

ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

COLLEGE OF NATURAL SCIENCES

CENTER FOR FOOD SCIENCE AND NUTRITION

Evaluating the influence of extrinsic iron from soil contaminated teff on hemoglobin regeneration of anemic rats: Indicator of bioavailability of extrinsic iron.

By

Habtamu Guja

Advisors: Kaleab Baye (PhD)

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Food Science and Nutrition.

June, 2015

Addis Ababa, Ethiopia

ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

COLLEGE OF NATURAL SCIENCES

CENTER FOR FOOD SCIENCE AND NUTRITION

Evaluating the influence of extrinsic iron from teff contaminated with soil on hemoglobin regeneration of anemic rats: Indicator of bioavailability of extrinsic iron.

By

Habtamu Guja

Advisors: Kaleab Baye (PhD)

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Food Science and Nutrition.

Approval by Examining Board:	**Signature**	**Date**
Dr. Zelalem Debebe (External Examiner)	_____	_____
Dr. Dawud Gashu (Internal Examiner)	_____	_____
Dr. Kaleab Baye (Advisor)	_____	_____
Mr. Tilahun Kebede (Chairman)	_____	_____

DECLARATION

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

Signature: _____ Date: _____

The Thesis has been approved for Submission by:

Name of Supervisor Signature Date

Dr. Kaleab Baye (Advisor) _____ _____

Acknowledgments

I would like to thank Almighty God for His Peace and filling me in Wisdom, Understanding and Knowledge as in Exodus 31 for executing all my daily activities including this work.

I would like to express appreciation and thanks to my advisor Dr. Kaleab Baye, for his unreserved support, brotherly coaching, encouragement and supervision throughout the work.

My appreciation also goes to all staffs of MOFAM Pharmaceuticals for providing me moral support, for their tolerance and gap filling at times when I was not at work. Special thanks to Mr. Tolha Bahru, Mrs. Tsehaynesh Abat, Mrs. Eleni Kassa, Mr. Tigistu Woldie & Mr. Hassen T.

Their Excellencies: Mrs. Tadelech Woldie (Eteyae's Prayer), Mrs. Alemnesh Assefa (Ma), Mr. Reshad Seman, Mrs. Marta Ageba, Mr. Getacher Kebede, Mr. Gizaw Getenet, Dr. Woyita Woza Mrs. Selamawit Guja (Emush), Mr. Wosenyeleh Ambaw and Mr. Debebe Hailu are strongly appreciated for their routine support and involvement in my research work in different ways.

I would like to thank Dytes Inc., USA for donating Iron Deficient AIN-93G Rat Diet and Ferrous sulfate for the research work.

I would like to thank all staffs of Center for Food Science and Nutrition for their idea, material support and positive interaction in one way or another.

I would like to thank Ethiopian Public Health Institute especially staffs at Nutrition department; Mr. Alemayehu Tadesse at Laboratory Animals Breeding unit; and Mr. Kelbessa Urga.

Finally, I thank all my family members and friends who were played a role in the accomplishment of this work and May God Bless them abundantly!

Table of Contents

Contents	**Page**
Declaration	i
Acknowledgments	ii
Table of Contents	iii
List of Abbreviations	vii
List of Figures	viii
List of Tables	ix
Abstract	x
CHAPTER ONE	1
1. INTRODUCTION	2
1.1 Background	2
1.2. Statement of the Problem	4
1.3. Significance of the study	5
1.4. Objective	6
1.4.1. General Objective	6
1.4.2. Specific Objectives	6
CHAPTER TWO	7
2. LITERATURE REVIEW	8
2.1. Iron	8
2.1.1. Historical Background and Importance of Iron	8
2.1.2. Dietary Sources and Human Body Regulation of Iron	9

2.1.3. Human Iron Requirements 10

2.1.4. Causes and Consequences of Iron Deficiency 11

2.1.5. Assessment of Iron Status 13

2.1.6. Prevalence of Iron Deficiency and Groups at High Risk 17

2.2. Methods of Assessing Iron Bioavailability and Bioaccessibility 18

2.2.1. In vitro Methods of Estimating Mineral Bioavailability 19

2.2.2. In vivo Methods of Estimating Mineral Bioavailability 25

2.3 Teff (Eragrostisis tef (Zucc) Trotter) 26

2.4. Intrinsic and Extrinsic to Food Iron Sources 29

2.4.1. Intrinsic to Food Iron Sources 29

2.4.2. Extrinsic to Food Iron Sources 31

CHAPTER THREE 34

3. MATERIALS AND METHODS 35

3.1. Study Site, Sample Collection and Preparation 35

3.2. Experimental Design 36

3.3. Analytical Methods of Dry Matter, Proximate and Mineral Content 38

3.3.1. Dry Matter Content 38

3.3.2. Proximate Composition Determination 38

3.3.2.1. Determination of Crude Protein 38

3.3.2.2. Determination of Crude Fat 40

3.3.2.3. Determination of Ash Content 40

3.3.2.4. Determination of Crude Fiber 41

3.3.3. Determination of Iron Content 42
3.4. Hemoglobin Repletion Method Using Rat Model 44
3.4.1. Study Animals and Screening 44
3.4.2. Animals Handling and Ethical Consideration 44
3.4.3. Experimental Design of Hemoglobin Repletion Method 45
3.4.3.1. Experimental Diets Composition and Preparation 45
3.4.3.2. Depletion Phase 47
3.4.3.3. Repletion Phase 48
3.4.3.4. Data Quality Management 49
3.4.3.5. Iron bioavailability measurements based on rat hemoglobin repletion assay 50
3.5. Statistical Analysis 51
CHAPTER FOUR 52
4. RESULTS AND DISCUSSIONS 53
4.1. Proximate Composition of Teff Flours 53
4.2. Total Iron Content in Teff Flours 55
4.3 Baseline Characteristics of Rats 57
4.4. Food Intake and Body Weight in Repletion Period 58
4.5. Hematologic Index and Iron Bioavailability 58
4.6. Bioavailability of Iron from Teff in comparison to Other Iron Sources 64
4.7. Consumption Trend of Teff in Ethiopia and Iron Status Paradox 66
4.8. Limitation of the Study 67

