsulated fortificants

Coating iron fortificants is done to physically separate iron from food components to prevent iron from causing organoleptic changes to the food it is added to [120]. Coatings are made primarily of hydrogenated vegetable oil, but mono- and diglycerides, maltodextrins, and ethyl cellulose can also be used. Encapsulating iron fortificants increases the cost by 10 times, and it is important to balance the stability of encapsulation while maintaining iron bioavailability. $FeSO_4$ and FeFum are most commonly encapsulated and can be as bioavailable as their unencapsulated counterparts when encapsulated.

Heme and organic fortificants

Heme iron and hemoglobin have been suggested as fortificants due to heme iron's high bioavailability, but due to their strong color, there are few vehicles that they would be acceptable in. When heme iron was used to fortify foods, it resulted in lipid oxidation like many nonheme

fortificants [132]. Despite these challenges, biscuits fortified with heme iron have been shown to be equally as effective as biscuits fortified with $FeSO_4$ in increasing hemoglobin in preschool aged children when fed for 10 weeks [133]. Additionally, chocolate flavored biscuits fortified with heme have been reported to be well accepted by adolescent girls and were effective at raising hemoglobin when fed for 13 weeks [134]. Heme as a food additive and fortificant has gained new interest due to its use in plant-based meat replacements like those made by Impossible Foods. Instead of bovine or porcine based heme iron concentrate, Impossible Foods has genetically modified yeast to produce heme using the genes for soy leghemoglobin [135]. However, research still needs to be done to assess the iron bioavailability from Impossible Foods' products.

Other alternative iron supplements include those produced by growing fungi or other organisms in iron rich media and processing the fungi to produce an iron rich powder. A radioisotope study in which *Aspergillus oryzae* was grown with $FeSO_4$ and processed into a powder showed that, in females of reproductive age, the iron from the *A. oryzae* product could be absorbed as well as iron from $FeSO_4$ [136]. Other research has shown that this same product in supplement form produces fewer gastrointestinal side effects, less non-transferrin bound iron, and potentially less oxidative stress than a conventional $FeSO_4$ supplement [137].

Methods to improve iron absorption

In addition to fortification, foods can be altered to improve iron bioavailability. Adding ASC and sodium EDTA to foods can improve iron bioavailability [120]. Additionally, removing phytate through milling, soaking, sprouting, and fermenting can also increase iron bioavailability if phytate is reduced to less than a 1:1 molar ratio with iron.

Existing methods for identification and quantification of iron fortificants

The American Association of Cereal Chemist's method 40-40 uses potassium thiocyanate (KSCN), along with 2 N hydrochloric acid (HCl) to identify ferric iron present in flour samples [138]. The method also suggests using dilute hydrogen peroxide, which will oxidize ferrous iron to ferric iron, to identify ferrous iron. While this method can identify oxidation state, it cannot identify specific fortificants by their solubility. This method is, however, useful for assessing uniformity of mixing as all added iron will appear as specks within the flour sample. Additionally, other methods that utilize potassium ferricyanide in conjunction with a magnet are able to identify $FeSO_4$, FeFum NaFeEDTA, and elemental iron [139].

Many methods exist for quantification. For example, atomic absorption spectroscopy and Inductively coupled plasma atomic emission spectroscopy are very sensitive but expensive. Devices like handheld photometers are less expensive than normal photometers and can be easily transported. The iCheck photometer from Bioanalyt, for example, uses bathophenanthroline to quantify iron levels. However, handheld photometers can still be too costly for use by agencies overseeing fortification programs in developing countries.

An alternative to photometers is smartphone applications that use the phone's camera to measure color intensity using an iron chromogen to quantify iron levels. Waller et al. recently developed a method that uses ferrozine to quantify FeFum and $FeSO_4$ in wheat flour, corn flour, and infant formula [140].

Conclusions

This literature review illustrates how iron deficiency is a significant global health problem with many challenges and no single path forward. Iron is an essential nutrient with many functions in the body and its metabolism in the body is highly regulated. Iron fortification,

supplementation, biofortification, and improved dietary diversity are all potential strategies to lessen the global burden of IDA each with advantages and disadvantages. Iron fortification can only be effective if there is adequate oversight ensuring that appropriate iron compounds are being used and that iron is added in appropriate amounts. Available methods to identify and quantify iron in flour are time consuming or require expensive analytical equipment and there is a need for inexpensive, rapid, and reliable methods to identify and quantify iron added to flour.

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CHAPTER 3. RAPID AND RELIABLE METHOD FOR QUALITATIVE AND QUANTITATIVE ASSESSMENT OF IRON FORTIFICANTS USED FOR FLOUR FORTIFICATION

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Abstract

Manufacturers are able to fortify flour with cheaper, less bioavailable iron compounds, or with inappropriate iron levels, leading to less impact on reducing the global burden of anemia. Currently, there is no quick, low-cost method for the identification or quantification of iron fortificants in cereal flours. Our objective was to develop a quick and simple method to identify and quantify iron compounds commonly used for flour fortification. Unfortified whole wheat, refined wheat, and yellow corn flours were fortified with 20–60 mg Fe/kg using ferric pyrophosphate (FePP), ferrous sulfate ($FeSO_4$), ferrous citrate (FeCit), ferrous fumarate (FeFum), sodium ferric EDTA (NaFeEDTA), and electrolytic iron (EFe). Using potassium thiocyanate (KSCN) with HCl with and without hydrogen peroxide (H_2O_2), we identified EFe, ferric, and ferrous fortificants. NaFeEDTA, FePP, $FeSO_4$, FeCit, and FeFum were identified based on their solubility in water using ferrozine with and without ascorbic acid (ASC). An alternative method for identification that uses only KSCN as a chromogen was also developed but was inferior to the ferrozine method. Four blinded samples were prepared with randomly selected fortificants (EFe, NaFeEDTA, FePP, FeFum) and all were correctly identified by four

personnel. For quantification, those blinded samples plus an additional sample with $FeSO_4$ were tested. The average of each person's reported iron levels for each sample were within 10 mg Fe/kg of actual iron levels 85% of the time. Estimated iron levels from the visual method were not significantly different than iron levels from two standard quantitative methods ($p > 0.05$) for all the fortificants tested suggesting reliability of simple visual testing. These quick, inexpensive, and reliable methods will be useful for agencies to identify the type and amount of iron added to flour to monitor the quality of iron fortification strategies.

Introduction

Iron deficiency is the most prevalent micronutrient deficiency in the world today and the most common cause of anemia. Globally, anemia affects 2.0 billion people [1], with the highest prevalence in preschool-age children and women of reproductive age [2]. If not prevented or corrected, IDA may cause impaired mental development, reduced physical performance, reduced work productivity, increased maternal and child morbidity and mortality, and referral to health-care professionals [3]. Food fortification can be a safe and effective strategy for reducing the incidence of iron deficiency. Iron fortification has been shown to increase serum ferritin and hemoglobin levels in females of reproductive age [4] and iron fortification of infant formula has been associated with a decrease in infancy and childhood anemia [5]. In 2006, the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) published recommendations on wheat and maize flour fortification [5]. Four iron sources, sodium ferric EDTA (NaFeEDTA), ferrous sulfate ($FeSO_4$), ferrous fumarate (FeFum), and electrolytic iron (EFe), which vary widely in bioavailability and cost, were listed as suggested iron fortificants for wheat and corn flour. Additionally, the Food Safety and Standards Authority of India allows for the use of those four fortificants plus ferrous citrate (FeCit), ferrous lactate,

ferric pyrophosphate (FePP), and ferrous bisglycinate to be added to Atta (whole wheat flour) and Maida (refined wheat flour) [6]. FeCit, ferrous lactate, and ferrous bisglycinate are more costly and not commonly used fortificants [5,7].

