ADDIS ABABA UNIVERSITY
COLLEGE OF NATURAL SCIENCES
CENTER FOR FOOD SCIENCE AND NUTRITION

Effect of Particle Size Reduction and Xylanase-Cellulase Treatment of Wheat on Rat Hemoglobin Regeneration Efficiency

By

Daniel Tsegaye

Advisor: Kaleab Baye (PhD)

A thesis submitted to the School of Graduate studies of Addis Ababa University in partial fulfillment of the requirement for the Degree of Master of Science in Food Science and Nutrition.

June, 2016

Addis Ababa, Ethiopia

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Approval by Examining Board:		**Signature**	**Date**
(External Examiner)	Mr. Kelbessa Urga	______________	______________
(Internal Examiner)	Dr. Dawud Gashu	______________	______________
(Advisor)	Dr. Kaleab Baye	______________	______________
(Chairperson)	Mrs. Hasset Tamirat	______________	______________

DECLARATION

I, the undersigned, declare that this thesis is my original work and that all sources of materials used for the thesis have been duly acknowledged.

Name	Signature	Date
Daniel Tsegaye Alemu	________________	________________

The thesis has been approved for submission by:

Name of Supervisor	Signature	Date
Dr. Kaleab Baye (Advisor)	________________	________________

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List of abbreviations and acronyms

AAS – Atomic Absorption Spectroscopy

AIN-93G – American Institute of Nutrition Formula for Growing Rats

ANOVA – Analysis of Variance

Caco-2 – Human Colon Carcinoma Cell Line

DM – Dry Matter

Fe – Iron

FS – Ferrous Sulfate

FAO – Food and Agriculture Organization

g – Gram

HRE – Hemoglobin Regeneration Efficiency

IAEA – International Atomic Energy Agency

IDA – Iron Deficiency Anaemia

Kg – Kilogram

L – Litre

Ml – Milliliter

MW – Micro milled Wheat

Hb – Hemoglobin

ID – Iron Deficiency

IDA – Iron Deficiency Anemia

RBV – Relative Biological Value

PPM – Parts Per Million

RBC – Red Blood Cells

RBV – Relative Biological Value

WHO – World Health Organization

WW – Wholegrain Wheat

XC – Xylanase + Cellulase

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Abstract

Iron deficiency is probably the most common nutritional deficiency present in the world population. Predominantly plant-based diets that are constituted of wholegrain cereals and legumes contain non-heme iron that is poorly absorbed due to susceptibility to mineral chelating agents such as phytate and polyphenols, all of which may adversely affect mineral bioavailability. When attempts are made to remove or minimize the effects of the anti-nutrients, the dietary fiber matrix is often altered or destroyed, which would make it possible to better target the inhibitors and to increase their degradation. The objective of this study was to investigate the effect of enzymatic treatment and particle size reduction on and evaluate their contribution to the bioavailability of iron using hemoglobin regeneration efficiency of rats. Thirty two (21-28 days old) male Wistar rats were fed with an iron-free diet for 21-28 days to induce anemia. Then the rats were divided into four groups (n=8 per group) and fed with a diet which provides 35 mg of iron per kg. Hb measurement was done by HemoCue. Hematologic indices were calculated and bioavailability of iron was determined. The hemoglobin regeneration efficiency (HRE), was significantly higher ($P< 0.05$) in the control group, ferrous sulfate, n=8 (46.93 ± 4.22), followed by the xylanase-cellulase treated (37.84 ± 3.99), followed by the micro-milled (31.75 ± 3.62) and wholegrain (23.66 ± 4.51) wheat flour groups. The HRE relative to ferrous sulfate group (control 100%), of xylanase-cellulase treated wheat flour was $81.56\pm14.02\%$, micro-milled wheat flour was $68.45\pm12.23\%$ and wholegrain wheat flour was $50.64\pm9.64\%$. The present in vivo study clearly showed that the treatments can lead to hemoglobin regeneration in iron deficient rats and is thus an important source of bioavailable iron.

Keywords: *Xylanase, Cellulase, Iron, Fiber, Wheat, Bioavailability, Hemoglobin Regeneration Efficiency, Relative Biological Value*

Chapter One

Introduction

1. Introduction

1.1. Background

Iron deficiency is probably the most common nutritional deficiency present in the world. Women of child-bearing age, pregnant and lactating women, and children are at an increased risk of iron deficiency. This is true because iron is poorly absorbed and because many diets, especially those consumed in low income countries, are poor in iron and hence are not adequate to meet requirements (Carolyn, 1998; David & Idamarie, 2003; Gershwin *et al.*, 2004; WHO, 2006). Predominantly plant-based diets that are constituted of whole grain cereals and legumes contain non-heme iron that is poorly absorbed and susceptible to mineral chelating agents such as phytate and polyphenols (Carolyn, 1998; WHO, 2004). These compounds make iron in the diet to be in a chemical form that is poorly or not at all absorbable. Thus, it may be present in very stable complexes or as an iron compounds with a low solubility in the gastrointestinal contents (Hallberg, 1981) and hence reduces its bioavailability (Baye *et al.*, 2014).

In many low-income countries, the prevalence of iron deficiency is high despite a high dietary intake of iron and anemia rates in children are high (above 50%) and the severity of anemia is marked. In many cases this is due to low availability of dietary iron rather than low intakes, as 90% of the total dietary supply in many of these countries comes from plants, which contain non-heme iron that is poorly absorbed and hence prevalence among those dependent on cereal-based diets containing few animal products is significantly higher (Thompson, 2011). Reported iron intakes of Ethiopian children exceed recommendations (Baye *et al.*, 2012), suggesting that if bioavailable and the amount supplied by these foods could be sufficient. Therefore, there is a need to conduct a bioavailability study by reducing iron absorption inhibitors and determining the actual availability by *in vivo* test.

Since none-heme iron is the main source of dietary iron in diets consumed by low income countries like Ethiopia, it is important to have knowledge on how to improve iron bioavailability by minimizing the effect of inhibitors. Treating whole grains with multiple processes, including physical, chemical, and biological, can release some of these nutrients into a free or soluble bound form that is much more bioavailable, making the whole grain products much more beneficial to health. Phytate can be degraded by endogenous phytases which can be activated by food processing techniques like fermentation, germination and to a lesser extent during cooking (Baye, 2014). The addition of ascorbic acid (vitamin C) or sodium ethylenediaminetetraacetic acid (sodium EDTA or Na_2EDTA) and the removal of phytates, all of which reduce the effect of the inhibitors, can be effective ways of increasing the total amount of iron absorbed from foods (WHO, 2006). Thus, such work should be done in a diet in which the iron is of low bioavailability while being good iron source.

Cereals constitute important sources of iron in human diet; however, much of the iron in wheat is lost during processing for the production of white flour (Latunde-Dada *et al.*, 2014). Wheat is a good source of essential fatty acids, fiber, minerals (especially calcium and iron), and phytochemicals such as polyphenols and phytates. The bioavailability of iron in wheat is likely to vary depending on processing. The bioavailability of iron in wheat can be studied by *in vitro* and *in vivo* methods for estimated on iron absorption.

The *in vitro* method showed a promising result in an estimation of nutrient bioavailability (Luciana *et al.*, 2013). Further investigation should be done using an *in vivo* method to get more information on this area of study. In an *in vivo* method, animals are fed with iron deficient diet to deplete their iron followed by feeding with diet prepared by treatment to librate iron from binding compounds in a repletion phase (Carolyn, 1998; Sturza *et al.*, 2008). In this study, food processing techniques to increase the bioavailability of iron in wheat was used. Size reduction and enzyme

treatment were employed in this work to investigate the bioavailability of iron from wheat in animal models (rats).

1.2. Statement of the problem

The fortification of flour with iron has additional challenges due to the presence of high levels of dietary inhibitors such as phytates, tannins, and dietary fiber, which have the potential to interact with iron and reduce bioavailability (Latunde-Dada, 1996). The studies on the bioaccessibility of naturally occurring iron in wheat by Latunde-Dada *et al.*, (2014) showed that enzymatic digestion with Driselase, and following mechanical disruption using micromilling enhanced iron bioavailability to both elemental iron and iron nano compounds.

Although micro-milling enhanced bioavailability of iron, the dietary fiber matrix could be altered or destroyed and bypass biological systems due to the maximal disruption. Consequently, losing the best possible nutritional advantages and may lead to other health problems. In this study, the wheat grains were minimally disturbed and treated with xylanase+cellulase to enhance mineral bioavailability.

1.3. Significance of the study

This study will consolidate the knowledge of food-based strategies to control micronutrient malnutrition. Particularly it will give information on how to improve the bioavailability of natural iron in the diet without any additional artificial fortification. Hence, it will minimize the effort and cost of iron fortification and provides an alternative for policy makers involved in iron fortification program. Furthermore, the study will serve as an input for researchers and students to fill the knowledge gaps in further iron bioavailability studies.

1.4. Research questions

This research was aimed to answer the following questions:

1. Will size reduction bring a significant difference in the bioavailability of iron from wheat flour?
2. Will enzyme treatment bring a significant difference in the bioavailability of iron from wheat flour?

1.5. Objectives of the study

1.5.1. General objective

To determine the extent of bioavailability of iron from wheat flour by micromilling and enzymatic treatments to liberate the iron from the binding compounds and to evaluate the contribution of the treatments in the bioavailability of iron using hemoglobin regeneration test.

1.5.2. Specific objective

- To evaluate the bioavailability of iron from micromilled wheat flour using rat Hb gain per gram of iron intake (% HRE)

- To evaluate the bioavailability of iron from xylanase+cellulase treated wheat flour using rat Hb gain per gram of iron intake (% HRE)

- To compare extent of biological value relative to Hb gain per gram of iron intake of wholegrain wheat flour (WW), micromilled wheat flour (MW) & enzyme treated wheat flour (XC) in comparison to the control (using ferrous sulfate as a control)

Chapter Two

Literature review

2. Literature Review

2.1. Iron (Fe)

Iron, the fourth most abundant element (after oxygen silicon and aluminum) and the second most abundant metal in the earth's crust (5.6% by mass). The most common oxidation states of iron are Fe^{2+} (ferrous) and Fe^{3+} (ferric). It is immensely important both in human civilization and in living systems (McMurry & Fay, 2003). It is an essential nutrient for nearly all living species. Iron is present almost exclusively as chelates with proteins. Iron plays many key roles in biological systems, including oxygen transport and storage in higher animals (hemoglobin and myoglobin), ATP generation (iron-sulfur proteins and cytochromes), DNA synthesis (ribonucleotide reductase), and chlorophyll synthesis (Fennema, 1996). The body of a healthy human adult contains about 4 g of iron, 65% of which is present in the oxygen-carrying protein hemoglobin (McMurry & Fay, 2003; Greenwood & Earnshaw, 1998).

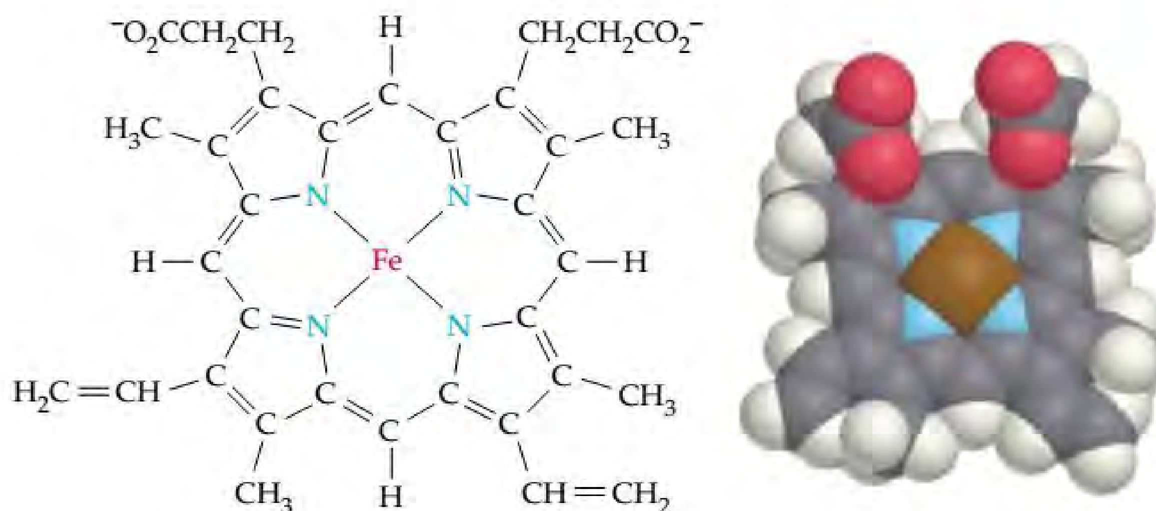

Figure 2.1. Heme - an O_2 molecule binds to the central iron atom. Adapted from McMurry & Fay, 2003.