CHAPTER FIVE 69

5. CONCLUSIONS AND RECOMMENDATIONS 70

REFERENCES 71

ANNEX 95

Annex I: Sample Collection site, Teff Type, Growing Condition 95

Annex II: Approval Letter from the Ethical Committee of the College of Natural Science, Addis Ababa University 95

Annex III: HemoCue Calibration using Bland and Altman (1986) method 97

Annex IV: Result of particle size determination of teff flours 98

List of Abbreviations and Acronyms

AIN-93G	American Institute of Nutrition formula for growing rats
ANOVA	Analysis of Variance
CD	Celiac Disease
Caco-2	Human colon carcinoma cell line
CRP	C-Reactive Protein
DM	Dry Matter
DMT1	Divalent Metal Transporter 1
EDHS	Ethiopian Demographic and Health Survey
EHNRI	Ethiopian Health and Nutrition Research Institute
EPHI	Ethiopian Public Health Institute
ESR	Erythrocyte Sedimentation Rate
FAAS	Flam Atomic Absorption Spectroscopy
Fe	Iron
FER	Feed Efficiency Ratio
FS	Ferrous Sulfate
FT	Field Threshed
Hb	Hemoglobin
HRE	Hemoglobin Regeneration Efficiency
ID	Iron Deficiency
IDA	Iron Deficiency Anemia
LT	Laboratory Threshed (Manual Threshing)
RBV	Relative Biological Value
SPSS	Statistical Package for the Social Sciences
TfR	Serum transferrin receptor

List of Figures

Figure 3.1.	Map of Ethiopia showing the sample collection site	36
Figure 3.2.	Experimental design from teff harvesting to animal study	37
Figure 3.3.	Process of pellets formulations: clump formation mixing powder diet with deionized water in stainless steel bowel (left), rolling the strips on a smooth surface slab (center) and the strips cut in to shorter length Pellets to be dried in an oven (right)	47
Figure 3.4.	Rat in individualized plastic cage (left), Rat in restrainer for blood collection via tail incision (center) and Hemoglobin reading using Hemocue machine (right).	48
Figure 3.5.	HemoCue machine calibration at Betezata Hospital Diagnostic Laboratory (left), uniform pellets formed from Fe deficient AIN-93G diet (center) and from Teff-AIN- 93G diet mix (right).	49
Figure 4.1.	Relative biological values (RBV) among treatment groups receiving equivalent amount of iron from LT, FT and FS sources.	61

List of Tables

Table 2.1.	Soil textural class and its local names around Eastern Shoa Zone	33
Table 3.1.	Series of Working Standard Solutions for Iron Determination	43
Table 3.2.	Composition of Experimental Diets (g/kg)	46
Table 4.1.	Proximate Composition of Laboratory and Field Threshed Teff Flours	54
Table 4.2.	Total Iron Content of Teff Flour (Lab & Field Threshed)	55
Table 4.3.	Iron Content of Red Teff from Previous Studies	56
Table 4.4.	Rats Characteristics at Baseline (End of depletion phase)	57
Table 4.5.	Data from food consumption, body weight and iron intake at the end of 14 days of repletion period	58
Table 4.6.	Hemoglobin Repletion of Rats Receiving 35mg Iron per Kg of Diet from LT, FT and FS groups	59
Table 4.7.	Relative Biological Value (RBV) of anemic rats fed diets fortified with graded levels iron in comparison to the RBV of iron in teff	65

Abstract

Iron deficiency is prevalent in Ethiopia despite high dietary intake. The contribution of iron from extrinsic sources from soil contamination during threshing is likely to be high. However, the actual bioavailability of contaminant iron remains unknown. The objective of this study was to determine the level of iron in field and laboratories threshed flours of teff from the same variety and investigate the iron bioavailability of the intrinsic and extrinsic iron associated with teff using rat hemoglobin repletion assay. Two samples of red teff grown in a similar condition were collected; one harvested and threshed in the laboratory and the other threshed traditionally. Flours from the two samples were analyzed for their dry matter, proximate composition, total iron contents and *in vivo* bioavailability was evaluated using rat hemoglobin repletion bioassay and was compared to the standard ferrous sulfate. Protein, fat and total carbohydrate contents of laboratory threshed and field threshed teff flours were shown to have no statistically significant difference ($p>0.05$). Field threshed teff (3.3$\pm$0.31 g/100g) has significantly higher ash content than the lab threshed (1.8$\pm$0.16 g/100g) ($p<0.05$). Total iron content of field threshed teff flour (29.44 $\pm$ 2.38 mg/100g) was more than fourfold greater than that of the lab threshed teff flour (6.65 $\pm$ 0.05). The hemoglobin regeneration efficiency relative to ferrous sulfate group (control ~100%), of lab and field threshed teff flours were 67.64$\pm$6.7% and 88.4$\pm$8.9%, respectively. Teff is a good source of bioavailable iron. Furthermore, additional soil iron contamination resulting from the traditional threshing of the grain contributes to the pool of bioavailable iron. The present *in vivo* study clearly illustrates that extrinsic iron can lead to hemoglobin repletion in iron deficient rats and is thus an important source of bioavailable iron.

Key Words: Teff, Threshing, Bioavailability, Contaminant Iron, Extrinsic iron, Hemoglobin Regeneration Efficiency, Relative Biological Value.