Cereal flours are the most widely used vehicles for iron fortification because they are staple food commodities in many parts of the world. Iron fortificants are broadly classified into three groups: water soluble, poorly water soluble but soluble in dilute acid, and water insoluble and poorly soluble in dilute acid. The criteria for selecting the form of iron to add to cereal flours include its bioavailability, effect on the quality of flour, and fortificant cost. $FeSO_4$ is well absorbed and is often used as the standard against which bioavailability of other iron fortificants is measured. $FeSO_4$ is the most commonly used water-soluble iron fortificant because it is inexpensive, but it can cause sensory changes due to fat oxidation or reaction with other natural substances present in the food matrix [5]. FeCit is poorly water soluble and soluble in dilute acid and it is believed that iron from FeCit is well absorbed (74% that of $FeSO_4$) [8]. FeFum is poorly soluble in water and soluble in dilute acid [5]. Because FeFum is poorly soluble in water, it causes fewer organoleptic problems in foods, but may also be poorly absorbed by those with low stomach acid production [7]. FePP is insoluble in water and poorly soluble in dilute acid and its bioavailability is low [5, 9]. However, FePP tends to have less effect on the sensory qualities of food than other fortificants [10]. Most commercially available elemental iron fortificants, including EFe, are water insoluble, poorly soluble in dilute acid [5]. EFe has a bioavailability that is up to 75% that of $FeSO_4$, which is high compared to other elemental fortificants, though particle size plays a role in the bioavailability of elemental fortificants [5, 11]. Recently, NaFeEDTA, a water-soluble iron compound, has been approved for use as a fortificant because of its promising effectiveness [5]. It is able to counteract the inhibitory effect on iron absorption

of phytic acid, which is present in whole wheat flour. The absorption of iron from NaFeEDTA when added to high phytate foods is 2–3 times greater than that of $FeSO_4$, and it also does not promote lipid oxidation in foods.

In terms of cost, the most inexpensive food-grade iron is hydrogen-reduced iron, followed by EFe, $FeSO_4$, FeFum, FePP, and finally NaFeEDTA [5]. However, the extent to which a national or regional food supply is fortified with iron varies considerably [12]. In a mandatory program, governments stipulate which iron fortificants are permitted, but in voluntary programs, industries may use the cheapest source of iron, which likely have low bioavailability. The public health impact of iron fortification programs depends on the amount and bioavailability of the iron fortificants added to foods. Although most countries rely on the 2006 WHO fortification guidelines, iron fortification programs appear to have marginal effects on reducing the burden of IDA, particularly in developing countries, due to lack of legislation and oversight of fortification programs [12]. Given the wide variety of iron fortificants, the ability to rapidly identify iron compounds in fortified foods allows program managers to readily determine if the fortified food complies with the technical specifications and is an objective measurement of program performance. In practice, the performance, complexity, and cost of fortification methods will depend on factors including food matrix, iron fortificant used, and levels of food enforcement desired. The objective of this study was to provide a cost-effective, rapid, and accurate test to identify and quantify iron fortificants in flour.

Methods

Flour, iron compounds, and chemicals

Whole wheat flour was obtained from a local market in India. Refined wheat flour and yellow corn flour were obtained from Archer Daniels Midland Company (Overland Park, KS,

USA and Jackson, TN, USA respectively). All iron compounds (electrolytic iron (EFe), sodium ferric ethylenediaminetetraacetate (NaFeEDTA), ferric pyrophosphate (FePP), ferrous sulfate ($FeSO_4$), ferrous fumarate (FeFum), and ferrous citrate (FeCit)) were obtained from Dr. Paul Lohmann (Emmerthal, Germany). L-ascorbic acid (ASC), sodium acetate trihydrate, hydrogen peroxide (H_2O_2), hydrochloric acid (HCl), and trichloroacetic acid (TCA) were from Fisher Scientific (Chicago, IL, USA). Ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt), potassium thiocyanate (KSCN), and thioglycolic acid (TGA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Iron standard solution for AAS was obtained from Fluka (Buchs, Switzerland).

Method and fortification of flour

The method presented here uses four steps (Figure 3.1). In step 1, iron fortificants were identified as ferrous, ferric, or electrolytic. In step 2, fortificants identified in step 1 as ferric were further tested to identify FePP and NaFeEDTA. In step 3, fortificants identified in step 1 as ferrous were further tested to identify $FeSO_4$, FeFum, and FeCit. In step 4, iron was quantified.

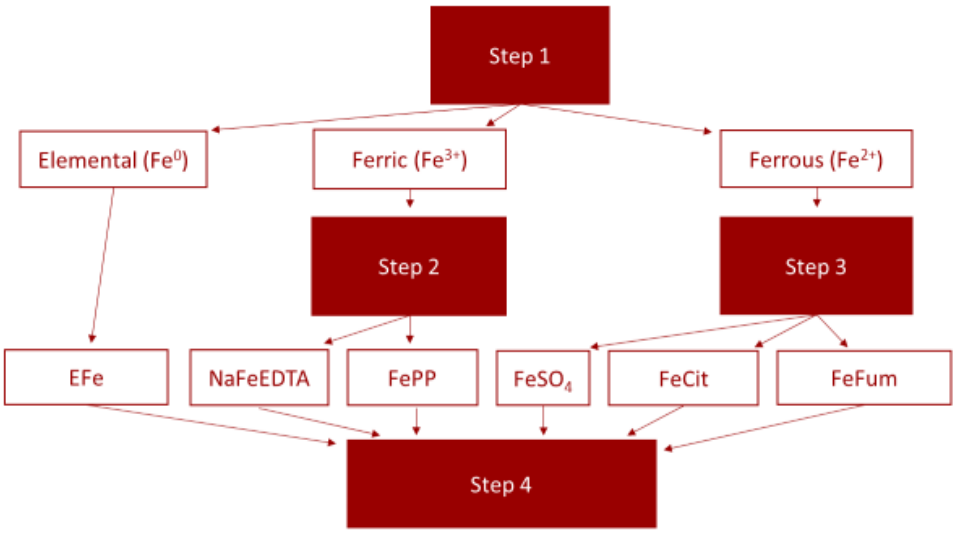

Figure 3.1. Method overview. In step 1, oxidation state (ferrous, ferric, or elemental) was identified. In step 2, FePP and NaFeEDTA were identified. In step 3, $FeSO_4$, FeFum, and FeCit were identified. In step 4, iron was quantified.

For use in development of steps 1–3, refined wheat, whole wheat, and yellow corn flour was fortified with NaFeEDTA, FePP, $FeSO_4$, FeCit, and FeFum to achieve 40 mg Fe/kg, and with EFe to achieve 60 mg Fe/kg iron (Table 3.1), based on WHO recommendations. For blinded sample testing of steps 1–3, four samples (A, B, C, and D) were prepared, and an additional blinded sample (E) was added for blinded testing for step 4. Type of fortificant and iron levels were randomly selected for samples A–D by assigning each fortificant (EFe, FePP, NaFeEDTA, FeFum, $FeSO_4$, FeCit) a number between 1–6, and each iron level (20, 25, 30, 35, 40, 45, 50, 55, and 60 mg Fe/kg) a number between 1–9. Numbers were randomly selected to determine iron levels and fortificants to be added to flour. For sample E, $FeSO_4$ was intentionally selected, but iron level was determined randomly. The resulting blinded samples are presented in Table 3.2. These samples were made with refined wheat flour. After iron was added, flour samples were mixed for at least 10 min using a hand crank mixer (OXO Softworks Egg Beater) and stored in airtight resealable plastic bags. Iron levels of all samples were verified using the established iron determination method (long method) described on pages 45–46.