Heme (or haem) consists of a complex organic ring structure, protoporphyrin, to which is bound a single iron atom in its ferrous (Fe^{2+}) form. The iron atom has six coordination bonds, four to nitrogen atoms that are part of the flat porphyrin ring system and two perpendicular to the porphyrin. The coordinated nitrogen atoms (which have an electron-donating character) help prevent conversion of the heme iron to the ferric (Fe^{3+}) state. Iron in the Fe^{2+} state binds oxygen reversibly; in the Fe^{3+} state it does not bind oxygen. Heme is found in a number of oxygen-transporting proteins, as well as in some proteins, such as the cytochromes, that participate in oxidation-reduction (electron-transfer) reactions. In free heme molecules (heme not bound to protein), reaction of oxygen at one of the two "open" coordination bonds of iron (perpendicular to the plane of the porphyrin molecule, above and below, Figure 2.1), can result in irreversible conversion of Fe^{2+} to Fe^{3+} (Nelson & Cox, 2004).

2.2. Iron deficiency and its consequences

Iron deficiency is the most common nutrient deficiency in the world. It has been estimated that approximately 1.62 billion people globally are anaemic, representing 24.8% of the world's population (WHO, 2008). Iron deficiency is thought to account for 50% of identified anaemia (WHO, 2008), and in total, 0.8 million (1.5%) of deaths worldwide are attributable to iron deficiency (WHO, 2002). Beard (2008) stated that a lack of sufficient iron intake may significantly delay the development of the central nervous system as a result of alterations in morphology, neurochemistry, and bioenergetics. In adults, iron deficiency and iron deficiency anaemia can adversely impact physical work capacity and productivity, variables that may have a detrimental impact on economic potential (IAEA, 2012). Clearly, the magnitude of adverse and often irreversible consequences associated with suboptimal iron status and iron deficiency anaemia highlight the need to develop strategies to combat deficiency of this trace element.

Iron deficiency is defined as a hemoglobin concentration below the optimum value in an individual. Iron deficiency anaemia implies that the hemoglobin concentration is below the 95th percentile of the distribution of hemoglobin concentration in a population (disregarding effects of altitude, age and sex, etc. on hemoglobin concentration) (WHO, 2004). Clinical iron deficiency anemia occurs in three stages. The first involves depletion of iron stores as measured by a decrease in serum ferritin which reflects the ferritin supply (iron stores) in the body, without loss of essential iron compounds and without any evidence of anemia. The second stage is characterized by biochemical changes that reflect the lack of iron sufficient for the normal production of hemoglobin and other iron compounds. This is indicated by a decrease in transferrin saturation levels and an increase in erythrocyte protophyrin, so-called iron deficiency without anemia. In the final stage, iron deficiency anemia occurs, with depressed hemoglobin production and a change in the mean corpuscular volume of the RBC to produce a microcytic hypochromic anemia. This is expressed clinically as pallor and weakness. There are also changes in the nails, which take on a spoon shape when the iron-deficient state is severe (Carolyn, 1998).

Nutritional iron deficiency implies that the diet cannot supply enough iron to cover the body's physiological requirements for this mineral. Worldwide this is the most common cause of iron deficiency. In many tropical countries, infestations with hookworms lead to intestinal blood losses that in some individuals can be considerable. The average blood loss can be reliably estimated by egg counts in stools. Usually the diet in these populations is also limited with respect to iron content and availability. The severity of the infestations varies markedly between subjects and regions (WHO, 2004).

Iron deficiency is probably the most common nutritional deficiency disorder in the world. A recent estimate based on WHO criteria indicated that around 600–700 million people worldwide have marked iron deficiency anaemia. And the bulk of these people live in low income countries. In low income countries, the prevalence of iron deficiency anaemia is much lower and usually varies between 2% and 8%. However, the prevalence of iron deficiency, including both anaemic and non-anaemic subjects, is much higher. In high income countries, for example, an absence of iron stores or subnormal serum ferritin values is found in about 20–30% of women of fertile age. In adolescent girls, the prevalence is even higher. Worldwide, the highest prevalence figures for iron deficiency are found infants, children, adolescents, and women of childbearing age, especially pregnant women. The weaning period in infants is especially critical because of the very high iron requirement needed in relation to energy requirement. During this period, iron nutrition is of great importance for the adequate development of the brain and other tissues such as muscles, which are differentiated early in life (IAEA, 2012; Beard, 2008).

Iron deficiency can cause reduced work capacity in adults and impact motor and mental development in children and adolescents. There is some evidence that iron deficiency without anaemia affects cognition in adolescent girls and causes fatigue in adult women. IDA may affect visual and auditory functioning and is weakly associated with poor cognitive development in children (Whitney, 2006; Killip *et al.*, 2007; WHO, 2001). Studies in animals have clearly shown that iron deficiency has several negative effects on important functions in the body. The physical working capacity of rats is significantly reduced in states of iron deficiency, especially during endurance activities. This negative effect seems to be less related to the degree of anaemia than to impaired oxidative metabolism in the muscles with an increased formation of lactic acid. Thus, the effect witnessed seems to be due to a lack of iron-containing enzymes which are rate limiting for oxidative metabolism. Further to this, several groups have observed a reduction in physical working capacity in

human populations with longstanding iron deficiency, and demonstrated an improvement in working capacity in these populations after iron administration (WHO, 2004).

The relationship between iron deficiency and brain function and development is very important to consider when choosing a strategy to combat iron deficiency. Several structures in the brain have high iron content; levels are of the same order of magnitude as those observed in the liver. The observation that the lower iron content of the brain in iron-deficient growing rats cannot be increased by giving iron at a later date strongly suggests that the supply of iron to brain cells takes place during an early phase of brain development and that, as such, early iron deficiency may lead to irreparable damage to brain cells (WHO, 2004).

Iron deficiency also negatively influences the normal defence systems against infections. In animal studies, the cell-mediated immunologic response by the action of T-lymphocytes is impaired as a result of a reduced formation of these cells (Gibney *et al*., 2009). This in turn is due to a reduced DNA synthesis dependent on the function of ribonucleotide reductase, which requires a continuous supply of iron for its function. In addition, the phagocytosis and killing of bacteria by the neutrophil leukocytes is an important component of the defence mechanism against infections. These functions are impaired in iron deficiency as well. The killing function is based on the formation of free hydroxyl radicals within the leukocytes, the respiratory burst, and results from the activation of the iron-sulfur enzyme NADPH oxidase and probably also cytochrome b (a haem enzyme). The impairment of the immunologic defence against infections that was found in animals is also regularly found in humans. Administration of iron normalizes these changes within 4–7 days (WHO, 2004).

2.3. Dietary iron sources, metabolism and absorption

Dietary iron sources are cereals, legumes, meat, vegetables, contamination from iron utensils and soil and enriched products (Fennema, 1996). Iron absorption is influenced by the combination of a number of factors: (a) Total iron content in the food ingested. The efficiency of iron is inversely proportional to the amount of iron in the food, i.e. the greater the amount, the lesser is the percent absorbed. However, total iron absorbed will still be higher with increased intake. (b) Amount of heme iron. Heme iron refers to the iron in hemoglobin and myoglobin and is much more readily absorbed than non-heme iron.

Approximately 40% of the iron from animal sources is heme iron and the remaining 60% is non-heme iron. All plant food iron is considered to be non-heme iron. Despite the relatively high meat consumption, heme iron contributes only 1 to 2 mg per day in the Western diet which usually contains 10 to 20 mg iron. Non-heme iron is thus the primary source of iron in the human diet. (c) Presence of enhancers and inhibitors. Non-heme iron absorption is greatly influenced by a number of dietary factors. Factors that increase non-heme iron absorption include meat, and ascorbic acid. Some of the known inhibitors are phytates, oxalates, phosphates, dietary fiber, soy protein and tannin.

Heme iron absorption is enhanced by the presence of meat in the diet, but is not influenced by the other factors that affect non-heme iron absorption. (d) Iron nutriture of the individual. More iron is absorbed by iron deficient individuals than by individuals with normal iron status. This is particularly true for non-heme iron. (e) Source of iron, type of iron compound and the food with which it is eaten. Breast fed infants absorb over 50% of iron from human milk compared to formula fed infants who absorb less than 12% of iron from cow milk-derived formula. The percentage of iron absorbed from soy formula is lower than that from cow milk formula. Iron found in meat sources is better absorbed than from nonmeat sources or in the pyrophosphate form (WHO, 2004).

With respect to the mechanism of absorption, there are two kinds of dietary iron: haem iron and non-haem iron. In the human diet, the primary sources of haem iron are the hemoglobin and myoglobin from consumption of meat, poultry, and fish whereas non-haem iron is obtained from cereals, pulses, legumes, fruits, and vegetables (WHO, 2004; Whitney, 2006). The average absorption of haem iron from meat-containing meals is about 25%. The absorption of haem iron can vary from about 40% during iron deficiency to about 10% during iron repletion. Haem iron can be degraded and converted to non-haem iron if foods are cooked at a high temperature for too long (WHO, 2004). Calcium is the only dietary factor that negatively influences the absorption of haem iron and does so to the same extent that it influences non-haem iron. Non-haem iron is the main form of dietary iron. The absorption of non-haem iron is influenced by individual iron status and by several factors in the diet (WHO, 2004).

For iron to be absorbed the presence of an acidic pH, (i.e. gastric acid or concurrent intake of vitamin C), is required. Ferric iron has to be reduced to its ferrous form before it can be adsorbed. Luminal iron binding proteins facilitate the absorption of ferrous iron across the intestinal mucosa (David & Idamarie, 2003). In spite of the fact that some foods have high iron levels, much of the population has frequently been found to be deficient in this element. Animal food products may have high levels that are well absorbed; liver may contain several thousand ppm of iron. The iron from other foods such as vegetables and eggs is more poorly absorbed. In the case of eggs the uptake is poor because the ferric iron is closely bound to the phosphate of the yolk phosphoproteins (DeMan, 1999).

The iron content of a typical 70 kg adult man is approximately 4–5 g. Of this content, approximately two-third is utilized as functional iron such as hemoglobin (60%), myoglobin (5%), and various heme (cytochromes and catalase) and non-heme (NADH hydrogenase, succinic dehydrogenase, aconitase) enzymes (5%). The

remaining iron is found in body storage as ferritin (20%) and hemosiderin (10%), the two major iron storage proteins. Only very minor quantities of iron (<0.1%) are found as a transit chelate with transferrin, the main iron transport protein in the body (Whitney, 2006; Gibney *et al*., 2009). The metabolism of iron differs from that of other minerals in one important respect: there is no physiological mechanism for iron excretion. The body has three unique mechanisms for maintaining iron balance and preventing iron deficiency and iron overload: (1) Storage of iron (with ferritin being an important reversible storage protein for iron). (2) Reutilization of iron (especially of iron in erythrocytes). (3) Regulation of iron absorption. In theory, therefore, when the body needs more iron, absorption is increased, and when the body is iron sufficient, absorption is restricted. This control is not perfect but is still of great importance for the prevention of iron deficiency and excess. Iron from food is absorbed mainly in the duodenum by an active process that transports iron from the gut lumen into the mucosal cell. When required by the body for metabolic processes, iron passes directly through the mucosal cell into the blood stream, where it is transported by transferrin, together with the iron released from old blood cells (i.e., the efficient iron recycling system, Figure 2.2), to the bone marrow (80%) and other tissues (20%). If iron is not required by the body, iron in the mucosal cell is stored as ferritin and is excreted in feces when the mucosal cell is exfoliated. Any absorbed iron in excess of needs is stored as ferritin or hemosiderin in the liver, spleen, or bone marrow. Iron can be released from these iron stores for utilization in times of high need, such as during pregnancy (Gibney *et al*., 2009).

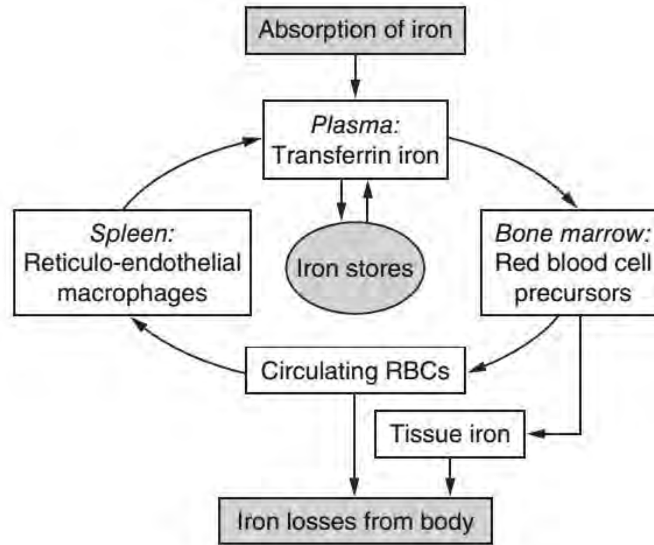

Figure 2.2. Metabolism of iron. There is a main internal loop with continous reutilization of iron and an external loop presentrd by iron losses from the body and absorption from the diet. Adapted from Gibney *et al*., 2009.

Heme iron is absorbed by a different mechanism from non-heme iron. The heme molecule is absorbed intact into the mucosal cell, where iron is released by the enzyme heme oxygenase. Its absorption is little influenced by the composition of the meal, and varies from 15% to 35% depending on the iron status of the consumer. Although heme iron represents only 10–15% of dietary iron intake in populations with a high meat intake, it could contribute 40% or more of the total absorbed iron, (Figure 2.3). Many poorer regions of the world consume little animal tissue and rely entirely on non-heme iron. The absorption of non-heme iron is strongly influenced by dietary components, which bind iron in the intestinal lumen. The complexes formed can be either insoluble or so tightly bound that the iron is prevented from being absorbed. Alternatively, the complexes can be soluble and iron absorption is facilitated. Under experimental conditions, non-heme iron absorption can vary widely from less than 1% to more than 90%, but under more typical dietary conditions it is usually in the region of 1–20% (Gibney *et al*., 2009).