Chapter One

Introduction

1. INTRODUCTION

1.1. Background

Iron-deficiency is the most widespread and common micronutrient deficiency throughout the world affecting more than 2 billion people (Zimmermann & Hurrell, 2007). Although young children and pregnant women are the most affected groups (Osorio, Lira & Ashworth, 2004), anemia affects all age groups, impairing mental as well as physical performance which leads to impaired psychomotor development in infants, impaired educational performance in schoolchildren, increased perinatal morbidity in pregnancy, and reduced work capacity in adults (Cook, 1999; Hall *et al*., 2000).

Nutritional iron deficiency arises when physiological requirements cannot be met by iron absorption from diet (Zimmermann & Hurrell, 2007). The most important factor for iron deficiency in developing countries is the low intake of bioavailable iron mostly as a result of a predominantly plant-based diet (Aragon, Ortiz & Pachon, 2012). Non-heme iron is the form of iron present in plant-based foods and its bioavailability is lower than heme-iron, the form found in animal source foods. Plant-based foods contain large amounts of constituents that form insoluble complexes with iron like phytic acid and hence reduce bioavailability of the mineral (Baye *et al*., 2013b).

Teff is one of the major and indigenous cereal crops in Ethiopia, where it is believed to have originated (Vinning & McMahon, 2006). Nutritionally, it is an excellent source of fiber, iron, and calcium than other cereal grains and is the most valuable grain in Ethiopia (Umeta *et al*., 2005). Nevertheless, the high iron content of teff has been a source of controversy as some authors have estimated it to be high (Umeta *et al*., 2005; Saturni *et al*., 2010), whereas others

reflect this high level to soil contamination during the traditional method of threshing (Abebe *et al*., 2007; Bokhari *et al*., 2012; Ambaw, 2013) and is thus of limited biological utility.

The bioavailability of iron in foods can be studied by *in vitro* and *in vivo* methods for estimating iron absorption. *In vitro* methods are relatively simple, rapid and inexpensive and can be carried out through simulating the gastrointestinal tract (Bueno *et al*., 2013).

The limited studies that evaluated the bioavailability of iron from teff in Ethiopia used different *in vitro* methods mainly phytic acid/Fe molar ratios (Umeta *et al*., 2005; Abebe *et al*., 2007); iron absorption algorithm and iron dialysability tests (Baye *et al*., 2013b). Since extrinsic iron has poor solubility relative to that of the iron intrinsic to the food, the validity of using phytate/Fe molar ratio to estimate iron bioavailability was questioned (Baye *et al*., 2013b).

Different unit operations of processing traditionally used to produce foods commonly consumed in Africa have shown to vary the content of iron with the process (Greffeuille *et al*., 2011). A significant proportion of the iron in the diets of such developing countries are extrinsic to food, usually referred to as contaminant iron, but the impact of this contaminant iron on iron status is controversial (Harvey *et al*., 2000).

In-vitro studies should be used for screening of foods with iron that is potentially bioavailable and should be succeeded by *in vivo* studies to confirm the bioavailability (Clark *et al*., 1997; Gudissa *et al*., 2011; Bueno *et al*., 2013). Comparison of *in vitro*, animal and clinical determinations of iron bioavailability study done by Forbes *et al*. (1989) showed that the hemoglobin depletion-repletion method in rat serves as the most reliable predictor of iron bioavailability in humans.

1.2. Statement of the Problem

In many of developing countries 90 % of the total dietary supply comes from plants and these plant-based foods are the principal source of iron in these countries (Aragon, Ortiz & Pachon, 2012). A national representative study carried out by Berhan *et al*. (2011) showed that there are six principal staples in Ethiopia: teff, wheat, maize, sorghum, barley, and enset (*Enset ventricosum*) which supplying more than 70 % of calories.

Among staple cereals consumed in Ethiopia, teff contains the highest iron; nevertheless part of the iron content was due to soil contamination (Umeta *et al*., 2005; Baye *et al*., 2013b). Contaminant iron significantly contributes to dietary iron intakes in developing countries (Galan *et al*., 1990). In Ethiopia reported iron intakes of children exceed recommendations (Baye *et al*., 2012), suggesting that if bioavailable and the amount supplied by these foods could be sufficient. However, according to the Ethiopian demographic and health survey 44% of children under five years of age are anemic (EDHS, 2011). Recent studies Umeta *et al*. (2008) Haidar and Pobocik (2009) and Haidar (2010) indicated moderate form of iron deficiency anemia become a problem in Ethiopia

There are limited bioavailability studies conducted in Ethiopia and the majority of them used *in vitro* methods showing bioaccessible fraction or potentially available fractions (Umeta *et al*., 2005; Abebe *et al*., 2007; Baye *et al*., 2013b). However, the use of *in vitro* methods to predict bioavailability may be less appropriate for iron-contaminated foods (Baye *et al*., 2013b) and there is still a need to assess the actual bioavailability of contaminant iron (Greffeuille *et al*., 2011); it was also indicated by Hooda *et al*. (2002) and Seim *et al*. (2013) that the impact of soil ingestion on human nutrition could be best assessed by *in vivo* studies.

1.3. Significance of the study

Strategies to control micronutrient deficiencies consists of food based strategies such as dietary diversification and food fortification, as well as nutrition education, public health and food safety measures, and supplementation (WHO, 2006). Among these approaches, dietary modification/diversification along with nutrition education is considered to be the most sustainable (Zimmermann & Hurrel, 2007).

Foods with high iron content may help consumers attain dietary recommendations for iron, which at the same time can reduce the risks of side effects and toxicity symptoms associated with taking high dose of iron supplements (Gautam *et al.*, 2008). However, it is important to determine bioavailability from iron rich plant sources before concluding the effectiveness of food based approach (Bokhari *et al.*, 2012). In this regard, teff as a good source of iron can contribute to the implementation of food-based approaches, but the bioavailability of this iron needs to be investigated first.

The information generated from this study will be useful to those developing programs designed to reduce iron deficiency and will make advances in human nutrition especially in the science and practice of nutrition by investigating the bioavailability of iron from soil contamination. This would be useful to most developing countries whose staples are often contaminated with soil (Harvey *et al.*, 2000). The study will thus uncover the controversial role of contaminant iron on iron status and provides a pace for further scientific studies in the area.