Table 3.1. Sample preparation for method development

Fortificant	mg Fe/kg flour	Flour (g)*	Fe (%)**	Fortificant (g)	Total Fe (mg)
EFe	60	100	99.6	0.006	6.0
NaFeEDTA	40	100	13.2	0.030	4.0
FePP	40	100	24.8	0.016	4.0
$FeSO_4$	40	100	32.7	0.012	4.0
FeFum	40	100	32.9	0.012	4.0
FeCit	40	100	21.0	0.019	4.0

*Refined wheat, whole wheat, and yellow corn flour
**Percent of iron in fortificant based on certificate of analysis, except for FeFum where chemical formula was used

Table 3.2. Blinded sample preparation

Sample	Fortificant	mg Fe/kg flour	Flour (g)*	Fe (%)**	Fortificant (g)	Total Fe (mg)
A	FePP	45	100	24.8	0.018	4.5
B	NaFeEDTA	20	100	13.2	0.015	2.0
C	FeFum	50	100	32.9	0.015	5.0
D	EFe	35	100	99.6	0.004	3.5
E	$FeSO_4$	60	100	32.7	0.018	6.0

*Refined wheat, whole wheat, and yellow corn flour
**Percent of iron in fortificant based on certificate of analysis, except for FeFum where chemical formula was used

Step 1: Identification of ferrous, ferric, and electrolytic fortificants

American Association of Cereal Chemists (AACC) method 40-40 [13] was modified to differentiate between ferrous and ferric fortificants, and EFe. A small amount of flour (~0.3 g) was mixed with 2 mL of 3 N HCl. One mL of 10% (w/v) KSCN was added to each sample and color developed for 10 min. One mL 3% (v/v) H_2O_2 was then added and color changes and specks were recorded. Samples that produced a dark pink or red color before the addition of H_2O_2 were identified as ferric and were subjected to step 2. Samples that produced a dark pink or red color only after the addition of H_2O_2 and did not have red specks were identified as ferrous and were subjected to step 3. EFe was identified if thin red specks were formed after addition of H_2O_2. The presence of EFe was also verified by running a magnet (Neodymium N52 grade) through a ~100 g flour sample and observing iron fragments on the magnet.

Step 2: Identification of sodium ferric EDTA and ferric pyrophosphate

Flours fortified with ferric fortificants identified in step 1 were further tested to differentiate between NaFeEDTA and FePP based on their solubility in water. Briefly, a small amount of flour (~0.3 g) and 2 mL iron free water were mixed for 30 min. One mL working ferrozine was added, which was prepared by mixing 1 part 0.25% (w/v) stock ferrozine solution,

5 parts water, and 5 parts 61.24% (w/v) sodium acetate trihydrate solution. After 10 min, 1 mL 5% (w/v) ASC was added and color was again allowed to develop for 10 min. If little to no color developed, the fortificant was identified as FePP, but if a strong purple color developed the fortificant was identified as NaFeEDTA. All tests were carried out in triplicates.

Step 3: Identification of ferrous sulfate, ferrous fumarate, and ferrous citrate

Flours fortified with ferrous fortificants identified in step 1 were further tested to differentiate between $FeSO_4$, FeCit, and FeFum. Again, a small amount of flour (~0.3 g) and 2 mL iron free water were mixed for 30 min. One mL working ferrozine, as described in step 2, was added and color developed for 10 min. At this stage, $FeSO_4$ and FeFum did not react to produce color, but FeCit reacted with ferrozine to produce a light purple color. To further differentiate $FeSO_4$ and FeFum, 1 mL 5% (w/v) ASC was added and color was again allowed to develop for 10 min. If a purple color developed, the iron was identified as $FeSO_4$, and if no color developed, the iron was identified as FeFum. Again, all tests were carried out in triplicates.

Step 4: Quantification of iron fortificants

Long method

Iron levels of all samples were verified using an established protocol [14]. In short, 0.1 g flour was mixed in 1 mL 10% (w/v) TCA in 3 N HCl and incubated at 65°C for 20 hours, cooled, and centrifuged at 750 × g for 15 min. Stock iron standard solutions were prepared by diluting 1 mg Fe/mL stock iron solution to 10, 20, 30, 40, 50, 60, 80, and 100 µg Fe/mL using iron free water. For standards without flour, these were further diluted to 1, 2, 3, 4, 5, 6, 8, and 10 µg Fe/mL using 10% (w/v) TCA in 3 N HCl. For standards with flour, 0.1 ± 0.01 g flour was weighed, 0.1 mL iron standard solution and 0.9 mL 10% (w/v) TCA in 3 N HCl were added, standards were incubated at 65°C for 20 hours, cooled, and centrifuged at 750 × g for 15 min.

Ferrozine chromogen was prepared by mixing 1 part 0.25% (w/v) ferrozine 1% TGA with 5 parts 61.24% (w/v) sodium acetate trihydrate and 5 parts water. Thirty µL standard or sample supernatant and 270 µL ferrozine chromogen were added to a ninety-six well microplate. After 10 min, absorbance was measured at 563 nm and iron levels were calculated based on the linear curve generated from standards.

Short method

To reduce time needed to perform the quantification assay, an established method for serum iron determination with modifications was used for flour iron determination [15]. A small amount of flour sample (~1 g) was placed in a 15 mL screw top centrifuge tube. One mL water and 9 mL 10% (w/v) TCA 3 N HCl were added. Samples were vortexed for 45 seconds and placed in a boiling water bath for 5 min. Samples were cooled and centrifuged for 15 min at 3200 × g to obtain clear supernatants.

For standards, a small amount of flour (~1 g) was placed in a 15 mL screw top centrifuge tube. Nine stock iron standard solutions were made by diluting 1 mg Fe/mL stock iron with water. Final concentrations of stock iron standard solutions were 10, 20, 30, 40, 50, 60, 80, and 100 µg Fe/mL. For each of the nine standards, 1 mL stock iron solution, and 9 mL 10% (w/v) TCA 3 N HCL of iron were added to ~1 g flour and were processed like samples. An additional set of standards were made that did not use flour. These were prepared by diluting 1 mL of stock iron with 9 mL 10% (w/v) TCA. Final concentrations of both standard curves were 1, 2, 3, 4, 5, 6, 8, and 10 µg Fe/mL, not including the iron contributed from the unfortified flour. Working ferrozine used in this step was slightly modified and consisted of 0.025% (w/v) ferrozine, 0.1% (v/v) TGA, and 61.2% (w/v) sodium acetate trihydrate. To accurately analyze iron level, 150 µL sample and 150 µL working ferrozine were mixed, color developed for 10 min, absorbance was

measured at 562 nm, and iron levels were calculated based on the linear curve generated from standards.

Visual quantification

To quantify visually, samples and standards were prepared using the short method and 1 mL of each sample or standard supernatant was added to a clear glass test tube. One mL working ferrozine was added and color developed for 10 min. Color intensity of samples was compared visually to color intensities of both standards (with and without flour) to estimate iron level.

Statistical analysis

Iron levels were reported as mean ± SD. Differences of mean values for each fortificant using three methods (separately with and without flour) were assessed using ANOVA with Tukey multiple comparisons and differences were considered significant at $p < 0.05$. Two tailed t-tests were performed to compare actual sample iron levels to intended iron levels (hypothesized mean); results were considered significant if $p < 0.05$. All statistical analyses were performed in JMP Pro 14 statistical software from SAS.

Alternative methods for fortificant identification and quantification

An alternative method was also developed for identification and quantification of EFe, NaFeEDTA, FePP, $FeSO_4$, and FeFum that requires only KSCN alone as a chromogen, due to difficulties acquiring ferrozine in some developing countries. Step 1 is the same as described above. In step 2, a small amount of flour (~0.3 g) was mixed in 2 mL water for 10 min. The flour mixture was then allowed to stand for 10 min or until the liquid on top was clear. One mL supernatant was transferred to a new dish. KSCN solution (10% w/v in 3 N HCl) was prepared immediately before use, 1 mL was added to flour supernatant, and color was recorded. In step 3, a small amount of flour (~0.3 g) was mixed in 2 mL 0.01 N HCl along with 1 mL 3% (v/v) H_2O_2.

This was mixed for 10 min and allowed to stand for 10 min or until the liquid on top was clear. One mL supernatant was removed and 1 mL 10% (w/v) KSCN in 3 N HCl prepared immediately before use was added. Color changes and the formation of red specks were recorded. In step 4, a small amount (~0.3 g) of sample flour and flour standards with 20, 30, 40, 50, and 60 mg Fe/kg iron were mixed in 4 mL 3 N HCl with 1 mL 3% (v/v) H_2O_2. Color intensity of samples was compared with color intensity of standards after 10 min after adding 1 mL 10% (w/v) KSCN.