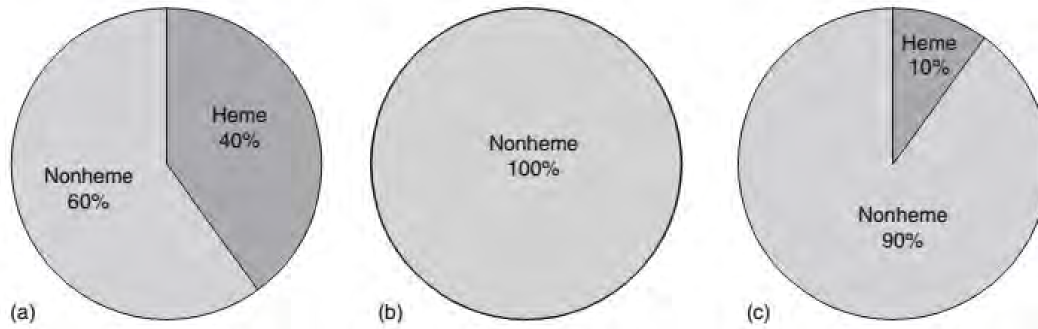

Figure 2.3. Heme and non heme iron in foods: (a) foods of animal origin; (b) foods of plant origin; (c) dietary iron intake from all foods, daily average. Adapted from Gibney *et al.*, 2009.

2.4. Iron bioavailability

Bioavailability is the fraction of an ingested nutrient that is available for utilization in normal physiological functions and/or for storage (LaFrano *et al.*, 2014). Iron in the diet must be absorbed before the biological effects of this trace element are evident. As most absorbed iron becomes incorporated into RBCs, the amount of absorbed iron that becomes incorporated into RBC hemoglobin is often used as a measure of iron absorption (Carolyn, 1998; Minten *et al.*, 2013). Iron bioavailability is determined almost totally by the efficiency of iron absorption in the intestine. Total iron intake, composition of the diet, and iron status of the individual consuming the diet all play a role in determining the amount of iron absorbed. Diets in industrialized countries like the United States consistently provide about 6 mg iron per 1000 kcal (4186 kJ). Iron in animal tissues, non-heme iron, is bound primarily to proteins but may also be complexed with citrate, phytate, oxalate, polyphenolics, or other ligands. The bioavailability of heme iron is relatively unaffected by composition of the diet and is generally significantly greater than that of non-heme iron. The bioavailability of non-heme iron varies markedly depending on composition of the diet. It is widely assumed that nonheme iron from all sources in a meal (foods as well as fortification iron) enters a common pool during digestion and that absorption of iron from this pool is determined by the totality of ligands present in the small intestine at the site of absorption. Several enhancers and

inhibitors of nonheme iron absorption have been identified. Enhancers include meat, poultry, fish, ascorbic acid, and EDTA (in diets where bioavailabilities are low). Inhibitors include polyphenolics (tannins in tea, legumes, and sorghum), phytates (presentin legumes and whole-grain cereals), some plant proteins (especially legume proteins), calcium, and phosphates. The overall bioavailability of iron in a diet is determined by complex interactions of the enhancers and inhibitors present. Iron absorption from diets composed primarily of roots, tubers, legumes, and cereals, with limited meat and ascorbic acid, may be only about 5% even in people with poor iron status. Such a diet would provide only about 0.7 mg absorbable iron per day, a quantity too small to meet the needs of many individuals. Iron absorption from diets based on roots, cereals, and legumes that contain some meat, poultry, or fish and some foods high in ascorbic acid may be about 10%. These diets provide about 1.4 mg of absorbable iron per day, an amount that is adequate for most men and postmenopausal women but inadequate for up to 50% of women of child-bearing age. Diets composed of generous quantities of meat, poultry, fish, and foods high in ascorbic acid provide over 2 mg absorbable iron per day, an amount sufficient to meet the needs of nearly all healthy persons (Fennema, 1996).

2.5. Cereals

Cereals are the edible seeds or grains of the grass family, Gramineae including rye, oats, barley, maize, millet and sorghum. On a worldwide basis, wheat and rice are the most important crops, accounting for over 50% of the world's cereal production. All of the cereals share some structural similarities and consist of an embryo (or germ) and an endosperm, which is packed with starch grains. Cerealbased foods are a major source of energy, protein, B vitamins and minerals (McKevith, 2004).

2.5.1. Wheat

Wheat is a major cereal crop in many parts of the world. It belongs to the Triticum family, of which there are many thousands of species, with *Triticum aestivum* subspecies Vulgare and the hard wheat *Triticum durum* being the most important commercially. Wheat is grown as both a winter and a spring cereal and, owing to the number of species and varieties and their adaptability, it is grown in many countries around the world (McKevith, 2004).

Ethiopia is the second largest producer of wheat in sub-Saharan Africa, following South Africa. About 900,000 ha of bread (*Triticum aestivum*) and durum (*Triticum turgidum* var. *durum*) wheats are grown in Ethiopia, primarily as highland rainfed crops (White *et al.*, 2001). In Ethiopia, wheat grain is used in the preparation of a range of products such as: the traditional staple pancake ("injera"), bread ("dabo"), local beer ("tella"), and several others local food items (i.e., "dabokolo","ganfo", "kinche"). Besides, wheat straw is commonly used as a roof thatching material, and as a feed for animals. Wheat contributes approximately 200 kcal/day in urban areas, compared to about 310 kcal in rural areas. It accounts for about 11% of the national calorie intake (Demeke & Marcantonio, 2013).

Wheat grains are generally oval shaped. The shape also ranges from almost spherical to long, narrow and flattened shapes in different wheat's. The grain is usually between 5 and 9mm in length, weighs between 35 and 50mg . The wheat grain (Figure 2.4) contains 2-3%germ, 13-17% bran and 80-85% mealy endosperm of the total dry matter. Bran is rich in B vitamins and minerals; it is separated from the starchy endosperm during the first stage of milling. In order to protect the grain and endosperm material, the bran comprises water-insoluble fiber. More than half of the bran consists of fiber components (53%). Chemical composition of wheat bran fiber is complex, but it contains, essentially, cellulose and pentosans, polymers based on xylose and arabinose, which are tightly bound to proteins. These

substances are typical polymers present in the cell walls of wheat and layers of cells such as aleurone layer. Proteins and carbohydrates each represent 16% of total dry matter of bran. The mineral content is rather high (7.2%). The two external layers of the grain (pericarp and seed coat) are made up of dead empty cells. The cells of the inner bran layer- aleurone layer are filled with living protoplasts. This explains the rather high levels of protein and carbohydrate in the bran. The inner endosperm, i.e. the endosperm without the aleurone layer, is referred to as mealy or starchy endosperm. The endosperm mainly contains food reserves, which are needed for growth of the seedling; it is rich in energy-yielding starch. Apart from carbohydrates, the mealy endosperm contains fats (1.5%) and proteins (13%): albumins, glubulins and the major proteins of the gluten complex- glutenins and gliadins- proteins that will form the gluten at dough making. The contents of minerals (ash) and of dietary fibers are low; 0.5% and 1.5%, respectively (Šramková *et al.*, 2009).

The germ lies at one end of the grain. It is rich in proteins (25%) and lipids (8-13%). The mineral level is also rather high (4.5%). Wheat germ is available as a separate entity because it is an important source of vitamin E. Wheat germ has only one half the glutamine and proline of flour, but the levels of alanine, arginine, asparagine, glycine, lysine and threonine are double (Šramková *et al.*, 2009).

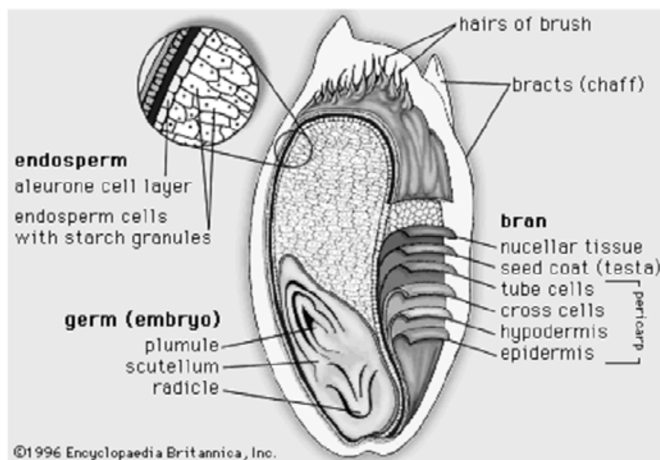

Figure 2.4. Wheat grain. Adapted from Šramková *et al.*, 2009.

2.5.2. Processing

Milling is the main process associated with cereals. Milling can basically be described as grinding, sifting, separation and regrinding. These steps are repeated to extract a particular part of the grain, the endosperm. Before milling begins, the cereal grains are cleaned. Most modern equipment uses differences in size, shape, colour, solubility, specific weight and response to magnetic force to separate foreign material from the grains. Prior to grinding, water may be added to the cereal, which is allowed to rest before milling (*tempering*). This allows absorption of water by the grains, toughening the pericarp and germ so they do not splinter during milling. If heat is also applied during tempering (to mellow the endosperm and make it easier to grind), then the process is referred to as *conditioning*. To ensure production of a uniform product, different grains may be blended prior to milling and this is referred to as *gristing* (McKevith, 2004).

2.6. Iron absorption inhibitors

Iron absorption inhibitors are compounds (ligands) that chelate iron to form insoluble complexes or complexes of very high affinity, so that iron is not released from the chelate for absorption. Iron absorption inhibitors include: phytate, polyphenols, calcium, fiber, and others (Hallberg *et al.*, l987; Fennema, 1996; WHO, 2004; Hurrell & Egli, 2010).

2.6.1. Phytates

Phytates are found in all kinds of grains, seeds, nuts, vegetables, roots (e.g., potatoes), and fruits. Chemically, phytates are inositol hexaphosphate salts and are a storage form of phosphates and minerals. Other phosphates have not been shown to inhibit non-heme iron absorption. In North American and European diets, about 90 percent of phytates originate from cereals. Phytates strongly inhibit iron

absorption in a dose-dependent fashion and even small amounts of phytates have a marked effect. Bran has a high content of phytate and strongly inhibits iron absorption. Whole-wheat flour, therefore, has a much higher content of phytates than does white wheat flour. In bread some of the phytates in bran are degraded during the fermentation of the dough. Fermentation for a couple of days (sourdough fermentation) can therefore almost completely degrade the phytate and increase the bio-availability of iron in bread made from whole-wheat flour (WHO, 2004, Hallberg *et al.*, l987, Hurrell & Egli, 2010). Oats strongly inhibit iron absorption because of their high phytate content, that results from native phytase in oats being destroyed by the normal heat process used to avoid rancidity (WHO, 2004). Sufficient amounts of ascorbic acid can counteract this inhibition (Siegenberg *et al.*, 1991, Hallberg *et al.*, 1989). By contrast, non-phytate-containing dietary fiber components have almost no influence on iron absorption(WHO, 2004, Hurrell & Egli, 2010).

2.6.2. Polyphenols

Almost all plants contain phenolic compounds as part of their defence system against insects, animals, and humans. Only some of the phenolic compounds (mainly those containing galloyl groups) seem to be responsible for the inhibition of iron absorption. Tea, coffee, and cocoa are common plant products that contain iron-binding polyphenols. Many vegetables, especially green leafy vegetables (e.g., spinach), and herbs and spices (e.g., oregano) contain appreciable amounts of galloyl groups, that strongly inhibit iron absorption. Consumption of betel leaves, common in areas of Asia, also has a marked negative effect on iron absorption (WHO, 2004), Hurrell & Egli, 2010).

2.6.3. Calcium

Calcium, consumed as a salt or in dairy products interferes significantly with the absorption of both heme and non-heme iron, which makes it different from other

inhibitors that affect nonheme iron absorption only. Because calcium and iron are both essential nutrients, calcium cannot be considered to be an inhibitor in the same way as phytates or phenolic compounds. The practical solution for this competition is to increase iron intake, increase its bio-availability, or avoid the intake of foods rich in calcium and foods rich in iron at the same meal. The mechanism of action for absorption inhibition is unknown, but the balance of evidence strongly suggest that the inhibition is located within the mucosal cell itself at the common final transfer step for heme and non-heme iron. Recent analyses of the dose-effect relationship show that no inhibition is seen from the first 40 mg of calcium in a meal. A sigmoid relationship is then seen, reaching a 60 percent maximal inhibition of iron absorption by 300–600 mg calcium. The form of this curve suggests a one-site competitive binding of iron and calcium. This relationship explains some of the seemingly conflicting results obtained in studies on the interaction between calcium and iron. For unknown reasons, the addition of soy protein to a meal reduces the fraction of iron absorbed. This inhibition is not solely explained by the high phytate content of soy protein. However, because of the high iron content of soy proteins, the net effect on iron absorption of an addition of soy products to a meal is usually positive. In infant foods containing soy proteins, the inhibiting effect can be overcome by the addition of sufficient amounts of ascorbic acid. Some fermented soy sauces, however, have been found to enhance iron absorption (WHO, 2004, Hurrell & Egli, 2010).