1.4. Objective

1.4.1. General Objective:

To determine the level of iron in field and laboratory threshed teff flours of same variety and evaluate the contribution of contaminant iron from soil to the hemoglobin regeneration in anemic rats.

1.4.2. Specific Objectives:

- To determine iron content and the extent of soil contamination during threshing of teff
- To determine the hemoglobin regeneration efficiency and relative iron bioavailability in teff
- To compare the relative bioavailability of iron from intrinsic and extrinsic sources
- To estimate the contribution of contaminant iron from soil to the relative bioavailability

Chapter Two

LITRATURE REVIEW

2. LITERATURE REVIEW

2.1. Iron

2.1.1. Historical Background and Importance of Iron

From ancient times man has recognized the special role of iron in health and disease. Iron had early medicinal uses; the Egyptians use rust (iron oxide) as a treatment of baldness, whereas the ancient Greeks used a mixture of wine and iron in attempt to restore male potency (Wood & Ronnenberg, 2005). A connection between dietary iron supply and disorders of blood was proposed in 16th century, but its physiological basis was not recognized until horse hemoglobin was shown to contain 0.335% iron by Zinoffsky in 1886 and this finding was confirmed in other species also (Suttle, 2010). In 1893 Stockman left controversial work showing hemoglobin concentration in anemic women could be rapidly increased by supplementation with ferrous salts (Stockman, 1893). During the 17th century, iron was used to treat chlorosis or green disease (historical names for hypochromic anemia), a condition often resulting from iron deficiency (Guggenheim, 1995). However, it was not until 1932 that the importance of iron was finally settled by the convincing proof that inorganic iron could be used for hemoglobin synthesis (Suttle, 2010).

Iron exists in one of two oxidation states: the ferrous form (Fe^{2+}) or the ferric form (Fe^{3+}), this chemical property of iron enabled its catalytic role in a multitude of redox reactions necessary to support basic metabolic functions for life. In fact, iron's central role in oxygen and energy metabolism underscores the biologic significance of this element and helps to explain why it is one of the best-studied metals in nutrition and health (Wessling-Resnick, 2014). Iron plays an important role in the proper structure of the hemoglobin molecule;

hemoglobin is the functional unit of the red blood cells and it is a complex protein structure inside the red blood cells that help in delivering oxygen from the lungs to other parts of the body. Even though the red blood cells are made within the bone marrow, many other factors are involved in their production; iron is a very important component of the hemoglobin molecule; erythropoietin, a molecule secreted by the kidneys, promotes the formation of red blood cells in the bone marrow (Abbaspour *et al*., 2014). Iron sufficiency has a role to play with improved cognitive development (learning ability), body's immune system and work capacity. Iron adequacy during pregnancy is associated with improved outcomes for both mother and infant through preventing morbidity and mortality (Abbaspour *et al*., 2014). Iron is essential for all tissues, present in the brain from very early life participating in the neural myelination processes, also involved in promoting growth (Iannotti *et al*., 2006).

2.1.2. Dietary Sources and Human Body Regulation of Iron

Iron is present in foods in two forms, as heme iron, which is derived from flesh foods (meats, poultry, and fish), and as non-heme iron, which is the inorganic form present in plant foods such as cereals, pulses, legumes, grains, nuts, and vegetables. In meat, 30–70% of iron is haem iron, of which 15–35% is absorbed (Hurrell & Egli, 2010); however, non-haem iron, in plant-based diets the absorption is much lower 2 - 20% which is strongly influenced by the presence of other food components (Abbaspour *et al*., 2014). The absorption of non-haem iron is increased by meat and ascorbic acid, but inhibited by phytates, polyphenols, and calcium (Zimmermann & Hurrell, 2007; Hurrell & Egli, 2010). The quantity of non-heme iron in the diet is many fold greater than that of heme-iron in most meals; thus despite its lower bioavailability, nonheme iron generally contributes more to iron nutrition than heme-iron (Abbaspour *et al*., 2014).

Iron balance is primarily controlled at the level of intestinal absorption because there is no regulated excretion of iron through the liver or kidneys (Andrews, 2008). Adequate amounts of iron during growth typically results in a 70 kg man accumulating about 4 g of body iron. About 2.5 g of body iron is within hemoglobin and about 1 g is stored as ferritin or haemosiderin, mainly in the liver. Nutritional iron deficiency arises when physiological requirements cannot be met by iron absorption from diet. Loss of blood depletes the iron store since 1 mL blood translated into a 0.5 mg loss of iron (Zimmermann & Hurrell, 2007).

2.1.3. Human Iron Requirements

During gestation, the fetus stores about 250 mg of iron; these stores are drawn on during breastfeeding because breast milk supplies only about 0.15 mg of absorbed iron per day, whereas requirements for absorbed iron are about 0.55 mg per day. Low birth weight infants do not store an adequate amount of iron during fetal life and are at high risk of developing iron deficiency while being breastfed (Zimmermann & Hurrell, 2007). During early infancy iron requirements are met by the little iron contained in the human milk. The need for iron rises markedly 4-6 months after birth and amounts to about 0.7-0.9 mg/day during the remaining part of the first year. Between 1 and 6 years of age, the body iron content is again doubled. Iron requirements are also very high in adolescents, particularly during the period of rapid growth (FAO/WHO, 2004).

Average adult stores nearly 4 grams of iron in his or her body, an exquisite balance between dietary uptake and loss maintains this balance (Abbaspour *et al.*, 2014). About 1 mg of iron is lost each day through sloughing of cells from skin and mucosal surfaces, including the lining of the gastrointestinal tract. Menstruation increases the average daily iron loss to about

2 mg per day in premenopausal female adults and a dietary intake of iron is needed to replace the basal iron loss (Zimmermann & Hurrell, 2007; Abbaspour *et al*., 2014).