Results and Discussion

Step 1: Identification of ferrous, ferric, and electrolytic fortificants

Taking advantage of chromogen reactivity with ferric and ferrous (KSCN and ferrozine) iron, solubility characteristics of iron fortificants, and using reducing and oxidizing agents (ASC and H_2O_2), we developed a step by step process to identify iron fortificants used to fortify flour. The first step differentiated ferric and ferrous fortificants, and also identified EFe. Testing with KSCN in an acidic solution has been used routinely in flour mills for at least 50 years to identify ferric and ferrous fortificants [13]. However, this method is not useful for identifying specific iron fortificants. KSCN can react only with ferric iron in an acidic condition to form a red colored complex. In the first step, as expected, a strong red color developed with NaFeEDTA and FePP samples when KSCN was added. After adding H_2O_2 to convert iron from ferrous to ferric, ferrous fortificants developed a uniform red color, as expected, while EFe formed thin red specks, which is likely due to its poor solubility. EFe was also confirmed using a magnet, but it was difficult to observe iron fragments on the magnet because of the low amount of iron added to fortified flour and because the iron particles are very small. This test could be improved by using a large amount of flour and utilizing a magnifying glass. The results of step 1 are summarized in Table 3.3.

Table 3.3. Results from steps 1–3 for identification of iron fortificants

Fortificant	Oxidation State Test KSCN and 3 N HCl		Magnetic	Solubility Test Ferrozine and water	
	- H_2O_2	+ H_2O_2		- ASC	+ ASC
NaFeEDTA	Dark	Dark	No	No color	Dark
FePP	Dark	Dark	No	No color	No color or light
EFe	No color or light	Dark with red specks	Yes		
$FeSO_4$	No color or light	Dark	No	No color or light	Dark
FeCit	No color or light	Dark	No	Medium color	Dark
FeFum	No color or light	Dark	No	No color	No color or light

Results were similar for all flour tested (refined wheat, whole wheat, and yellow corn flour), but whole wheat flour samples generally produced darker color than refined wheat and yellow corn flours.

Step 2: Identification of sodium ferric EDTA and ferric pyrophosphate

Because of the low solubility of FePP in water compared to NaFeEDTA, we expected to see more color development with NaFeEDTA when NaFeEDTA fortified flour was mixed with KSCN in water instead of acid. However, adding KSCN to ferric fortified flour mixed in water did not produce color, which may be due to the inability of KSCN to react with ferric iron at a neutral pH. Because ferrozine works at a wider pH range including at a neutral pH [16], we were able to use ferrozine to test the solubility of fortificants in water. Due to ferrozine reactivity with only ferrous iron, adding ASC to reduce iron allowed us to use ferrozine with ferric fortificants. As expected, NaFeEDTA produced a more intense purple color with ferrozine plus ASC than FePP because of NaFeEDTA's solubility in water. The results of step 2 are also presented in Table 3.3.

Step 3: Identification of ferrous sulfate, ferrous fumarate, and ferrous citrate

To differentiate the three ferrous fortificants, we utilized ferrozine, with and without ASC, using the flours mixed in water. Before adding ASC, only FeCit reacted with ferrozine. After adding ASC, both FeCit and $FeSO_4$ reacted with ferrozine to produce a purple color. These results may be due to the solubility and stability of $FeSO_4$ and the FeCit complex in water. We believe that, when dissolved at a neutral pH, $FeSO_4$ dissociates. Free ferrous iron in a neutral pH is oxidized to ferric [17] meaning the iron from $FeSO_4$ is unable to react with ferrozine. This is supported by the fact that adding ASC to $FeSO_4$ samples, which converts the ferric iron to ferrous, caused the $FeSO_4$ samples to then produce dark purple color with ferrozine. However, we believe that FeCit when dissolved in water does not dissociate to the same degree as $FeSO_4$ and the iron from FeCit is protected and is not oxidized allowing it to react with ferrozine. As expected, FeFum did not react with ferrozine and ASC due to its poor solubility in water. In this step, we were able to differentiate all three ferrous fortificants and the results of step 3 are presented in Table 3.3. If FeCit, which is not commonly used for fortification, is not included as a sample, adding ferrozine and ASC together in a single step will be enough to identify $FeSO_4$ and FeFum.

Step 4: Quantification of iron fortificants

Results from the long method with spectrophotometer, short method with spectrophotometer, and short method with visual analysis, each with the two sets of standards, for samples A–E are summarized in Figure 3.2. To assure that the presence of flour was not interfering with measurements, analyses where performed using standards with and without flour. Visual analysis is purely qualitative and comparing those values to more accurate results attained with a spectrophotometer assures that our quick method will provide accurate results in

a field setting. For each sample, the mean iron level using each of the three methods and measured against two standards (with and without flour) were not significantly different from each other ($p > 0.05$), suggesting that our visual method can accurately quantify iron fortificants in flour.

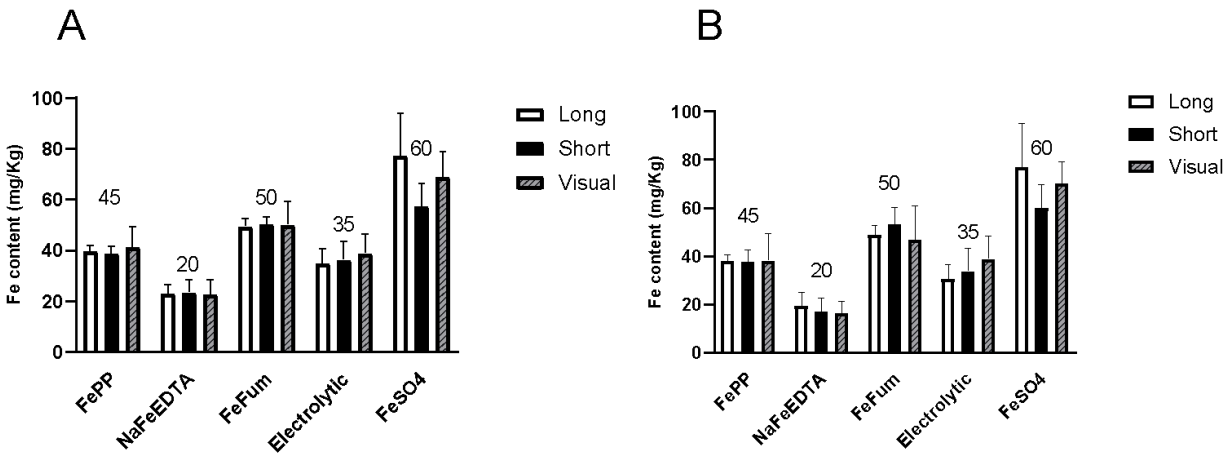

Figure 3.2. Comparison of quantification methods. Quantification methods using 3 different methods that are measured against standards made without (A) and with (B) flour. Values are means ± SD and values above bars are intended iron levels. For long and short methods with spectrophotometer n = 5–7. For visual method, means represent values measured by 4 personnel who analyzed each sample in duplicate (n = 8). Short method with visual analysis and spectrophotometer analyses are as accurate at estimating iron levels as the long method. Within each sample, means among the methods are not significantly different (P = 0.05).

For sample A, intended and actual iron level were significantly different (P = 0.05). For samples B–E, intended and actual iron levels were not significantly different (P > 0.05). Fortifying flour in a lab setting is likely different than fortification in an industrial setting due to differences in scale. Mixing with a hand mixer for 10 minutes was likely insufficient in creating homogenous samples. Because of this and the small size of samples, there were large standard deviations for some samples tested with the long method.

Identification and quantification of blinded samples

We were able to differentiate six iron fortificants with simple methods using only a few reagents. This method worked with fortified wheat flour, refined wheat flour, and yellow corn flour suggesting usefulness of this for universal testing of many flour types. To assure the reliability and reproducibility of our methods, blinded samples (A, B, C, and D) were tested by four laboratory personnel. All four personnel correctly identified the iron fortificant used in all four blinded fortified flour samples using steps 1–3.