2.6.4. Fiber

Dietary fiber is a portion of a food carbohydrate which cannot be digested by enzymes secreted by the host and normally present in the gastrointestinal tract. Unrefined cereal grains have been shown to be the best single food source of both dietary fiber and minerals. The refining of grains, essential for edibility, and desirable for palatability, reduces both the mineral and dietary fiber content of the grains. Minerals are found in highest concentrations in the germ and outer layers of

the grains. Thus, with a higher extraction rate, more minerals and dietary fiber would remain undisturbed, presumably affording the best possible nutritional advantages. However, these minimally disturbed grain products contain anti-nutrients as well : oxalates, tannins (polyphenols), and phytates, all of which may adversely affect mineral bioavailability. When attempts are made to remove or minimize the effects of the anti-nutrients, the dietary fiber matrix is often altered or destroyed. Moreover, if dietary fiber fractions are removed, concentrated and then added back, the original food takes on new characteristics, eliciting different metabolic responses (Harland, 1989)

2.7. Iron absorption enhancers

2.7.1. Ascorbic acid

Ascorbic acid is the most potent enhancer of non-heme iron absorption. Synthetic vitamin C increases the absorption of iron to the same extent as the native ascorbic acid in fruits, vegetables, and juices. The effect of ascorbic acid on iron absorption is so marked and essential that this effect could be considered as one of vitamin C's physiologic roles. Each meal should preferably contain at least 25 mg of ascorbic acid and possibly more if the meal contains many inhibitors of iron absorption. Therefore, a requirement of ascorbic acid for iron absorption should be taken into account when establishing the requirements for vitamin C, that are set only to prevent vitamin C deficiency (especially scurvy) (WHO, 2004).

2.7.2. Muscle tissue

Meat, fish, and seafood all promote the absorption of non-heme iron. The mechanism for this effect has not been determined. It should be pointed out that meat also enhances the absorption of heme iron to about the same extent. Meat promotes iron nutrition in two ways: it stimulates the absorption of both heme and

non-heme iron and it provides the well-absorbed heme iron. Epidemiologically, the intake of meat has been found to be associated with a lower prevalence of iron deficiency. Organic acids, such as citric acid, have in some studies been found to enhance the absorption of non-heme iron. This effect is not observed as consistently as is the effect of ascorbic acid. Sauerkraut and other fermented vegetables and even some fermented soy sauces enhance iron absorption. The nature of this enhancement has not yet been determined (WHO, 2004, Hurrell & Egli, 2010).

2.8. Effect of size reduction on iron bioavailability

Iron in wheat is confined in the aleurone layer, a single layer of cells located between the endosperm and outer pericarp of the wheat grain (Figure 2.4). This layer is removed as part of the bran component during the production of white flour. Since the aleurone layer contains approximately 70% of the iron in wheat grain, the bioavailability of endogenous iron in wheat can be increased by mechanical disruption of wheat to rupture the cell walls comprising the aleurone layer and thereby enhance iron bioavailability. Remarkably, particle size reduction enhanced iron bioavailability to both elemental iron and iron nanocompounds (Latunde-Dada *et al.*, 2014).

An advantage of the mechanical approach to iron release from wheat aleurone is that the bioaccessibility of the endogenous aleurone iron reservoir could be increased through modified food processing technique. Potentially this could enhance the bioaccessibility of iron from wholegrain flour. Furthermore, micromilled aleurone could offer a natural, stable, bioavailable iron fortificant or complement in foods (Latunde-Dada *et al.*, 2014).

2.9. Effect of enzyme treatment on iron bioavailability

Applying exogenous enzymes to disrupt the food matrix could be an effective way of assessing the relative effects of different iron absorption inhibitors, which would make it possible to better target the inhibitors and to increase their degradation (Baye *et al.*, 2015). The addition of exogenous phytase or its activation during food processing, or the addition to a meal just before human consumption, has been shown to improve iron absorption significantly (Hurrell & Egli, 2010; Hallberg *et al.*, l987). According to (Baye *et al.*, 2013, Hallberg *et al.*, l987), the use of exogenous enzymes to hydrolyse fibers or oxidize polyphenols after dephytinization of flours resulted in significant increase in iron bioavailability ($P<0.05$).

According to Baye *et al.*, (2015), the effect of different enzyme treatments targeting phytate, iron-binding polyphenols, and plant cell wall constituents (hemicelluloses + cellulose) on iron bioaccessibility were evaluated. Treatments with phytase or xylanase + cellulase also reduced iron-binding polyphenols, although to a lesser extent. There are several possible reasons for this reduction: treatment with xylanase + cellulase leads to the disruption of plant cell wall integrity and hence to the release of endogenous polyphenol oxidases trapped in the vacuoles and plastids of the grains. The use of xylanase + cellulase showed that the hydrolysis of native fibers increased iron *in vitro* bioaccessibility. The hydrolysis of dietary fibers with xylanase + cellulase after dephytinisation improved iron bioaccessibility, suggesting the effect of the fiber is independent of phytate content.

2.10. Methods of assessing iron bioavailability

There are a number of techniques developed to assess iron bioavailability. Several of them can be combined creating a vast number of methods with varying accuracy when it comes to accomplish results relevant in humans. However, all techniques

and methods have limitations and advantages, and the balance between these differs depending on the context they are used in. Some of iron bioavailability measurement methods are discussed below:

2.10.1. Solubility and dialyzability

Solubility is the first step in covering the bioavailability definition mentioned above, and thus a necessity for iron absorbability. In order to simulate the gastrointestinal environment a commonly used procedure is to measure the solubility in dilute hydrochloric acid (HCl) (Wienk et al., 1999). To further mimic the iron absorption taking place in vivo, methods also introducing the aspect of dialyzability have been used. The principle of the dialyzability method is that following an in vitro enzymatic digestion of a test meal the dialyzable iron passes over a dialysis membrane, which then can be quantified by spectrophotometry (Wienk et al., 1999).

2.10.2. Caco-2 cells

A commonly used *in vitro* model is the uptake of iron by Caco-2 cells. Although these cells are derived from human colon adenocarcinoma, they exhibit many features of small intestinal cells, including the influence from dietary factors on iron absorption (Sandberg, 2010). One indicator of the iron uptake in Caco-2 cells is the ferritin formation in the cells. Another approach to study Caco-2 uptake is to use radioactive iron isotopes. The Caco-2 cell model has in some studies proved to be a rapid screening procedure of the potential maximum bioavailability for iron compounds (Sandberg, 2010).

2.10.3. Hemoglobin regeneration efficiency

Hemoglobin regeneration anemic rat assay has been used to study the bioavailability of iron from human foods. The only logical and complete measure of

iron availability using the hemoglobin regeneration anemic rat model was to divide the total gain in hemoglobin iron during the repletion period by the total intake of iron over the same period (Hazell, 1987).

2.11. Assessment of iron status

Iron status can be determined by several well-established tests in addition to measurement of hemoglobin or haematocrit. Unfortunately, however, there is no single standard test to assess iron deficiency without anaemia. The use of multiple tests only partially overcomes the limitation of a single test (WHO, 2001) and is not an option in resource-poor settings. Moreover, iron-related tests do not all correlate closely with one another because each reflects a different aspect of iron metabolism (Gibney *et al*., 2009). In anaemic individuals, such tests are used to confirm or help clarify the type or cause of anaemia. The most commonly used methods to assess iron status include: serum ferritin, transferrin saturation, erythrocyte protoporphyrin, mean corpuscular volume, serum transferrin receptor hemoglobin or packed cell volume.

2.11.1. Serum ferritin

The serum ferritin level is the most specific biochemical test that correlates with relative total body iron stores. A low serum ferritin level reflects depleted iron stores and hence is a precondition for iron deficiency in the absence of infection. Serum apoferritin is an acute-phase reactant protein and is therefore elevated in response to any infectious or inflammatory process. Consequently, serum ferritin in the normal range reflects only iron sufficiency in the absence of these conditions. Interpretation of serum ferritin levels is thus problematic in populations in which, with the exception of parasitic infections and malaria, the incidence of infection or inflammation is high. Interpretation of serum ferritin as an indicator of the relative

extent of depletion of iron stores is presented in Table 2.1. The generally accepted cut-off level for serum ferritin, below which iron stores are considered to be depleted, is <15 µg/l. Kits used for serum ferritin determination should be carefully calibrated against the WHO standard shown in Table 2.1 (WHO, 2001).

Table 2.1. Relative extent of iron stores on the basis of serum ferritin concentration.

Iron stores	Serum ferritin (µg/l)			
	Less than 5 years of age		More than 5 years of age	
	Male	Female	Male	Female
Depleted iron stores	< 12	< 12	< 15	< 15
Depleted iron stores in the presence of infection	< 30	<30	-	-
Severe risk of iron overload	-	-	> 200 (adult male)	> 150 (adult female)

Significant variations in serum ferritin levels relating to vulnerability to iron deficiency occur across age and gender groups. Infants, young children, and pregnant women usually have serum ferritin values near or in the range reflective of depletion; however, a low level per se does not imply functional iron deficiency. Only when the mobilizable iron supply for physiological function is inadequate is iron deficiency considered present. Serum ferritin measurement is the preferred method for detecting depleted iron stores. However, it is of limited usefulness during pregnancy because it diminishes late in pregnancy, even when bone marrow iron is present (WHO, 2001).

2.11.2. Transferrin saturation

Iron deficiency results in a reduction in serum iron (SI) levels, an elevation in transferrin (total iron-binding capacity [TIBC]) levels, and hence a net reduction in transferring saturation (i.e. SI/TIBC). However, the diurnal variation both in serum iron and transferrin saturation is considerable. In addition, there is a marked overlap in these indices between normal and iron-deficient subjects. This overlap

diminishes the usefulness of these indices in establishing or rejecting a diagnosis of iron deficiency. Transferrin saturation is of great value, however, as the first screening step for hereditary haemochromatosis. Cut-off values of between 60% and 70% have been widely used for this purpose. In screening for iron deficiency, individuals with more marked anaemia (responding to iron therapy with a hemoglobin increase >20 g/l) usually have a transferrin saturation <16% (WHO, 2001).

2.11.3. Erythrocyte protoporphyrin

Levels of erythrocyte protoporphyrin, the precursor of haem, become elevated when the iron supply is inadequate for haem production. With adequate iron, erythrocyte protoporphyrin levels, like those of hemoglobin, are maintained within a well-defined normal range in healthy individuals. Table 2.2, on the following page, reflects the several equivalent units in which erythrocyte protoporphyrin cut-off levels can be expressed. In general, an elevated erythrocyte protoporphyrin level correlates well with low serum ferritin, and can serve to screen for moderate iron deficiency without anaemia. Three commonly encountered conditions, in addition to iron deficiency, can cause a significant elevation of erythrocyte protoporphyrin: infection or inflammation, lead poisoning, and haemolytic anaemia. For this reason, the measurement of erythrocyte protoporphyrin is most useful in settings where iron deficiency levels are common and where infections, lead poisoning and other forms of anaemia are rare. Until recently, erythrocyte protoporphyrin was measured by a complex and costly procedure that limited its use to that of a reference method. A simplified haematofluorometer that directly measures erythrocyte protoporphyrin fluorescence is now available. This device has enabled the widespread use of erythrocyte protoporphyrin testing in outpatient settings in the USA. The severity of iron deficiency on the basis of erythrocyte protoporphyrin measurement is reflected in Table 2.2, below. Erythrocyte protoporphyrin levels are considered normal if only mild iron depletion is present (i.e. with serum ferritin

levels of 12-24 mg/l). In the absence of infection, measurement of erythrocyte rotoporphyrin is the preferred method for detecting iron deficiency once serum ferritin drops below the cut-off value, indicating inadequate iron supply to tissues (WHO, 2001).

Table 2.2. Changes in iron status by age group on the basis of erythrocyte protoporphyrin.

Iron status	Erythrocyte protoporphyrin	
	Less than 5 years of age	More than 5 years of age
Iron overload or excess	Normal	Normal
Normal	Normal	Normal
Mild iron deficiency without anaemia	Normal	Normal
Moderate iron deficiency without anaemia	>70 µg/dl red blood cell >2.6 µg/g hemoglobin >61 mmol/mol haem	>80 µg/dl red blood cell >3.0 µg/g hemoglobin >70 mmol/mol haem
Severe iron deficiency with anaemia	>70 µg/dl red blood cell >2.6 µg/g hemoglobin >61 mmol/mol haem	>80 µg/dl red blood cell >3.0 µg/g hemoglobin >70 mmol/mol haem

2.11.4. Mean corpuscular volume

Among all the red cell indices measured by electronic blood counters, mean corpuscular volume and mean corpuscular hemoglobin are the two most sensitive indices of iron deficiency. Reduction in mean corpuscular volume occurring in parallel with anaemia is a late phenomenon in the development of iron deficiency (WHO, 2001).