2.1.4. Causes and Consequences of Iron Deficiency

Iron deficiency results from depletion of iron stores and occurs when iron absorption cannot keep pace over an extended period with the metabolic demands for iron to sustain growth and to replenish iron loss (Abbaspour *et al*., 2014). The primary causes of iron deficiency include low intake of bioavailable iron which is considered to be responsible for around 50% of anemia (Allen *et al*., 2006), inflammatory and infectious diseases, especially malaria, blood loss from parasitic infections, and other nutrient deficiencies (vitamin A, riboflavin, folic acid and vitamin B12) are important causes (Brabin *et al*., 2001). Increased iron requirements as a result of rapid growth, pregnancy, menstruation and impaired absorption of iron (Zimmermann & Hurrell, 2007; Abbaspour *et al*., 2014) are also documented causes. The frequency of iron deficiency rises in female adolescents because menstrual iron losses are superimposed with needs for rapid growth (Harvey *et al*., 2005). Other risk factors for iron deficiency in young women are high parity, use of an intrauterine device, and vegetarian diets (Abbaspour *et al*., 2014).

Consequences of iron deficiency result in functional changes that may occur with or without anemia. Anemia is described as a condition of low hemoglobin level in blood cells (Abbaspour *et al*., 2014). Anemia is actually a sign of a disease process rather than a disease itself. It is usually classified as either chronic or acute. This also helps predict how severe the symptoms of anemia may be. In chronic anemia, symptoms typically begin slowly and progress gradually; whereas in acute anemia symptoms can be abrupt and more distressing.

Nutritional anemia includes anemia due to deficiency in iron plus deficiencies in folate, vitamins A and B12, and certain trace elements involved with red blood cell production even though about half of the global burden of anemia is due to iron deficiency (Ouedraogo *et al*., 2010; Abbaspour *et al*., 2014). Since iron plays an important role in the proper structure of the hemoglobin molecule, if iron intake is limited or inadequate due to poor dietary intake, anemia may occur as a result and this is called iron deficiency anemia. Iron deficiency anemia can also occur when there are stomach ulcers or other sources of slow, chronic bleeding (like colon cancer, uterine cancer, intestinal polyps, and hemorrhoids) (Abbaspour *et al*., 2014).

Mild iron deficiency may be compensated by more efficient oxygen delivery from hemoglobin to tissues, redistribution in blood flow to protect brain and heart, and enhanced cardiac output (Wessling-Resnick, 2014). Work performance can become significantly impaired by reduced oxidative energy production as iron deficiency anemia evolves, however, under severe iron deficiency conditions, acidosis can occur (Wessling-Resnick, 2014). Another characteristic of anemia is lack of body temperature regulation under cold conditions that is associated with decreased thyroid hormone synthesis resulting from reduced activity of thyroid peroxidase, which is a heme-dependent enzyme (Zimmermann, 2006). Restless legs syndrome arises from impaired dopaminergic function and is also linked to iron deficiency (Salas, Gamaldo & Allen, 2010). Iron deficiency is thought to impair immune function secondary to the important role of iron in generating free radical response to infection (e.g., myeloperoxidase activity), and low iron status is associated with changes in lymphocyte and neutrophil function (Bhaskaram, 2001). Respiratory infections are observed to be more frequent and to last longer in iron-deficient children (De-Silva *et al*., 2003).

During early childhood, low brain levels of iron can also create developmental issues that may not be reversed on iron repletion (WHO, 1992). Iron deficiency increases the incidence of preterm delivery and low birth weight, and patients with very severe cases are at increased risk of maternal and child mortality (Allen *et al*., 2008).

2.1.5. Evaluation of Iron Status

Iron deficiency and eventually anemia develop in stages and can be assessed by measuring various biochemical indices (Abbaspour *et al*., 2014). The assessment of Fe status can be made by using specific laboratory measurements; this technology is constantly evolving, and no single method or combination of methods is suitable for all purposes (Cook, 1999). The optimal laboratory approach depends on the anticipated severity of the iron lack, the age and sex of the target population, the potential for associated disease, the feasibility of venous sampling, and the available economic and laboratory resources. The preferred methods are listed below in approximate order of increasing severity of the iron lack which they reflect (Cook, 1999). Sensitivity and specificity of methods are necessary to characterize changes in iron status (Mei *et al*., 2005).

Serum ferritin

Transferrin Saturation

Erythrocyte Protoporphyrin

Mean corpuscular volume

Serum transferrin receptor

Hemoglobin/ packed cell volume

A variety of measurements have been used to identify milder form of Fe deficiency, including serum ferritin, transferrin saturation, erythrocyte protoporphyrin, mean corpuscular volume, and more recently the concentration of the soluble fragment of transferrin receptor in serum. Laboratory evidence of milder Fe deficiency without anemia will be referred to as Fe deficiency (ID). More advanced deficiency is accompanied by a reduction in the packed cell volume or hemoglobin concentration, and thus referred to as iron deficiency anemia (Cook, 1999).

***Hemoglobin (Hb)*:** is most commonly used because it is inexpensive, easy to perform, and rapid. However, Hb levels are affected by factors other than iron deficiency because Hb is a function of red blood cells (RBC) production and turnover. Hb levels therefore lack specificity for categorizing iron status. Moreover, mild iron deficiency may not affect Hb levels (Mei *et al*., 2005)

Although it lacks sensitivity, Hb is a useful screen for iron deficiency in women and children in western societies because iron deficiency is the predominant cause of anemia in these populations and it is less useful in developing countries where anemia is multifactorial (Lynch, 2012). Other micronutrient deficiencies besides iron, such as vitamin B12, folate and vitamin A, can also cause anemia (Ouedraogo *et al*., 2010). Because of these limitations hemoglobin measurement cannot be used in isolation to indicate of iron status (Cook, 1999).