Samples A–E were processed via step 4 and four laboratory personnel quantified the iron by visually comparing the color intensity of samples in duplicate to the color intensity of standards with and without flour. Duplicates from the reported iron level for each sample were averaged and data for each sample was combined (n = 20). When standards without flour were used, the averaged reported iron values were 85% of the time within 10 mg Fe/kg and 70% of the time within 5 mg Fe/kg of the actual iron level of samples determined with the long method using standards without flour (Table 3.4). The values were lower when standards with flour were used. Averaged reported iron values were 60% of the time within 10 mg Fe/kg and 45% of the time within 5 mg Fe/kg of the actual iron level (Table 3.4). These results suggest that when comparing the color intensity visually for quantification, standards should be prepared without flour for more accurate and precise results. Additionally, some fortificants were more accurately predicted than others. When using standards without flour, the average reported iron level for each person from NaFeEDTA samples was within 5 mg Fe/kg of the actual iron level 100% of the time, 75% of the time for FePP, EFe, FeFum, and 25% of the time for $FeSO_4$. For all samples, the average reported iron level using standards without flour was within 10 mg Fe/kg of

the actual iron level between 75–100% of the time, except for sample E ($FeSO_4$), which was within 10 mg Fe/kg only 50% of the time.

Table 3.4. Visual analysis method

	Standards without flour			Standards with flour		
Sample (Fortificant)	Within 5 mg Fe/kg of actual*	Within 10 mg Fe/kg of actual*	Within 15 mg Fe/kg of actual*	Within 5 mg Fe/kg of actual*	Within 10 mg Fe/kg of actual*	Within 15 mg Fe/kg of actual*
A (FePP)	3/4	4/4	4/4	2/4	2/4	4/4
B (NaFeEDTA)	4/4	4/4	4/4	2/4	3/4	4/4
C (FeFum)	3/4	3/4	4/4	2/4	2/4	3/4
D (EFe)	3/4	4/4	4/4	1/4	3/4	4/4
E ($FeSO_4$)	1/4	2/4	3/4	2/4	2/4	3/4
Total	14/20	17/20	19/20	9/20	12/20	18/20

Fraction of laboratory personnel whose average reported iron level from blinded sample testing using the short method with visual analysis was within 5, 10, and 15 mg Fe/kg of the sample's actual iron level (n=4).
*Actual iron level of each sample determined via the long method using standards without flour rounded to the nearest whole number.

Alternative methods for fortificant identification

In the alternative methods we utilized an extraction step to extract iron that is soluble in water or dilute acid. We then added KSCN dissolved in 3 N HCl to produce color. In step 2 of the alternative methods, NaFeEDTA produced a dark color with KSCN, while FePP produced little or no color, likely due to NaFeEDTA being more soluble in water than FePP. In step 3 of the alternative methods, $FeSO_4$ produced no specks or large red specks, while FeFum produced many small red specks, likely due to differences in particle size. For quantification, similar to the ferrozine method, we were able to quantitate the iron level of unknown samples when standards of the same flour type were used and prepared in the same manner as samples.

Comparison to other identification and quantification methods

To our knowledge no other methods exist that allow for the identification of six iron fortificants without the use of analytical instruments. The AACC method 40-40 [13] and variations of it have been able to differentiate ferrous and ferric fortificants, but not identify fortificants more specifically. Other methods that utilize potassium ferricyanide in conjunction with a magnet are able to identify $FeSO_4$, FeFum NaFeEDTA, and elemental iron [18]. Many methods exist for quantification of iron in foods, many of which utilize atomic absorption spectrometry (AAS), a spectrophotometer, or photoelectric colorimeter, which are used along with iron chromogens like ferrozine, KSCN, bathophenanthroline (4,7-Diphenyl-1,10-phenanthroline), and 2,2′-Bipyridine [14, 19–21]. In many methods, the process for preparing samples can be time consuming and may require ashing of samples [14, 20]. Spectrophotometers, as well as instruments for AAS, can be expensive and not portable. A portable photometer like the iCheck handheld photometer from BioAnalyt could be a potential solution, but the photometer and its required kits can still be costly. An even more affordable alternative for quantification uses a smartphone camera and application to quantify iron based on the color intensity using ferrozine [22]. These methods, however, appear to only have been used with $FeSO_4$ and FeFum, while our methods for quantification have been tested with EFe, FePP, NaFeEDTA, $FeSO_4$, and FeFum.

Limitations and future research

This work presents a novel method for the identification and visual quantification of iron fortificants. The ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end users) criteria can be used to assess the usefulness of diagnostic and analytical tools [23]. The method described here is user-friendly in that it requires

training in only basic laboratory techniques. The method is also affordable and equipment-free in that it does not require costly analytical tools like spectrophotometers, except an analytical balance. A centrifuge is also used in step 4 of these methods. Future work could standardize a method in which gravity is used for this step instead of a centrifuge or where separation is not required. Lastly, because these methods rely on visual analysis for both identification and quantification, we recognize that those with visual impairments or who are color blind may be unable to use these methods. Future methods could be developed that utilize a smartphone camera and application to judge color and color intensity similar to those presented in Waller et al. [22].

Conclusions

Using the knowledge of simple iron chemistry, like solubility and oxidation states, and using appropriate iron chromogens, we developed an inexpensive, rapid, and reliable qualitative test to identify iron fortificants added to flour. Additionally, modifying existing iron quantification protocols allowed us to develop methods to quantify iron visually. While we were able to identify and quantify iron fortificants using only KSCN as a chromogen, we recommend using our methods with both KSCN and ferrozine as the iron-ferrozine complex and its resulting color are more stable than the color produced by iron-SCN complex, and the method with ferrozine does not require the additional extraction step. Additionally, we developed methods that can be used to quantify iron visually, though we recommend using a spectrophotometer or other quantitative equipment if available for more accurate and reliable results. We also recommend using standards that do not contain flour for more accurate quantification of total sample iron. Still, our methods can be easily used for monitoring and evaluation of iron fortification programs, especially in low income countries.

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CHAPTER 4. GENERAL CONCLUSIONS

The research presented here focused on the development of inexpensive, rapid, and reliable methods for the identification of iron fortificants in flour. Iron is the most common nutrient deficiency globally and cereal flours are commonly fortified with iron, but without appropriate methods to identify and quantify iron compounds in flour, oversight of fortification programs is not possible.

We were able to develop methods that can identify six of the most commonly used iron fortificants in flour: EFe, $FeSO_4$, FeFum, FeCit, NaFeEDTA, and FePP. When lab assistants tested blinded flour samples containing either EFe, FeFum, NaFeEDTA, or FePP, they were able to correctly identify the fortificant 100% of the time. When quantifying iron from samples with EFe, $FeSO_4$, FeFum, FeCit, NaFeEDTA, or FePP they were able to correctly identify the iron level of the sample within 5 mg Fe/kg of the actual level 70% of the time and within 10 mg Fe/kg of the actual iron level 85% of the time. Additionally, we were able to develop alternative methods using only KSCN as a chromogen that are able to identify and quantify iron fortificants in flour. Lastly, we had some success being able to identify iron samples that contained more than one fortificant (Appendix A). Future research should focus on refining the quantification protocols to produce more accurate results and that do not require the use of a centrifuge so they can be used in a field setting. Additionally, quantification methods should be developed using a smartphone camera and application which are able to quantify all iron fortificants.

APPENDIX A. METHODS FOR IDENTIFICATION OF MORE THAN ONE FORTIFICANT IN A SINGLE SAMPLE

Occasionally, more than one fortificant is used to fortify a single batch of flour. This may be done because it is mandated in a fortification program, as in Venezuela, where precooked corn flour is fortified with 30 mg Fe/kg of iron as ferrous fumarate (FeFum) and 20 mg Fe/kg of electrolytic iron (EFe) [1]. This is done to lessen the organoleptic problems that would be caused by adding 50 mg Fe/kg iron as only FeFum. Mixing inexpensive low bioavailable iron fortificants with costly high bioavailability fortificants may also be done to save money. Due to this, a method is needed that can identify more than one iron fortificant in a single sample.

Methods

Mixed samples were prepared to create samples with 40 mg Fe/kg iron total with 20 mg Fe/kg as EFe and 20 mg Fe/kg iron as either FeFum or NaFeEDTA. These samples were not tested blindly, and they were not tested quantitatively except to verify accuracy of fortification using the long method as described in chapter 3. Preparation of standards with 40 mg Fe/kg added iron as NaFeEDTA, FeFum, and EFe is described in chapter 3.