2.11.5. Serum transferrin receptors

The measurement of serum transferrin receptors is a recent addition to the available selection of tests for iron deficiency. However, epidemiological studies have yielded limited information concerning the usefulness of this test in discriminating between iron-deficient and iron-replete subjects. An increase in

serum transferrin receptors is a sensitive response during the early development of iron deficiency. Serum transferrin receptor levels increase progressively as the supply of iron to the tissues becomes progressively more deficient. Major advantages of measuring serum transferrin receptors involve the facts that the assay is not significantly affected by infection or inflammatory processes, and it does not vary with age, gender, or pregnancy. However, serum transferrin receptor levels may be elevated when there is increased red cell production, turnover, or both, such as in the case of haemolytic anaemia. There are several methods for measuring serum transferrin. The most commonly used method is based on the ELISA assay (enzyme-linked immunosorbant assay). The values obtained will vary according to the method used, however, since there is no uniform standard available for their measurement. Similarly, there is currently no universally agreed reference value for serum transferring (WHO, 2001).

2.11.6. Hemoglobin measurement

The prevalence of anaemia in a population is best determined by using a reliable method of measuring hemoglobin concentration. Compared with the cost and difficulty of biochemically assessing the prevalence of iodine deficiency and vitamin A deficiency, the determination of the prevalence of anaemia in a population is relatively simple and inexpensive. The only methods generally recommended for use in surveys to determine the population prevalence of anaemia by hemoglobinometry are the cyanmethemoglobin method in the laboratory and the HemoCue system. There exists a range of other quantitative and semi-quantitative methods for determining hemoglobin concentration. A report of the strengths and weaknesses of the various methods that may have an application in clinical practice is available. Again, however, only the two methods described above are generally recommended (WHO, 2001).

The cyanmethemoglobin method: for determining hemoglobin concentration is the best laboratory method for the quantitative determination of hemoglobin. It serves as a reference for comparison and standardization of other methods. A fixed quantity of blood is diluted with a reagent (Drabkins solution) and hemoglobin concentration is determined after a fixed time interval in an accurate, well-calibrated photometer (WHO, 2001).

The HemoCue system: is a reliable quantitative method for determining hemoglobin concentrations in field surveys, based on the cyanmethemoglobin method. The HemoCue system consists of a portable, battery-operated photometer and a supply of treated disposable cuvettes in which blood is collected. The system is uniquely suited to rapid field surveys because the one-step blood collection and hemoglobin determination do not require the addition of liquid reagents. Survey field staff without specialized laboratory training have been successfully trained to use this device. The HemoCue system gives satisfactory accuracy and precision when evaluated against standard laboratory methods. Long-term field experience has also shown the instrument to be stable and durable. These features make it possible to include hemoglobin determinations in multipurpose health and nutrition surveys (WHO, 2001).

2.11.7. Haematocrit or packed cell volume

Haematocrit or packed cell volume is a commonly performed clinical assessment frequently used in surveys of anaemia because of its simplicity and the widespread availability of the necessary equipment. Haematocrit measurement is an acceptable and recommended method for anaemia determination, but has no advantage compared to hemoglobin measurement. Moreover, reliable haematocrit determination requires a stable power supply. For haematocrit determination, blood is collected in anticoagulant-treated capillary tubes and spun in a small, specially designed centrifuge. The volume of packed cells as a portion of the total volume of

blood is measured and expressed as l/l whole blood. Many referral hospitals have electronic cell counters. Provided that they are well calibrated and maintained, these devices can yield rapid and reliable indications of mean cell volume (MCV) and the number of red blood cells (RBC) from which a "calculated haematocrit" (MCV x RBC concentration) can be obtained. In general, centrifuge haematocrit and calculated haematocrit based on electronic counters are closely matched. Although in most populations the prevalence of anaemia determined by using haematocrit or hemoglobin concentration (using the cut-off values given in Table 2.1), will be similar, results may not be identical. This difference in anaemia prevalence, obtained by using these two methods, may add to the complexity of a survey report and make the results more difficult for decision-makers to interpret. Accordingly, there is little advantage in determining haematocrit as well as hemoglobin during surveys. A potential source of error in hemoglobin and haematocrit determination lies in inadequate technique in obtaining capillary blood. Care must be taken to ensure adequate puncture of the tissue and spontaneous blood flow from the wound. The key to accuracy is blood sampling from finger- or heel-prick (WHO, 2001).

2.11.8. Bone marrow iron stain

A bone marrow stain for iron has been regarded as the reference against which to evaluate other iron tests. Absence of stainable iron reflects absent iron stores. For this reason, the bone marrow stain correlates best with serum ferritin, which is another measure of iron stores. For obvious reasons, bone marrow iron-staining is not useful in simple population-based surveys (WHO, 2001).

2.12. Assessing population iron status by using hemoglobin distributions

Estimates of hemoglobin are commonly included in nutrition surveys of children, whereas surveys specific for anaemia usually examine both children and women. The prevalence of anaemia serves as an index of the severity of iron deficiency in

the whole population. Where multiple factors may contribute significantly to anaemia, it is possible to differentiate anaemia attributable to iron deficiency from anaemia due to other factors. The latter include deficiencies of folic acid, vitamins A, B 12, and C; or infections including malaria, hookworm, and schistosomiasis. This differentiation can be achieved by observing blood smears, by means of a small supplementation trial, or by conducting specific tests. Where poor availability of dietary iron is the main etiologic factor, children and women are disproportionately affected, while the hemoglobin levels of adult men are virtually unaffected. Where other factors contribute significantly, adult men are less likely to be spared. A useful approach inolves comparison of the hemoglobin distribution among children, women, and men from the population under study with a non-anaemic reference population. This approach allows inferences to be made as to which factors are likely to be responsible for a high anaemia prevalence. One example involves the comparison of hemoglobin distributions among children, women, and men in a Palestinian refugee population in which iron deficiency was the sole cause of anaemia. Another involves an Ethiopian refugee population in which a combination of both iron and vitamin C deficiencies coexisted, with the vitamin C deficiency affecting men as well as women and children. Figures 2.5a 2.5b and 2.5c on the following page compares the hemoglobin distributions for each of these two refugee populations with US reference distributions (WHO, 2001).

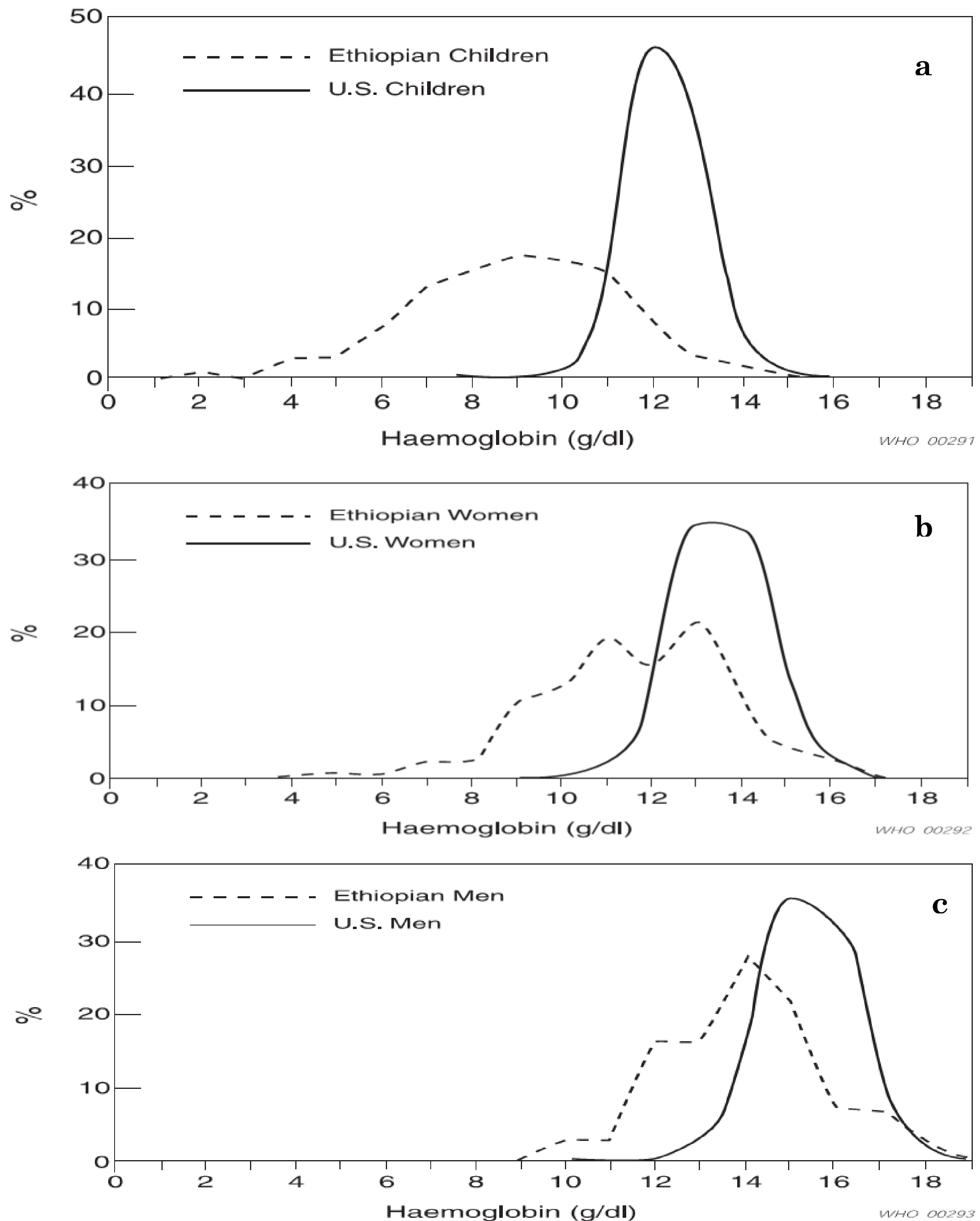

Figure 2.5. Hemoglobin distribution in Ethiopian vs US children, women, and men (WHO, 2001).

Chapter Three

Materials and methods

3. Materials and Methods

3.1. Location of Study Area

The study was carried out in research laboratory of Center For Food Science and Nutrition and Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia.

3.2. Experimental Design

The experimental design was completely randomized controlled design, and the study was carried out in three phases as shown in Figure 3.1.
This study includes three major experiments:

1st Phase : Proximate composition and analysis of iron in the samples.

2nd Phase: Further particle size reduction, enzymatic treatment and experimental diet formulation.

3rd Phase: Evaluation of bioavailability of iron by hemoglobin regeneration efficiency test in rats.

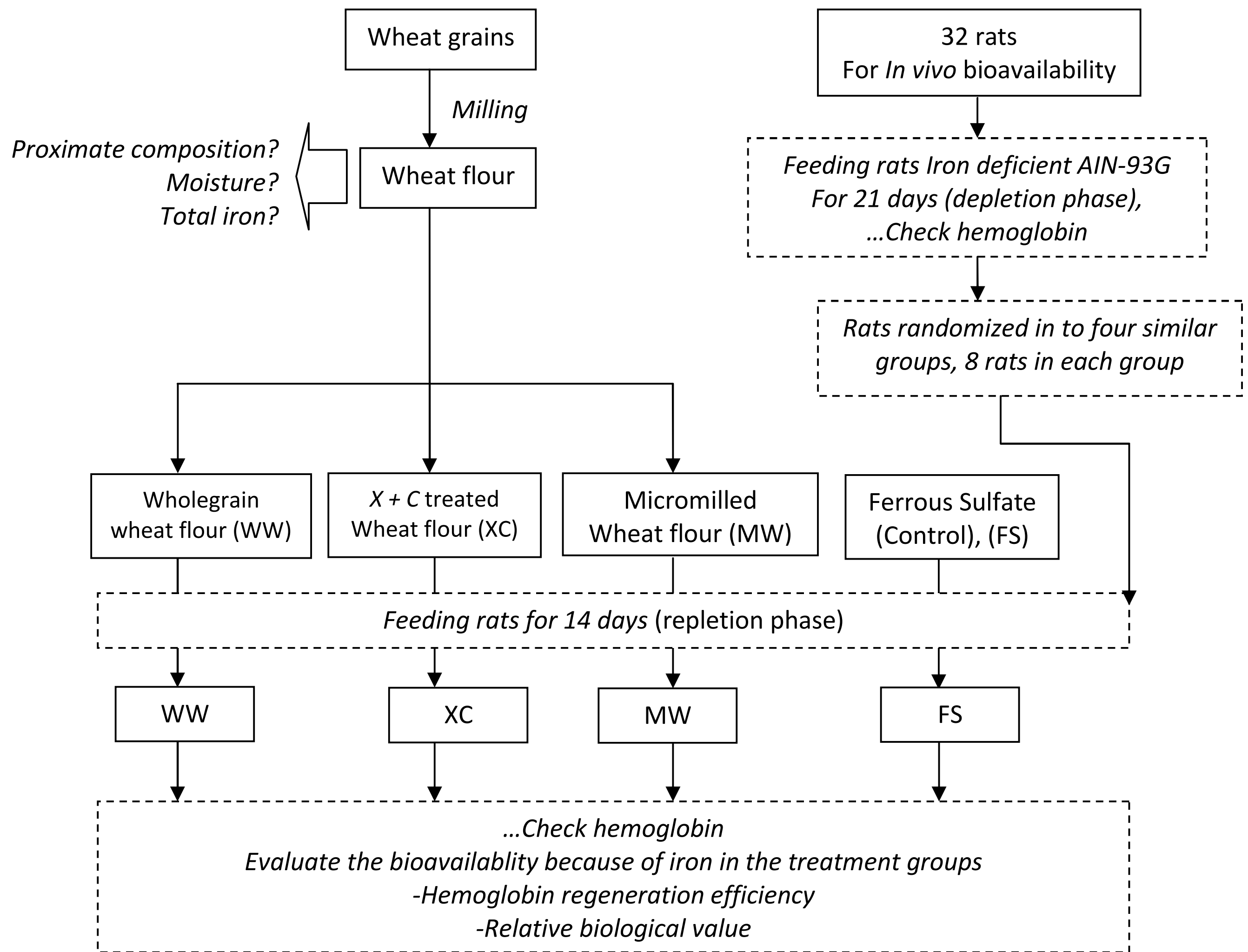

Figure 3.1. Experimental design

3.3. Sample Collection

Sample of bread wheat grain (*Triticum aestivum*) was collected from Holeta Agricultural Research Center (HARC). The center is located in the West Showa Zone of Oromia Regional State, in Holeta town, 45 km west of Addis Ababa, it has a latitude and longitude of 9°3′N 38°30′E and an altitude of 2391 meters above sea level. The average annual rainfall of the area is 1040 mm and the average maximum and minimum daily temperatures are 21 °C and 6 °C, respectively. The collected sample was kept in a closed metal free

polyethylene bags and stored at cool and dry place for subsequent use throughout the study.