Low hemoglobin concentration is a measure of anemia, which is the end stage of iron deficiency (Abbaspour *et al*., 2014). The Hb determination is in wider use at present because of the availability of a hand-held battery-operated instrument called Hemocue, which gives immediate and highly-accurate result (Cook, 1999).

***Serum Ferritin*:** Mild iron deficiency is better explained by serum ferritin level (Cook, 1999). Importance of the serum ferritin is that a low value in an anemic individual identifies IDA without a doubt; when the concentration is low (<12 µg/L for <5 years of age and <15 µg/L for >5 years of age) iron stores are depleted. Similarly, when the concentration of serum ferritin is ≥15 µg/L indicates the presence of adequate iron stores which is useful for excluding IDA as the cause of anemia (Cook, 1999; Abbaspour *et al*., 2014). Serum ferritin level 1µg/L approximately as equivalent to 8mg storage of iron in adults (Lynch, 2012), and as an acute-phase reactant, serum ferritin is elevated by acute and chronic infections, inflammatory diseases, malignancies and liver disorders. Thus, the utility of the serum ferritin is enhanced if these disorders are excluded by clinical examination or other laboratory tests such as the erythrocyte sedimentation rate (ESR) or C-reactive protein (Cook, 1999).

Inflammatory disorders also increases circulating hepcidin concentrations, a peptide hormone that blocks iron release from enterocytes and reticuloendothelial system, resulting in iron deficiency (Zimmermann & Hurrel, 2007), because iron balance is primarily controlled at the level of intestinal absorption (Andrews, 2008).

A widely used marker of inflammation is the C-reactive protein (CRP), but the extent of increase of CRP concentration that invalidates the use of serum ferritin to diagnose iron deficiency and CRP values higher than 10–30 mg/L have been used. The problem with CRP is that during the acute-phase response, the increase of CRP concentration is typically of shorter duration than the increase of serum ferritin (Zimmermann & Hurrel, 2007), but still it is preferred over ESR to determine systemic inflammation (Costenbader, Chibnik & Schur, 2007).

Transferrin saturation: calculated serum Fe/total Fe-binding capacity, partly compensates for the limitations of individual measurements, but does not overcome the fundamental problem that plasma iron transport is affected by a wide range of disorders (Cook, 1999).

Mean corpuscular volume: a hematological method that has been extensively used to detect Fe-deprived erythrocytes, indicates the average size of circulating erythrocytes (Cook, 1999).

Serum transferrin receptor (TfR): transferrin receptor is mostly derived from developing RBCs and therefore reflects the intensity of erythropoiesis and the demand for iron (Abbaspour *et al*., 2014). Enhanced synthesis of the transferrin receptors represent the cellular response to a declining iron supply, indicating sever iron insufficiency provided that there are no other causes of abnormal erythropoiesis (Abbaspour *et al*., 2014). Unlike other methods, it is not affected by chronic inflammation or infections which are often confused with iron deficiency (Cook, 1999; WHO/CDC, 2004).

Finally, the ratio of TfR to ferritin (TfR/ferritin) is designed to evaluate changes in both stored iron and functional iron and thought to be more useful than either TfR or ferritin alone (Cook *et al*., 2003). TfR/ferritin has been used to estimate body iron stores in both children and adults (Cook *et al*., 2005). However, the high cost and the lack of standardization of the TfR assay so far have limited the applicability of the method (Yang *et al*., 2008).

2.1.6. Prevalence of Iron Deficiency and Groups at High Risk

Iron deficiency is widespread and globally about 1.62 billion people are anemic, with the highest prevalence among preschool children 47% followed by pregnant women 42% (Benoist *et al*., 2008). A lack of iron causes over 600,000 stillbirths or neonatal deaths and over 100,000 maternal deaths during pregnancy (Rowe & Dodson, 2012). According to Ethiopian Demographic and Health Survey (EDHS) (2011), the prevalence of anemia in women is 17% and in children under five years 44%.

EDHS data has limitation since it uses only hemoglobin level to determine iron status and no other iron specific tests are done and such surveys have rarely measures iron deficiency or any of the other factors that contribute to the development of anemia and therefore the contributing factors frequently remain unknown (McLean *et al*., 2008). But country representative good quality study done by Umeta *et al*. (2008), that used a combination of methods employing iron specific test, serum ferritin, along with hemoglobin levels has identified in women of reproductive age in Ethiopia the prevalence of mild to moderate form of anemia was (30.4%), iron deficiency (49.7%) and iron deficiency anemia (17%).

Groups at highest risk of suffering from iron deficiency are found in those parts of a population that have inadequate access to foods rich in absorbable iron during stages of high iron demand. These groups correspond to children, adolescents, and women of reproductive age, in particular during pregnancy. In the case of infants and adolescents, the increased iron demand is the result of rapid growth. For women of reproductive age the principle reason is the excessive blood loss during menstruation. During pregnancy, there is a significant increase in iron requirement due to the rapid growth of the placenta and the fetus and the

expansion of the globular mass. In contrast, adult men and postmenopausal women are at low risk of iron deficiency and the amount of iron in a normal diet is usually sufficient to cover their physiological requirements (FAO/WHO, 2004; Abbaspour *et al*., 2014).

2.2. Methods of Assessing Iron Bioavailability and Bioaccessibility

The quantity of a nutrient that is absorbed from a food can be measured at different levels, which can be described by different terms. Fair-weather-Tait *et al*. (2007) and Bokhari *et al*. (2012) have proposed the following definitions. Bioaccessibility corresponds to the proportion of iron that can be released from food matrix into the lumen of the gastrointestinal tract. This fraction of nutrient potentially available for absorption mostly relies on food composition. Bioaccessibility is measured by *in vitro* methods, either by solubility or dialysability. On the other hand, bioavailability of iron corresponds to the proportion of the ingested iron that can be used for normal body functions and thus is measured by *in vivo* methods. Unlike bioaccessibility, bioavailability measurement is not only depends on food composition but also take into account the iron status of the host.