Samples with NaFeEDTA + EFe and controls with 40 mg Fe/kg NaFeEDTA and 40 mg Fe/kg EFe were subjected to steps 1 and 2 of our identification protocol as described in chapter 3. Samples with FeFum + EFe and controls with 40 mg Fe/kg FeFum, and 40 mg Fe/kg EFe were subjected to steps 1 and 3 as described in chapter 3. All tests were performed in triplicate.

Results and Discussion

In step 1, before adding H_2O_2, NaFeEDTA produced a red color, NaFeEDTA + EFe produced a dark pink color, and EFe, FeFum, and FeFum + EFe produced a light pink color. After adding H_2O_2, NaFeEDTA maintained a dark pink color, NaFeEDTA + EFe became

slightly lighter and developed red specks, EFe and FeFum + EFe became slightly darker and developed red specks, and FeFum became darker. In step 2 before adding ASC, NaFeEDTA, EFe, and NaFeEDTA + EFe all had no color. After adding ASC, NaFeEDTA produced a very dark purple color, NaFeEDTA + EFe produced a dark purple color, and EFe produced only a light purple color. In step 3 before adding ASC, EFe, FeFum, and FeFum + EFe, all had no color. After adding ASC, they all produced a light purple color. Results for steps 1–3 are in table A-1.

Table A-1. Steps 1–3 on samples with more than one fortificant

Step		NaFeEDTA	NaFeEDTA + EFe	EFe	FeFum + EFe	FeFum
Step 1	Before H_2O_2	Dark pink	Pink	Light pink	Light pink	Light pink
	After H_2O_2	Dark pink	Pink with red specks	Pink with red specks	Pink with red specks	Pink
Step 2	Before ASC	No color	No color	No color	———	———
	After ASC	Very dark purple	Dark purple	Light purple	———	———
Step 3	Before ASC	———	———	No color	No color	No color
	After ASC	———	———	Light purple	Light purple	Light purple

Samples with a single fortificant (NaFeEDTA, EFe, or FeFum) were fortified with 40 mg Fe/kg added iron. Samples with two fortificants (NaFeEDTA+EFe and FeFum+EFe) were fortified with 20 mg Fe/kg added iron from EFe and 20 mg Fe/kg added iron from either NaFeEDTA or FeFum.

When controls with 40 mg Fe/kg NaFeEDTA, 40 mg Fe/kg FeFum, and 40 mg Fe/kg EFe were used alongside samples for comparison, samples with NaFeEDTA + EFe could be identified. In step 1, NaFeEDTA + EFe formed a dark color with KSCN before the addition of

H_2O_2 indicating the presence of a ferric iron compound. After adding H_2O_2, red specks are formed indicating the additional presence of EFe. In step 2, a dark purple color is formed with ferrozine and ASC indicating the presence of a soluble iron compound like NaFeEDTA. Samples with FeFum + EFe could not be identified. In step 1, samples with FeFum + EFe can be identified as containing EFe iron because of the red specks. In step 3 however, FeFum and EFe produced the same color.

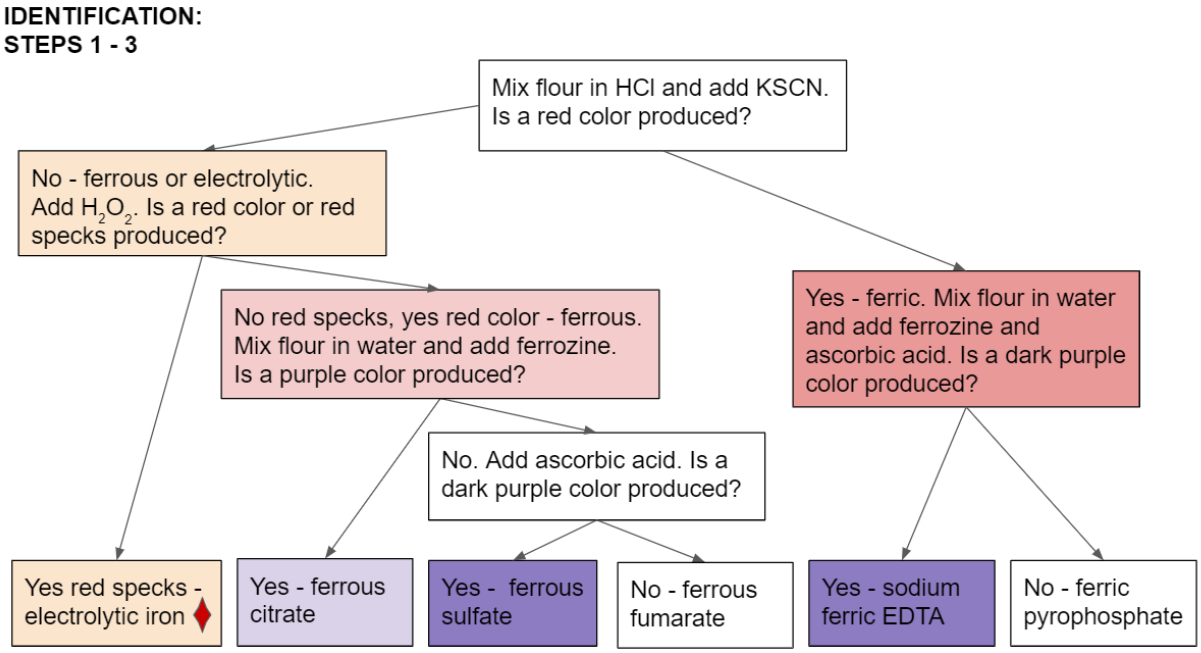
Conclusions

Using steps 1–3, samples that contain both NaFeEDTA and EFe can be identified as having these two iron fortificants. However, because EFe and FeFum produce very similar results in our methods, only being differentiated by the red specks produced by EFe, a sample that contained both EFe and FeFum would likely be misidentified as just having EFe unless it was already known that the sample contained two iron fortificants.

Reference

1. García-Casal, M.N.; Layrisse, M. Iron fortification of flours in Venezuela. *Nutr Rev* **2002**, *60*, S26–S29.

APPENDIX B. COMPLETE INSTRUCTIONS FOR IDENTIFICATION AND QUANTIFCATION



Notes

- Identification methods have been tested with whole wheat, refined wheat, and yellow corn flour.
- In general, flour is fortified with 40 ppm iron, except for electrolytic Fe with 60 ppm. These methods have been tested with 6 fortificants with iron levels between 20–60 ppm (ppm = mg Fe/kg).

Fortificant Abbreviations:

NaFeEDTA: Sodium ferric ethylenediaminetetraacetate

FePP: Ferric pyrophosphate

EFe: Electrolytic iron

FeCit: Ferrous citrate

FeFum: Ferrous fumarate

$FeSO_4$: Ferrous sulfate

Steps

Step 1: Differentiates by oxidation state

Identifies Ferrous (Fe^{2+}), Ferric ($Fe3^{+}$), and Elemental (Fe^{0})

Step 2: Differentiates ferric fortificants by solubility in water

Identifies NaFeEDTA and FePP

Step 3: Differentiates ferrous fortificants by solubility in water

Identifies $FeSO_4$, FeFum, and FeCit

Step 4: Quantification

Supplies Needed for Identification

- Iron free measuring spoons that measure ~0.3 g flour (slightly less than 1/8 tsp.)
- 12 well plates or other small dishes that hold ~5–10 mL
- Pipettes that can accurately measure 100 µL and 1000 µL
- Volumetric flasks with 1000 mL and 50 mL volumes (all solutions are 50 or 1000 mL but volumes can be adjusted as needed)
- Hot plate
- Scale or balance

Chemicals for Identification

- Iron free water
- Concentrated hydrochloric acid (12.1 N)
- Potassium thiocyanate (FW = 97.18 g/mol) or ammonium thiocyanate (FW = 76.12 g/mol)
- Hydrogen peroxide (H_2O_2)
- Ferrozine 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate (FW = 492.46 g/mol)
- Sodium Acetate Trihydrate (FW = 136.08 g/mol)
- L-Ascorbic Acid (FW = 176.13 g/mol)

Additional Chemicals for Quantification

- Trichloroacetic acid (FW = 163.38 g/mol)
- Thioglycolic acid (FW = 92.11 g/mol)

QS = quantity sufficient (fill to line on volumetric flask)

Solutions Needed for Identification

Hydrochloric acid solution (3 N)
In a 1 L volumetric flask add ~500 mL iron free water. Add 250 mL concentrated hydrochloric acid (12.1 N). QS to 1000 mL.