3.4. Sample Preparation

Wheat grain sample was washed with running distilled-deionized water and dried in an oven at $50^{o}C$ for two days as shown in Figure 3.2. Whole grain wheat flour was prepared by milling the wheat grain sample using stainless steel laboratory miller (FW 100, china) in a manner that does not add any adventitious contamination and flour was kept in a dry polyethylene bags and closed.

Figure 3.2. Process of washing and drying: washing the sample with deionized water in plastic bowel (left), soil residues from washed sample (center), washed samples being dried in an oven (right).

Micromilled wheat flour was prepared by further milling the wholegrain wheat flour using stainless steel laboratory ball miller (Retsch PM100, Planetary ball mill, Germany).

3.5. Method of Analysis

Moisture content; determination of proximate composition (crude fiber, fat, ash content etc…) were carried out by using AOAC official methods of analysis. Determination of iron content were done using atomic absorption spectrophotometer method after wet digestion and a series of standard solutions were prepared by serially diluting metal stock solution with deionized water (Osborne & Voogt, 1978).

3.5.1. Moisture content

Moisture content was determined according to AOAC (2000) using the official method 925.09. A crucible was dried in an oven at 105°C for 1 hour and placed in desiccators to cool. The weight of the crucible (W1) was determined. 5gm sample was weighed in the dry crucible (W2) and dried at 105°C for 3 hours and after cooling to room temperature in desiccators it was again weighed (W3). The moisture content was determined by using Eq. (1).

$$\%\text{Moisture} = \left(\frac{W2-W3}{W2-W1}\right) \times 100 \qquad (1)$$

3.5.2. Determination of crude protein

Protein content was determined according to AOAC (2000) using the official method 979.09. **Digestion:** About 0.5 g of fresh samples (in triplicate) was added to a Tecator tube and 6ml of acid mixture (5parts of concentrated ortho-phosphoric acid and 100 parts of concentrated sulfuric acid) were added and mixed, and 3.5 ml of 30% hydrogen peroxide was added step by step. As soon as the violet reaction is ceased, the tubes were shaken and placed back to the rack. Three gram of catalyst mixture (ground 0.5 g of selenium metal with 100 g of potassium sulfate) were added into each tube, and allowed to stand for about 10 minutes before digestion. When the temperature of the digester attained 370°C, the tubes were lowered into the digester. The digestion was continued until a clear solution is obtained

(about 4 hour). The tubes in the rack were cooled in a fume hood; 25 ml of de-ionized water were added, and shake to avoid precipitation of sulfate in the solution.

Distillation and titration: The digested and diluted sample solution were distilled using boric acid and the distillate were titrated using 0.1N hydrochloric acid until reddish color appeared. The crude protein was determined by using Eq. (2) and (3).

$$\%\mathbf{Nitrogen} = \frac{\mathbf{V\ HCl \times N\ HCl \times 14.0 \times 100}}{\mathbf{1000 \times Wo}} \quad (2)$$

$$\%\mathbf{Protein} = \mathbf{6.25} \times \%\mathbf{Nitrogen} \quad (3)$$

Where;

V-volume of HCl consumed (ml) to the endpoint of titration

N-the normality of the HCl used

Wo-Sample weight on dry matter basis

14-the molecular weight of atomic nitrogen

6.25-conversion factor

3.5.3. Determination of crude fat

The crude fat was extracted according to AOAC (2000) official method 4.5.01. About 2g of flour were extracted with 50 ml diethyl ether for a minimum period of 4 hours in the soxhlet extractor (2010 05 025, China). The solvent were then evaporated and the extracted fat was dried in the oven and cooled in a desiccator. The crude fat was determined using Eq. (4):

$$\%\text{Fat} = \frac{W2 - W1}{W} \times 100 \quad (4)$$

Where;

W_1 = Weight of the extraction cylinder

W_2 = Weight of the extraction cylinder plus the dried crude fat

W = Weight of the sample

3.5.4. Determination of ash content

The ash content was determined by AOAC (2000) using official method 923.03. Washed porcelain dishes with distilled and deionized water was placed in a muffle furnace for 30min at 550 ^oC. The dishes were cooled in desiccators (with granular silica gel) for about 30 minutes at room temperature and was weighed to the nearest milligram (M1). About 2.5g of fresh sample were weighed in a dish (M2). The dishes were placed on a hot plate under a fumehood and the temperature was slowly increased until smoking ceases and the samples became thoroughly charred. The dishes with the samples were placed inside a muffle furnace (Carbolite CSF 12/13, England) at 550^oC for 5 hours and cooled in desiccators for 1 hour. The ash should be clean and white in appearance. When cooled to room temperature, each dish with ash was reweighed to the nearest milligram (M3). The total ash content was determined using Eq. (5):

$$\text{Total ash } \% = \frac{\text{M3}-\text{M1}}{\text{M2}-\text{M1}} \times 100 \quad (5)$$

Where:

(M2-M1) is sample mass in g on dry base and (M3-M1) mass of ash in g.

3.5.5. Determination of crude fiber

Four crucibles were cleaned and dried with 0.001 gm celite in an oven at 105^oC for 1 hour. The crucibles were taken out and cooled in a desiccator. About 1 gm of wheat sample was weighed in to pre-dried crucibles (**W_1**). The crucibles were placed in the digestor using a holder. 1.25% H_2SO_4 solution was added in to each column by pressing the button for R_1. The time was set for 37 minutes and the temperature between 6 and 8. 3-5 drops of n-octanol antifoaming agent was dropped into each column to prevent loss of sample. The acid was drained by using a vacuum pump after 37 minutes. The samples were cooled for 5 minutes and washed with distilled water twice. 1.25% NAOH solution waas added in to each column by pressing the button for R_2. The time and temperature was set as previous, the base was drained by using a vacuum pump after the set time. The samples were washed again with distilled water twice. The crucibles containing residue were dried at 130^oC for 2

hours. The crucibles were cooled in a desiccator and weighed (**W_2**). The residues were ashed in a muffle furnace at 550 for 3 hours. The crucibles were left to cool down to below 250°C before removing from the furnace. The crucibles were cooled in a desiccator to room temperature and their weight was measured using analytical balance (**W3**). The crude fiber content was determined using Eq. (6):

$$\%\textbf{Crude fiber} = \frac{\mathbf{W1-W2}}{\mathbf{W1}} \times \mathbf{100} \qquad (6)$$

Where

W_1 = Sample weight
W_2 = Crucible + residue
W3 = Crucible + ash residue

3.5.6. Determination of iron

Iron content was determined using atomic absorption spectrophotometer method of Osborne & Voogt, (1978). Ash was obtained from dry ashing of food samples. The ash was wetted completely with 5ml of 6N HCl, and dried on a low temperature hot plate. 7ml of 3N HCl was added to the dried ash and heated on the hot plate until the solution boiled. The ash solution was cooled to room temperature in a hood and filtered into a 50ml graduated flask using a filter paper (whatman 45,125mm). 5ml of 3N HCl was added into each crucible dishes and heated until the solution boiled, was then cooled and filtered into the flask. The crucible dishes were again washed three times with deionized water; the washing was filtered into a flask. Then, the solution was cooled and diluted to 50ml with de-ionized water. A blank which contains 12ml 3N HCl and deionized water in 50ml volumetric flask was also prepared for FAAS reading. Standard solutions: Four series of working standard metal solution (Table 3.1) was prepared by appropriate dilution of the metal stock solution (nitrate of the metal) with deionized water containing 2.4ml 3N HCl in 10ml volumetric flask. Calibration curve (concentration versus absorbance) for each element using the prepared standard solution was prepared. The sample

concentration was analyzed using FAAS by aspirating de-ionized water. Sample blank solution was run with the sample solution.

Table 3.1 Series of working standard solution for iron determination

Series no.	Standard concentration(ppm)
1	0.0
2	0.5
3	1
4	3
5	4

$$\text{Iron content} \left({}^{mg}/_{100g}\right) = \frac{[(a-b)-V]}{10W} \quad (9)$$

Where; W= weight (g) of samples V= 50ml = volume (V) of extract a= concentration (μg/ml) of sample solution b= Concentration (μg/ml) of blank solution

3.5.7. Determination of particle size

A representative weighed sample is poured into the top sieve which has the largest screen openings of 1.8mm. Each lower sieve in the column has smaller openings than the one above. At the base is a round pan, called the receiver. The column is typically placed in a mechanical shaker. The shaker shakes the column, usually for 15-20 minutes. After the shaking was complete the material on each sieve was weighed. The weight of the sample in each sieve was then divided by the total weight to give a percentage retained on each sieve. The size of the average particles on each sieve then being analysis to get the cut-point or specific size range captured on screen. To find the percent of aggregate passing through each sieve, first find the percent retained in each sieve. To do so, equation (10) was used.

The next step is to find the cumulative percent of aggregate retained in each sieve. To do so, add up the total amount of aggregate that is retained in each sieve and the amount in the previous sieves. The cumulative percent passing of the aggregate is found by subtracting the percent retained from 100%, equation (11). The values are

then plotted on a graph with cumulative percent passing on the y axis and sieve size on the x axis according to the procedure described by Sonaye & Baxi, (2012).

$$\% \text{ Retained} = \frac{\text{Wsieve}}{\text{Wtotal}} \times 100 \qquad (10)$$

Where W_{sieve} = weight of aggregate in the sieve and
W_{total} = total weight of the aggregate.

$$\%\text{Cumulative Passing} = 100\% - \%\text{Cumulative Retained} \qquad (11)$$

3.6. Enzymatic Degradation

The enzymes used in this study were fungal, xylanase from Non-pathogenic, non-gene-modified *Aspergillus niger* strain (Stern E.C.3.1.3.26 H 14009, activity 54000-67500 u/g solid) and cellulase from Non-pathogenic, non-gene-modified *Aspergillus niger* strain (Stern E.C.3.1.3.26 C 13030, activity 220-270 u/g solid).

3.6.1. Enzyme solution preparation

To prepare 1000 ml of 0.1 M acetate buffer at pH: 5.6, we prepared 200 ml of 0.2 M acetic acid (CH_3COOH), add 2.31 ml acetic acid from the stock solution and completed to 200 ml with deionized water. Then, 500 ml of 0.2 M Sodium acetate ($CH_3COONa.3H_2O$) was prepared by weighing 13.6 g of sodium acetate from the stock and complete to 500 ml with deionized water.
Finally a mixture of 48 ml of 0.2 M acetic acid and 452 ml of 0.2 M Sodium acetate were mixed and completed to 1000 ml with deionized water. The pH of the buffer was checked.

Enzymatic solution of XC was prepared by adding 0.62g of celluloae and 0.0018 g of xylanase for every one gram of flour respectively. The mixture was homogenized. Solutions were kept at 4 ^{o}C for a maximum of 24 hours as described in by Baye *et al.*, (2014).

3.6.2. Enzyme (Xylanase+Cellulase) treatment

Treatment with xylanase and cellulase (XC) was performed on the wheat flour by suspending in 0.1 M acetate buffer (pH=5.6) in 1:3 (W/V) flour: buffer properties. For every gram of flour 0.0018 g of xlynase (~50 XU) and 0.062 g of cellulase (6.8 CU) was added, and the mixture was incubated in a shaking water bath for 3 hour at 35 ^{o}C, (according to the procedure described by Baye *et al*., 2014).

3.7. Hemoglobin Regeneration Efficiency Test in Animals (*in vivo*)

3.7.1. Ethical consideration

Animals were handled as per the National Research Council of National Academies Guide for the care and use of laboratory animals (2011) and this study was carried out after obtaining ethical approval from Addis Ababa University Ethical Clearance Review Board (Annex II).