Estimating iron absorption is mainly studied by *in vitro* methods since it is relatively simple, rapid and inexpensive and carried out through simulating the digestion in gastric and duodenum, followed by dialysis. The proportion of the element diffused through a semi-permeable membrane during the process, is the dialysability of element after an equilibration period, being used as an estimate the relative bioavailability of nutrients (Bueno *et al*., 2013).

Studies aimed at the optimization of iron in nutritional formulations should include *in vitro* methods followed by an assessment of iron absorption *in vivo* in order to better investigate their metabolic behavior (Bueno *et al*., 2013). Laboratory animals have contributed greatly to the knowledge of biological structure and function and are essential tools in biomedical research; they are used extensively in a broad variety of biological investigations to evaluate different therapeutic drugs, foods, and chemicals (Clark *et al*., 1997; Gudissa *et al*., 2011). There are different *in vivo* and *in vitro* methods of determining iron bioavailability.

2.2.1. *In vitro* Methods of Estimating Mineral Bioavailability

In vitro methods have been used to estimate mineral bioavailability for more than 50 years (Ismail, 1999). The *in vitro* procedures have been very useful for identifying and characterizing factors that affect non-heme iron absorption, but direct comparisons between absorption predicted from the *in vitro* tests and measurements in human volunteers have only been made in a limited number of published studies (Lynch, 2005). Generally, it is advantageous if human bioavailability testing could be based on preliminary *in vitro* screening methods to efficiently identify the promising food substance of interest (Beiseigel *et al*., 2007). There are different methods of determining the *in vitro* bioavailability of minerals including iron: iron solubility, dialysability, Coca-2 (Frontela *et al*., 2011), and other *in vitro* methods include phytic acid/mineral molar ratio, and absorption prediction algorithm (Baye *et al*., 2013b).

i. Iron Solubility: This method is employed through exposing foods to a stimulated gastric acid environment and with subsequent measurements of the soluble iron released by the digestion i.e., measuring mineral solubility (Frontela *et al*., 2011).

ii. *In Vitro* Iron Dialysability: This method is used to determine the percentages of dialyzable iron after simulated gastrointestinal digestion of foods; the fraction represents the proportion of iron that can be released from the food matrix into the lumen of the gastrointestinal tract and thus potentially available for absorption i.e. bioaccessible fraction (Frontela *et al*., 2011; Baye *et al*., 2013b). Hence, dialyzability methods are limited to modeling luminal interactions among the factors as such interactions affect mineral availability (Hotz, 2005).

In iron contaminated foods, no difference in iron dialysability was observed irrespective of the processing status; tef flour & its fermented product (injera) as shown in the study by Baye *et al*. (2013b), the authors pointed out that mineral absorption inhibitors other than phytate might contributed for that. Earlier findings from human iron absorption studies also showed that dephytinisation in the presence of polyphenols did little to improve iron absorption (Hallberg & Hulthen, 2000).

Improvement in the methodology of dialyzability has been obtained when the human colon carcinoma (Caco-2) cell line has been incorporated (Frontela *et al*., 2011). It is more current approach in the *in vitro* study of mineral bioavailability which makes use of brush border membrane vesicles and cultured epithelial cells (Ismail, 1999).

iii. Caco-2 Cell Model: The human intestinal Caco-2 cell line originally isolated by J. Fogh in Sloan Kettering Institute, New York, from a human colon adenocarcinoma (Fogh *et al*., 1977), this human colon adenocarcinoma, undergoes in culture a process of spontaneous differentiation that leads to the formation of a monolayer of cells, expressing several morphological and functional characteristics of a mature enterocyte (Sambuy *et al*., 2005).

To better represent the steric conditions existing in the intestine *in vivo*, Caco-2 Cells are cultured on permeable filter supports that allow free access of ions and nutrients to the two sides of the cell monolayer (Sambuy *et al*., 2005). Caco-2 cell line has been extensively used over the last twenty years as a model of intestinal barrier (Sambuy *et al*., 2005); it is still the best available cell culture model of absorptive small intestinal enterocytes (Ferruzza *et al*., 2012).

However, attempts to establish differentiated enterocytic cell lines in culture have not been fully successful though expressing several brush border enzymes; some of them are expressed at the level higher or lower than human enterocytes do (Ismail, 1999; Sambuy *et al*., 2005). For instance; oxidative metabolic enzymes are lower than in the human enterocytes, limiting the use of this line as an *in vitro* model of intestinal oxidative metabolism. On the contrary, the ferric reductase activity associated with brush border that reduces Fe^{3+} for uptake by the Fe^{2+} transporter was found to be high in differentiated Caco-2 cells (Ekmekcioglu *et al*., 1998). The divalent metal transporter (DMT1) is located on the brush border membrane that transports Fe^{2+}; DMT1 expression and activity is regulated mainly by iron but since it has a broad substrate activity, it can also transport other physiologically relevant divalent cations such as Zn^{+2}, Mn^{+2}, Cu^{+2} and the toxic metals Cd^{+2}, and Pb^{+2}. Studies by Andrerle *et al*. (2003) and Fleet *et al*. (2003) were confirmed up-regulation of DMT1 during Caco-2 differentiation. Similarly, Caco-2 Cells have an elevated activity of P-glycoproteins (a trans-membrane protein responsible for apical efflux of several substances), higher than the human intestine *in vivo*, that should be taken in account when these cells are used as a model of intestinal bioavailability (Sambuy *et al*., 2005). This great

variability of Caco-2 cells model arises from cell- and culture related factors and these factors again show huge variation in differentiation of Caco-2 Cells (Sambuy *et al*., 2005).