Thiocyanate Solution (1 M)
Add 5.0 g potassium thiocyanate or 3.9 g ammonium thiocyanate to a 50 mL volumetric flask. QS to 50 mL. Make fresh on day of assay. 1 M KSCN = 10% (w/v) KSCN

Hydrogen Peroxide (3% v/v)
Can purchase at 3% (over the counter hydrogen peroxide is 3%). Can also buy 30% hydrogen peroxide and dilute. Add 100 mL 30% hydrogen peroxide to 1000 mL volumetric flask and QS to 1000 mL.

Stock Ferrozine
Add 0.125 g ferrozine to a 50 mL volumetric flask and QS to 50 mL. Can keep in dark (wrap it with foil) for 2 weeks in the refrigerator.

Saturated Sodium Acetate (4.5 M)
Add 30.62 g sodium acetate trihydrate to 50 mL volumetric flask and QS to 50 mL with iron free water. Heat on hot plate to dissolve. Crystals should form after the solution cools to room temp.

Working Ferrozine
Mix together 10 mL saturated sodium acetate, 10 mL iron free water, and 2 mL stock ferrozine. Make fresh on day of assay.

Ascorbic Acid (ASC) (5% w/v)
Add 2.5 g to a 50 mL volumetric flask. QS to 50 mL with iron free water. Make fresh on day of assay.

Step 1. Identifies EFe, Ferric, and Ferrous

1. Add ~0.3 g flour per well x 3 replicates for each flour sample
2. Add 2 mL 3 N HCl to each well
3. Few minutes on shaker (could also mix by hand) until flour is well mixed
4. Add 1 mL 10% (w/v) KSCN to each well
5. Allow color to develop 10 minutes
6. Record color (red/pink) in table below
7. Add 1 mL 3% (v/v) H_2O_2 to each well
8. Record color (red/pink) in table below

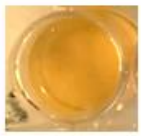
	H_2O_2	NaFeEDTA	FePP	EFe	FeCit	FeFum	$FeSO_4$
Replicate 1	-	Color	Color	No color	No color	No color	No color
	+	Color	Color	Color with red specks	Color	Color	Color
Replicate 2	-	Color	Color	No color	No color	No color	No color
	+	Color	Color	Color with red specks	Color	Color	Color
Replicate 3	-	Color	Color	No color	No color	No color	No color
	+	Color	Color	Color with red specks	Color	Color	Color

Example table with expected color for each fortificant.

Step 1 Expected Results Before Adding H_2O_2

Ferric fortificants → red color

EFe and ferrous fortificants → no color



EFe

FePP

NaFeEDTA

$FeSO_4$

FeCit

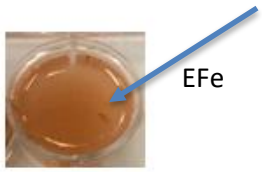
FeFum

Step 1 Expected Results After Adding H_2O_2

EFe → forms red color with specks

Ferrous fortificants → red color

Ferric fortificants → no change or lighter



EFe

FePP

NaFeEDTA

$FeSO_4$

FeCit

FeFum

Step 2. Identifies NaFeEDTA and FePP

1. Add ~0.3 g ferric flour per well x 3 replicates for each flour sample
2. Add 2 mL water to each well
3. Mix on shaker for 30 minutes
   - Alternatively, mix with nonmetal utensil and allow to sit for 30 minutes
4. Add 1 mL working ferrozine to each well
5. Allow color to develop 10 minutes
6. Record color (purple) in table below
7. Add 1 mL 5% (w/v) ascorbic acid to each well
8. Allow color to develop 10 minutes
9. Record color (purple) in table below

	ASC	NaFeEDTA	FePP
Replicate 1	-	No color	No color
	+	Color	No color
Replicate 2	-	No color	No color
	+	Color	No color
Replicate 3	-	No color	No color
	+	Color	No color

Example table with expected color for each fortificant.

Step 2 Expected Results After Adding ASC

NaFeEDTA → dark color (because it is water soluble)

FePP → no or light color (will likely not be completely white or clear due to some iron from fortificant or flour being soluble in water)

FePP

NaFeEDTA

Step 3. Identifies $FeSO_4$, FeFum, and FeCit

1. Add ~0.3 g ferrous flour per well x 3 replicates for each flour sample
2. Add 2 mL water to each well
3. Mix on shaker for 30 minutes (timing is important)
    - Alternatively, mix with nonmetal utensil and allow to sit for 30 minutes
4. Add 1 mL working ferrozine to each well
5. Allow color to develop 10 minutes
6. Record color (purple) in table below
7. Add 1 mL 5% (w/v) ASC to each well
8. Allowed color to develop 10 minutes
9. Record color (purple) in table below

	ASC	$FeSO_4$	FeCit	FeFum
Row 1 (replicate 1)	-	No color	Light color	No color
	+	Color	Color	No color
Row 2 (replicate 2)	-	No color	Light color	No color
	+	Color	Color	No color
Row 3 (replicate 3)	-	No color	Light color	No color
	+	Color	Color	No color

Example table with expected color for each fortificant.

Step 3 Expected Results Before Adding ASC

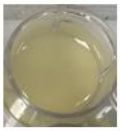
FeCit → light color

$FeSO_4$ → no color (soluble ferrous iron oxidizes at neutral pH)

FeFum → no color (not soluble in water)

$FeSO_4$

FeCit



FeFum

Step 3 Expected Results After Adding ASC

FeCit → dark color

$FeSO_4$ → dark color

FeFum → light or no color (will likely not be completely white or clear due to some iron from fortificant or flour being soluble in water)

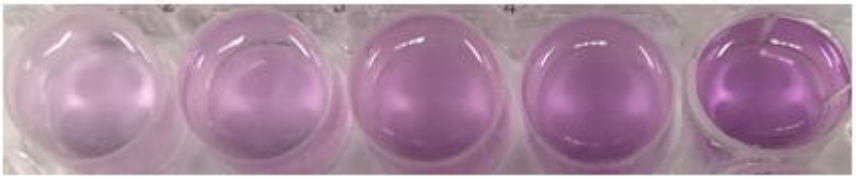
$FeSO_4$

FeCit

FeFum

Step 4. Quantification (quick and visual)

1. Prepare standards with iron of same oxidation state (see below) or use atomic absorption standard (dilute 1 mg/mL to 0.1 mg/mL)
    - Add ~0.3 g flour to wells in triplicate
    - Add 60, 90, 120, 150, and 180 µL 0.1 mg/mL iron solution to wells to produce 20, 30, 40, 50, and 60 ppm standards
2. Weigh 0.3 g ± 0.01 g sample flour
3. Mix samples and standards in 3 mL 3 N HCl
    - Makes all iron soluble
4. Let settle and transfer 0.5 mL supernatant to new well
5. Add 4.5 ml working ferrozine reagent and 1 mL 5% (w/v) ASC to each well
    - Same working ferrozine as identification protocol
6. Compare color intensity of samples to standards



20 ppm | 30 ppm | 40 ppm | 50 ppm | 60 ppm

Preparation of Iron Standards Solution (0.1 mg Fe/mL) in 3 N HCl

1. Prepare 1 mg Fe/mL 3 N HCl solution by adding amount of iron in table below to 50 mL volumetric flask and QS to 50 mL with 3 N HCl.
2. Prepare 0.1 mg Fe/mL 3 N HCl solution by adding 5 mL 1 mg Fe/mL 3 N HCl solution and QS to 50 mL with 3 N HCl.