3.7.2. Study animals screening and handling

A total of thirty two weaned, male wistar rats, 21-28 days old with initial weight ranging from 40–60 gram was obtained from Ethiopian Public Health Institute (EPHI) and were housed individually in stainless steel covered plastic cages at room temperature with 12 hrs light and dark cycles.

3.7.3. Composition and preparation of experimental diets

Diets were prepared based on AIN-93G composition, according to (Reeves, et al 1993) as shown in Table 3.2. Ingredients were weighed and mixed using plastic tools. Since variation in the levels of vitamin B2, B12, folate and vitamin A affect

hemoglobin levels, Vitamins and mineral contents of the mixes among groups were made similar (Ouedraogo *et al.*, 2010).

Iron absorption enhancer (Vitamin C) and minerals that can compete with iron absorption like, zinc, and calcium (Zimmermann & Hurrell, 2007) and other micronutrients were also adjusted by adding vitamin mix (AIN-93G VX; Harlan Tekladiet, 46 Madison, WI, USA) and minerals mix (AIN-93G MX, MP Biomedicals, LLC; Cat No: 960400, Lot No: 9634, Illkirch Cedex, France). Total energy of the treatment diets were made isocaloric (Weber *et al.*, 2010), which was adjusted by adding 13.47 g of soybean oil per Kg to the XC, MW and WW containing diets; as soy bean oil is the only single source of fat in AIN-93G diet (Reeves *et al.*, 1993). Finally, the prepared diets were stored in polyethylene bags at refrigerator at 4 ^{o}C until the preparation of pellets (Reeves *et al.*, 1993).

Table 3.2: Composition of experimental diets

Components **g/kg**	Depletion Period	Repletion Period			
	Fe def AIN-93G	WW + AIN-93G	MW + AIN-93G	XC + AIN-93G	$FeSO_4.7H_2O$ + AIN-93G
Total Iron (mg)	--	35	35	35	35
Total Lipid (Soy bean oil)	70	36.7*	36.7*	36.7*	70
Total Protein	200	140.62	140.62	140.62	200
Total Carbohydrate	629.5	745.79	745.79	745.79	629.5
Iron-Free Mineral Mix	35	35	35	35	35
Vitamin Mix	10	10	10	10	10
Total Energy (k.cal/kg)	**3945.45**	**3945.45**	**3945.45**	**3945.45**	**3945.45**

Fe def: iron deficient, AIN-93G: American Institute of Nutrition Formula for test rats, WW: Wholegrain wheat flour, MW: Micro milled wheat flour, XC: xylanase+cellulase treated wheat flour *Addition of soy bean oil for calorie adjustment

Pellets were formed through agglomerating powdered diet by adding deionized water of 160- 180ml to 500g diet powder, and made in to paste with the right consistency manually in plastic bowl and the formed clumps forced to pass through a hollow plastic tube to have a uniform cylindrical shape (diameter of 15mm) and then the strip was cut in to shorter length (about 5cm) with stainless steel cutting knife or braking to the edge of the tube by hand as shown in Figure 3.3. Finally, pellets placed in an oven to dry at 50 ^{o}C overnight, then removed from an oven and allowed to cool, then placed within air tight polyethylene zipped bag in refrigerator until provided to rats as per the method elucidated by Green and Turner (1974).

Figure 3.3: Process of making pellets: adding deionized water (a) and mixing powder diet in a plastic bowel (b), extruding the dough through a plastic tube (c), the strips cut in to shorter length pellets (d), preparing pellets to be dried in an oven (e) and dried pellets (f).

3.7.4. Depletion phase

The rats were fed iron deficient AIN-93G diet (Dytes Inc., USA) to induce iron deficiency anemia, as recommended by the American Institute of Nutrition for 21-28 days, until they become anemic, with Hb lower than 6g/dl . During the whole study period, the rats received deionized water and rations *ad libitum* (Bokhari *et al.*, 2012). Dietary intake by individual rat was recorded daily. Hb measurement was done by HemoCue after collecting blood through incision at the terminal portion of the tail. HemoCue method is easy for operation and has high sensitivity & specificity relative to the gold standard Hb assessment method, direct cyanmethemoglobin method (Sari *et al.*, 2001).

3.7.5. Repletion phase

After inducing iron-deficiency anemia, eight rats were randomized in to four similar groups according to Hb level to obtain groups with similar mean. The 1st group was fed with diet containing 35mg of iron mixed with per Kg of the standard diet; the 2nd group was fed with XC treated wheat flour; the 3rd group was fed with micro milled wheat flour and the 4th group was fed with wholegrain wheat flour. The rats were fed diet as per the randomly assigned experimental diet and given deionized water *ad libitum* for 14 days. Amounts of diet fed by the rats were recorded daily. At the end of repletion period, blood sample was collected by tail incision for determination of Hb and also final body weight recorded.

Figure 3.4. Rat in individualized plastic cage (a, b), Body weight being measured (c), Rat in restrainer for blood collection via tail incision (d, e) and reading Hemoglobin using Hemocue machine (f).

3.7.6. Analysis of Hemoglobin

Hemoglobin concentration was measured by HemoCue method, and blood was obtained from the tail tip. To calculate total hemoglobin content in the blood, the mass of blood assumed to be 67gm/Kg body mass, and hemoglobin is assumed to contain 3.35mg Fe/Kg. And hemoglobin efficiency was calculated according to the method of (Lucia *et al.*, 2013; Miyada *et al.*, 2011). Iron (Fe) consumption was calculated considering the total amount of diet consumed and the iron content of the

specific diet, which was calculated for each animal according to the following formula:

Fe consumption = [total diet consumption during repletion period (g) x Iron in the diet. (mg/1000 g)] / 1000 (12)

Fe pool (mg) = [Fe Hb (final)–Fe Hb (initial) x 100] / Fe intake (mg) (13)

Hb Regeneration Efficiency (HRE):

% HRE = [Hb Fe pool (final)–Hb Fe pool (initial) x 100] / Fe intake (mg) (14)

Where: Hb Fe (initial) = [weight (g) (initial) x Hb (g / dL) (initial) x 6.7 x 0.335] /1000 Fe Hb (final) = [weight (g) x (final) x Hb (g / dL) (final) x 6.7 x 0.335] / 1000

Relative Biological Value (RBV):

RBV = 100 x (% HRE test group / % HRE FS group) (15)

3.8. Statistical Analysis

Considering ferrous sulfate as the control group, the four groups (FS, XC, MW and WW) were compared for hemoglobin levels, hemoglobin regeneration efficiency, relative biological value, weight, iron consumption and other parameters using one way analysis of variance (ANOVA). Significant ($p < 0.05$) differences between means were tested by the Duncan's *post-hoc* multiple comparisons test. Descriptive statistics were used and results are shown in terms of mean and standard deviation. Data were analyzed with Microsoft Office Excel 2010 and Statistical Package for the Social Sciences (SPSS), version 20.0.

Chapter Four

Results and discussion

4. Results and Discussion

A sample of wheat grain was washed, dried and milled. The flour was analyzed for its proximate composition, particle size and total iron. The *in vivo* bioavailability was evaluated using hemoglobin regeneration efficiency in rats fed with XC treated, Untreated and micro-milled wheat flour pellets. The bioassay results were compared to the standard ferrous sulfate (control group).

4.1. Proximate Composition and Total Iron Content of Wheat Flour

The proximate composition in protein, fiber, fat, ash and iron contents of the wheat sample analyzed in this study agrees with previous reports by, Charalampopoulos *et al* (2002), McKevith, (2004), Šramková *et al.*, (2009), Baye, (2014) and the Ethiopian food composition table (ENRHI, 1997).

Table 4.1. Proximate composition and iron of wholegrain wheat flour

Parameters determined	**Value (mean ± sd)**
Moisture (g/100 g)	7.40 ± 0.20
Fat (% DM)	2.07 ± 0.20
Ash (g/100g DM)	1.33 ± 0.23
Fiber (% DM)	2.32 ± 0.59
Protein (% DM)	13.74 ± 0.07
Carbohydrate (% DM)	75.21 ± 1.07
Iron (mg/100 g DM)	3.69 ± 0.04

4.2. Baseline Characteristics of the Experimental Rats

The rats (21 -28 days old) were placed on depletion phase for 21 -28 days and at the start of the repletion phase, they were 42-54 days old. The initial hemoglobin, body weight and age were not significantly different (P>0.05) statistically among the four groups as shown in Table 4.2. This shows that the groups were effectively randomized and homogeneity was kept among groups. Hemoglobin measurement considered altitude of Addis Ababa (2,300m above sea level) and for adjustment of 1.1 g/dL deducted from every hemoglobin measurement (WHO, 2011).

Table 4.2: Baseline characteristics of rats at end of depletion phase

	Treatment Groups			**Control**
	Wheat			**AIN-93G**
Indices	**WW (n=8)**	**MW (n=8)**	**XC (n=8)**	**FS (n=8)**
Initial Hb (g/dL)	5.26 ± 0.54[a]	5.40 ± 0.34[a]	5.35 ± 0.53[a]	5.40 ± 0.31[a]
Age (Days)	45.5 ± 3.7[a]	48.25 ± 3.2[a]	48.5 ± 4[a]	47.5 ± 2.7[a]
Weight Initial (g)	159.61 ± 11.85[a]	158.25 ± 8.08[a]	176.64 ± 37.08[a]	155.46 ± 6.19[a]

Values are mean ± standard deviation and different superscripts across a row represent statistically significant difference (P<0.05)

4.3. Food Intake and Body Weight during the Repletion Period

The total food and iron intake in ferrous sulfate group was significantly higher than the other groups (P<0.05) since higher efficiency of converting dietary iron in to hemoglobin in the ferrous sulfate group improved appetite (Kalantar-Zadeh *et al.*, 2004; Khan *et al.*, 2014). Whereas, the total food intake was the least in the micro-milled wheat flour group and the lowest iron intake (Table 4.3).

Table 4.3: Food intake, body weight and iron intake at the end of repletion period

Indices	Treatment Groups			Control
	Wheat			AIN-93G
	WW (n=8)	**MW (n=8)**	**XC (n=8)**	**FS (n=8)**
Total food intake (g)	259.05 ± 16.07^{b}	236.72 ± 9.43^{a}	255.88 ± 21.45^{b}	280.78 ± 6.62^{c}
Weight Gain (g)	62.25 ± 9.71^{ab}	70.45 ± 4.55^{bc}	54.45 ± 13.26^{a}	73.51 ± 5.36^{c}
Iron intake (mg)	9.56 ± 0.59^{b}	8.74 ± 0.35^{a}	9.44 ± 0.79^{b}	10.36 ± 0.24^{c}
Feed Efficiency Ratio	0.24 ± 0.03^{ab}	0.30 ± 0.01^{c}	0.22 ± 0.06^{a}	0.26 ± 0.02^{bc}

Values are mean ± standard deviation and different superscripts across a row represent statistically significant difference ($P<0.05$)

The feed efficiency ratio value of the micro-milled wheat flour group was significantly higher than the other groups ($P<0.05$) since efficiency of weight gain from total food intake was improved.

4.4. Hematologic Indices and Iron Bioavailability

The hemoglobin gain, hemoglobin iron and the hemoglobin regeneration efficiency (HRE) of rats consuming diets composed of wholegrain wheat flour, micro milled wheat flour, xylanase+cellulase treated wheat flour and AIN-93G enriched with ferrous sulfate, all supplying 35mg/kg iron are described in Table 4.4. The Relative Biological value (RBV) is also shown in Figure 4.1.

Table 4.4: Hemoglobin regeneration of rats taking 35mg iron per Kg of diet from the three treatment groups and the control group

Indices	**Treatment Groups**			**Control**
	Wheat			**AIN-93G**
	WW (n=8)	**MW (n=8)**	**XC (n=8)**	**FS (n=8)**
Initial Hemoglobin (g/dL)	5.26±0.54[a]	5.40±0.34[a]	5.35±0.53[a]	5.40±0.31[a]
Final Hemoglobin (g/dL)	8.34±0.50[a]	9.13±0.72[b]	10.97±0.39[c]	13.12±0.69[d]
Hemoglobin Gain (g/dL)	3.08±0.82[a]	3.73±0.48[a]	5.62±0.51[b]	7.72±0.77[c]
Initial Hemoglobin Iron (mg)	1.90±0.32[a]	1.92±0.21[a]	2.14±0.57[a]	1.89±0.15[a]
Final Hemoglobin Iron (mg)	4.16±0.31[a]	4.70±0.53[a]	5.69±0.70[b]	6.75±0.56[c]
Hemoglobin Regeneration Efficiency (HRE) %	23.66±4.51[a]	31.75±3.62[b]	37.84±3.99[c]	46.93±4.22[d]

Values are mean ± standard deviation and different superscripts across a row represent statistically significant difference (P<0.05).

The initial hemoglobin concentrations among treatment groups were similar, but hemoglobin gain was significantly higher (P< 0.05) in the control group (7.72±0.77), followed by the XC (5.62±0.51) and both the MW and WW (3.73±0.48, and 3.08±0.82) wheat flour groups. The hemoglobin gain between MW and WW wheat flour groups were not significantly different (P> 0.05).

This study showed that 47% of iron in the ferrous sulfate diet was converted to hemoglobin and this result is consistent with those observed by Weber *et al.* (2010), which was 43.0+9.46. Hemoglobin Regeneration Efficiency (HRE) of the ferrous sulfate (control) diet was about 50% higher than the wholegrain wheat flour group, 32% higher than the micro-milled wheat flour group and 18% higher than the

xylanase+cellulose treated wheatflour group. The difference was statistically significant among groups (P<0.05).

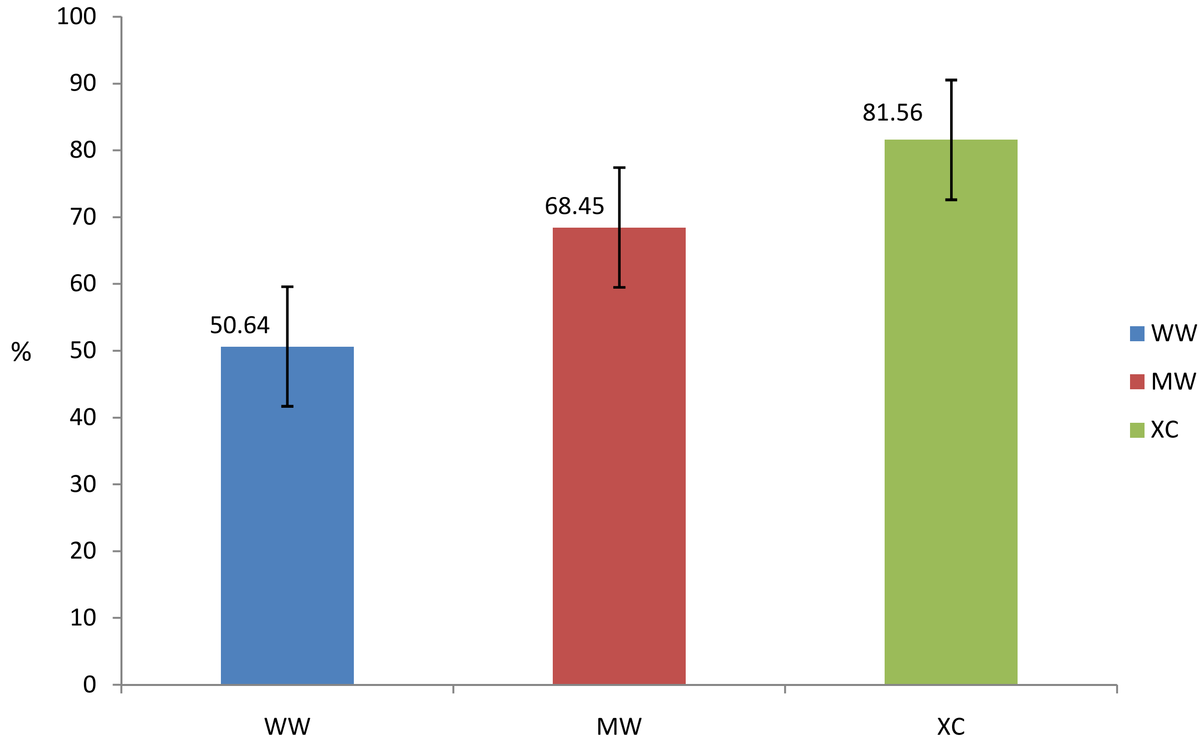

Figure 4.1. Relative biological values (RBV) among treatment groups.

The Relative Biological Values (RBV) for xylanase+cellulase treated, micro-milled and wholegrain wheat flour were 81.56±14.02%, 68.45±12.23% and 50.64±9.64% respectively (Figure 4.1.).

4.5. Bioavailability of Iron from Wheat in Comparison to other Iron Sources

The RBV of xylanase+cellulase treated wheat flour (81.56±14.02%) agrees with the previous report of 82.5% for soybean-fortified wheat flour bread (Urga *et al*., 1999) and 83% for whole wheat bread (Abdullah *et al*., 1987). The RBV of wholegrain wheat flour (50.64±9.64%) agrees with the previous report of 55% for wholegrain

wheat flour (Miguel *et al.,* 2003). The bioavailability of iron from wheat in comparison to other iron sources is shown in Table 4.5.

Table 4.5. Relative biological value (RBV) of anemic rats fed diets fortified with graded levels iron in comparison to the RBV of iron in wheat

Iron Source	Supplemental Iron Level (mg Fe/kg Diet)	*RBV %	Statistical Assay Method Used	Reference
Fe fortified whey protein concentrate diet	45	47.2±4.5	Mean Ratio	Nakano *et al.,* 2007
Enriched White Bread	30	64	Slope Ratio	Abdullah *et al.*, 1987
Whole Wheat Bread	30	83	Slope Ratio	Abdullah *et al.*, 1987
Cocoa Powder	20	35.2	Mean Ratio	Katsuhiko *et al.*, 2009
Beef	30	91	Slope Ratio	Abdullah *et al.*, 1987
Electrolytic Iron Poweder (EIP)	12	64.5	Slope Ratio	Swain *et al.*, 2013
$FeSO_4$+Na_2EDTA	35	86±0.03	Mean Ratio	Whittaker & Vanderveen 1990
NaFeEDTA	30	128±0.05	Mean Ratio	Whittaker & Vanderveen 1990
Difo Dabbo (sour dough bread) from soybean-fortified wheat flour	25	82.5±0.1	Mean Ratio	Urga *et al.*, 1999
Porridge from soybean-fortified wheat flour	25	35.8	Mean Ratio	Urga *et al.*, 1999
Teff Injera (Fermented teff product)	35	94.76±0.16	Mean Ratio	Urga *et al.*, 1998
Whole wheat flour	20	39.3	Mean Ratio	Grewal *et al.*, 2000
Wholegrain wheat flour	30	55	Mean Ratio	Miguel *et al.,* 2003
Wholegrain Wheat Flour	35	50.64±9.64	Mean Ratio	This study
Micro milled Wheat Flour	35	68.45±12.23	Mean Ratio	This study
XC treated Wheat Flour	35	81.56±14.02	Mean Ratio	This study

*The RBV of these iron sources from different studies were calculated considering similar iron level of ferrous sulfate group of that specific study.

Chapter Five

Conclusions and recommendations

5. Conclusions and Recommendations

In comparison to the ferrous sulfate control group, the efficiency of converting iron from XC treated wheat flour in to hemoglobin was 81.56±14.02%, 68.45±12.23% for micro milled wheat flour and 50.64±9.64% for wholegrain wheat flour. The present *in vivo* study clearly showed that XC treatment and particle size reduction can lead to hemoglobin repletion in iron deficient rats and is thus an important source of bioavailable iron. This suggests that wheat can be a good source of bioavailable iron if the mentioned treatments are applied.

The study showed that enzymatic degradation of fiber using XC significantly decreased fiber contents of the food matrix. The particle size reduction as well significantly increased the surface area of the flour which increases the rate of extraction of iron from the flour.

This study therefore recommends that further investigations to be made whether the treatments have no side effects particularly in connection to diabetes. Furthermore, the country's future fortification plan needs to consider the contribution of particle size reduction and enzymatic treatments.

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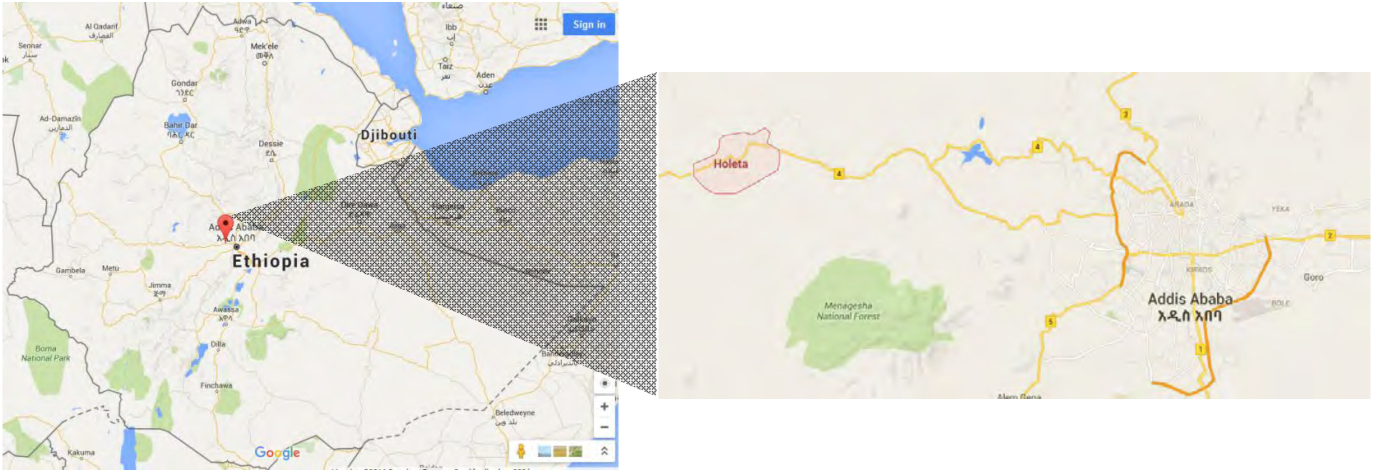
ANNEX

Annex-I: Sample Collection Site, Wheat Type, Growing Condition



Sample Collection Site:

Holeta Agricultural Research Center (HARC).
West Showa Zone of Oromia Regional State, in Holeta town,
45 km west of Addis Ababa,
Latitude and longitude 9°3′N 38°30′E
Altitude of 2391 meters above sea level
Average annual rainfall 1040 mm
Average maximum and minimum daily temperatures are 21 °C and 6 °C

Wheat Type:

Bread wheat grain (*Triticum aestivum*), white, meraro variety

Soil Type:

Nitosols (Local name: *Key Afer*)

Type of weed:

Broadleaf weeds

Weed killer used:

2,4 D (200 liter water and 1 liter weed killer per Hectar used)

Water Source:

Rain water

Type and amount of Fertilizers used:

DAP 125kg and UREA 75kg used per Hectar

Annex-II: Approval Letter from College of Natural Science Institutional Review Board (CNS-IRB), Addis Ababa University

COLLEGE OF NATURAL SCIENCES
Addis Ababa University

OFFICE OF THE DEAN
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Ref. / ቁጥር CNSDO/177/08/2016
Date. / ቀን January 12, 2016

To Whom It may Concern

College of Natural Science Institutional Review Board (CNS-IRB) has reviewed an MSc thesis project proposal entitled "Effect of particle size reduction and xylanase-cellulase treatment of wheat on rat hemoglobin regeneration efficiency" by Daniel Tsegaye from Center for Food Science and Nutrition.

The proposal was approved for implementation.

With regards,

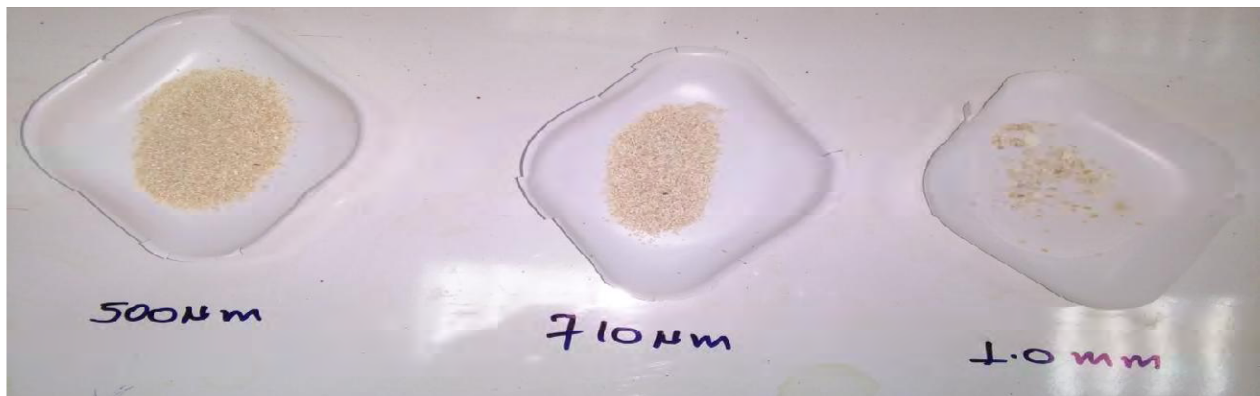
Dr. Shibru Temesgen
Dean College of Natural Science

ስልክ Tel: +251-11-123 94 72
ፋክስ Fax: +251-11-123 94 69
Please quote our reference number in your correspondence.
ፖ.ሣ.ቁ P.O.Box: 1176 Addis Ababa, Ethiopia
ኢሜል/Email: dean_cns@aau.edu.et

"Examine all things; hold fast that which is good"
"ሁሉን መርምሩ መልካሙን ያዙ"

Annex -III: Result of Particle Size Determination of Wheat Flour.

Sieve size	% passed (MW)	% passed (WW)
1.0 (mm)	99.83	98.32
710 (μm)	98.85	97.11
500 (μm)	97.17	72.61
180 (μm)	96.11	49.32



Annex -IV: Equipments Used.

FW 100, lab miller, China

VIVAGEN,
EXL-3 water purification system, Korea

Retsch PM100, Planetary ball mill, Germany.

Analytical balance, sieves of different size openings with pan and mechanical shaker for determination of particle size