	Percent Iron*	**Amount of Iron needed for 50 mL 1 mg Fe/mL solution**	**Amount of Iron Salt needed for 50 mL 1 mg Fe/mL solution**
Electrolytic iron	100%	50 mg	50 mg (0.0500 g)
NaFeEDTA	15.215%	50 mg	328.62 mg (0.3286 g)
Ferrous Sulfate	36.762%	50 mg	136.01 mg (0.1360 g)

*Percent iron based on chemical formula. Use percent iron from certificate of analysis if available.

Additional Solutions for Quantification

10% (w/v) TCA in 3 N HCl (Protein Precipitate Solution)
Weigh 5 g trichloroacetic acid (TCA). QS to 50 mL with 3 N HCl.

Stock ferrozine for quantification
Weigh 0.125 g ferrozine and mix in ~40 mL iron free water. Add 0.5 mL ~100% (v/v) thioglycolic acid (TGA) and QS to 50 mL.

Working ferrozine for quantification
Weigh 30.6 g sodium acetate, add 5 mL stock ferrozine, and QS to 50 mL with iron free water. Mix well. (Sodium acetate may not completely dissolve.)

Step 4. Quantification (accurate measurement of iron)

1. Weigh 1 g fortified flour for samples
2. Weigh 1 g unfortified flour for standards
3. Make stock iron standards with 0, 10, 20, 30, 40, 50, 60, 80, and 100 µg Fe/mL by diluting stock iron with 1 mg Fe/mL. Dilute with water.
4. Add 1 mL stock iron standard to standards and add 1 mL water to samples
5. Add 9 mL protein precipitant solution (10% trichloroacetic acid in 3 N HCl) to samples and standards
6. Boil for 5 min (short method) or incubate for 20 h at 65^{O}C (long method)
7. Centrifuge for 15 min
    - We use 3750 rpm with the Beckman Coulter Allegra 6R centrifuge for large volumes and 3000 rpm with the Eppendorf 5415C centrifuge for small volumes.

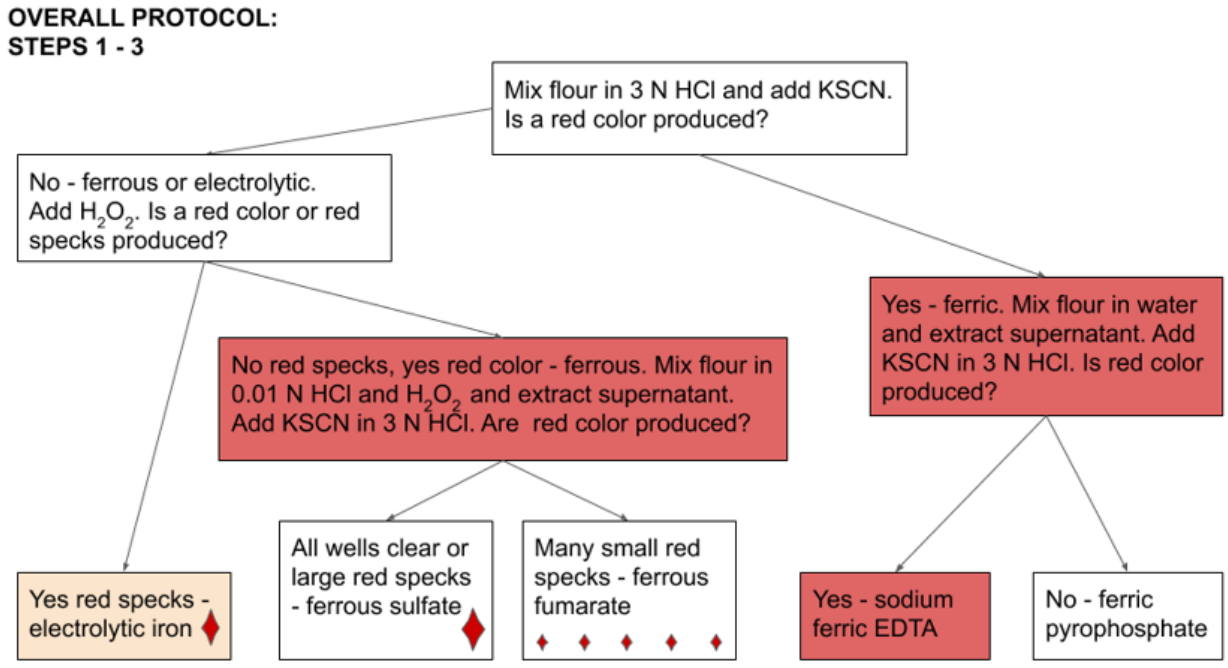
For visual analysis:

8. Add 1 mL of supernatant + 1 mL working chromogen to a test tube
9. Allow color to develop for 10 min
10. Compare color intensity of samples to standards

For spectrophotometer analysis:

8. Mix 1 part supernatant with 1 part working chromogen (150 µL supernatant + 150 µL working chromogen for 96 well plate with 300 µL wells)
9. Allow color to develop 10 minutes
10. Read absorbance at 562 nm

Alternate Protocol (if ferrozine is unavailable)



Steps

Step 1: Same as original method

Steps 2–4: Use KSCN instead of Ferrozine

Note: KSCN color is less stable
KSCN requires acidic pH for color formation
Additional extraction steps required

Additional Solutions for Alternative Methods

10% (w/v) KSCN in 3 N HCl

Weigh 5 g KSCN. QS to 50 mL with 3 N HCl. Prepare immediately before use.

0.01 N HCl

Add ~900 mL water to a 1000 mL volumetric flask. Add 3.33 mL 3 N HCl. QS to 1000 mL with water.

Alternative Step 2. Identifies NaFeEDTA and FePP

1. ~0.3 g flour x 3 replicates
2. Mix in 2 mL water
   - Only water soluble iron will react
3. Mix for 10 minutes or until well mixed
4. Let stand until liquid on top is clear
5. Remove 1 mL liquid and put in new well
6. Add 1 mL 10% (w/v) KSCN in 3 N HCL prepared immediately before use
7. Record color (red/pink)

Alternative Step 2 Expected Results

FePP → Light or no color
NaFeEDTA → Dark

FePP

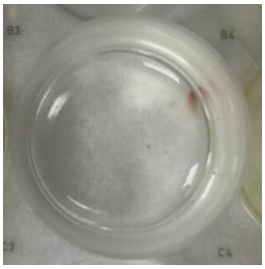
NaFeEDTA

Alternative Step 3. Identifies $FeSO_4$ and FeFum

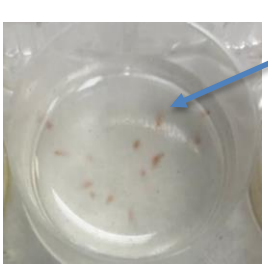
1. ~0.3 g flour x 3 replicates
2. Add 2 mL 0.01 N HCl
3. Add 1 mL 3% (v/v) H_2O_2
4. Mix 10 minutes or until well mixed
5. Let stand until liquid on top is clear
6. Remove 1 mL liquid and put in new well
7. Add 1 mL 10% (w/v) KSCN in 3 N HCl prepared immediately before use
8. Record color (red specks)

Alternative Step 3 Expected Results

$FeSO_4$ → No specks or large specks
FeFum → Many small specks



$FeSO_4$



FeFum

Alternative Step 4. Quantification

1. Weigh 0.3 g flour $\pm$ 0.01 g for samples
2. Prepare standards
    - ~0.3 g flour
    - Add 60, 90, 120, 150, and 180 µL 0.1 mg/mL iron solution (stock can be 1 mg/mL and dilute it to 10X) (These are equivalent to 20, 30, 40, 50, and 60 ppm Fe)
3. Add 4 mL 3 N HCl to samples and standards
    - To make all iron soluble
4. Add 1 mL 3% (v/v) H_2O_2 to samples and standards
    - Converts ferrous to ferric
5. Add 1 mL 10% (w/v) KSCN in water
6. Compare color to standards
    - Can also read the absorbance at 490 nm using a microplate reader or spectrophotometer.

20 ppm | 30 ppm | 40 ppm | 50 ppm | 60 ppm

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