According to the study by Beiseigel *et al*. (2007), indirect comparisons with human data indicated that the Caco-2 model can predict the influence of ascorbic acid and polyphenolic compounds on relative iron bioavailability. However, this model has not been directly validated by simultaneously testing identical meals in both humans and the in vitro system. Authors have shown that though providing similar magnitude of total iron from maize varieties to both human and Caco-2 cell model, iron absorption difference was not significant in human and was only marginally significant in Caco-2 cell. The authors then suggested that, the variation might be because in humans the efficiency of iron absorption correlates inversely with body iron status but relative bioavailability measurements (as in Caco-2 cell) appear to be independent of the host's iron status (Beiseigel *et al*., 2007).

The Caco-2 system is limited to measuring the entrance of iron from a food digestate into the mucosal cell, whereas iron bioavailability for humans also involves serosal transfer from the mucosal cell and subsequent transport and utilization (Beiseigel *et al*., 2007). Additional testing of *in vitro* conditions is necessary, because cell passage and differentiation, incubation medium, time, pH, method of cell protection from digestive enzymes, and use of radio-iron variables compared with Caco-2 cell ferritin response variables may all influence *in vitro* results (Beiseigel *et al*., 2007). Generally, the method lacks standardized protocol and therefore, reproducibility of results carried out in different settings may vary due to variation in culture, maintenance of the Caco-2 cell line and for specific nutrient/toxicant test to be carried out has no protocol (Sambuy *et al*., 2005).

iv. Absorption Prediction Algorithms: The amount of iron absorbed from a meal is determined by iron status, the content of heme and non-heme iron, and the bioavailability of the two kinds of iron, which in turn is determined by the balance between dietary factors enhancing and inhibiting the absorption of iron, especially for non-heme iron. It is known that the variation in dietary iron absorption from meals is due to more to differences in the bioavailability of the iron, which can lead to a >10-fold variation in iron absorption, than to a variation in iron content (Bovell-Benjamin *et al*., 2000; Hallberg & Hulthen, 2000). Algorithm is used to predict the bioavailability of iron in a meal and it is a product of all factors present in the meal that inhibit or enhance iron absorption (Hallberg & Hulthén, 2000).

The algorithm models include all the factors in calculations assuming their effect was independent and additive rather than interactive, which is an important issue in addressing iron bioavailability. The accuracy of the estimations is of concern due to lack of quantitative measurements of bioavailability modifiers, inability to consider interactive effects, and the use of non-iron status measurements (Reddy, 2005). Therefore, algorithm method is more qualitatively than quantitatively useful as it classify meals or diets as being of high, medium, or low bioavailability and requires further development to accurately predict the influence of polyphenols on human iron absorption (Lynch, 2005; Beiseigel *et al*., 2007).

Study done by Baye *et al*. (2013b), have shown that in foods rich in iron but a high proportion of iron was associated with soil contamination, the knowledge of the exact amount of contaminant iron and its bioavailability may be a prerequisite for proper interpretation of algorithm to predicted absorptions unless the predictions only apply to intrinsic iron.

V. Phytic Acid/Mineral Molar Ratios: Previous attempts to assess or estimate relative mineral bioavailability in Ethiopian staples were based on phytate/mineral molar ratios (Umeta *et al*., 2005; Abebe *et al*., 2007). The level of Phytate/ mineral molar ratios have different values to indicate adequacy for different minerals, the desirable level suggested for mineral absorption i.e., phytate:iron < 1, phytate:zinc < 18, phytate:calcium < 0.17 (Ma *et al*., 2007; Gibson *et al*., 2010). As phytic acid is strongly inhibitory at low concentrations, complete enzymatic degradation is recommended (>90%). If this is not possible, the phytic acid to iron molar ratio should be decreased to below 1 and preferably below 0.4 (Hurrell, 2004).

Phytate/iron molar ratio >1 is an indication of poor iron bioavailability (Tamanna *et al*., 2013); however, according to Lestienne *et al*. (2005), molar ratio of phytate to iron is not a sufficient indicator of iron availability if the level of anti-nutritional factors other than phytate is high. Besides phytates, polyphenols and to some extent calcium and fibers have negative effects on mineral absorption (Hurrell & Egli, 2010).

Moreover, in cereals with large amount of contaminant iron, the validity of using phytate/iron molar ratio to estimate relative bioavailability of the mineral is in question; knowing the exact amount of contaminant iron and its bioavailability may thus be a prerequisite for the use of phytic acid/Fe molar ratios to correctly predict bioavailability in foods containing contaminant iron (Baye *et al*., 2013b).

2.2.2. *In Vivo* Methods of Estimating Iron Bioavailability

i. Human Study: human trials are the gold standard for evaluating non-heme iron bioavailability (Aragon, Ortiz & Pachon, 2012). However, determining the bioavailability of a mineral in human is complicated by the fact that once a food is consumed, it mixes in the gastrointestinal tract with other foods that are consumed at about the same time or may be because the minerals are present in a mixture of sources available in the diet or meal. The use of extrinsic radio-iron tagging and stable isotopes in humans has partly offers a solution to this problem. Since human iron absorption studies are mainly done in healthy adults using isotopic techniques based on erythrocyte incorporation of either stable or radioactive isotopes (Fidler, 2003), this method adversely expose subjects to radiation and the use of radioisotopes may require monitoring the fate of radioisotopes or stable isotopes of iron in blood (Wienk, Marx, & Beynen, 1999). Moreover, human studies are cumbersome, complicated, costly, and time-consuming to perform (Ismail, 1999; Aragon, Ortiz & Pachon, 2012). These limitations have restricted the use of mineral absorption studies in human for the purpose of identifying specific biochemical components of the diet that explain the inhibitory or enhancing nature of certain foods (Ismail, 1999).

ii. Animals Study: in animals, the hemoglobin-repletion bioassay was most often used (Wienk, Marx, & Beynen, 1999). Unlike humans, they are good for efficacy study because their environmental conditions can be controlled properly; however, animal studies have limitations because of different mineral requirements, metabolism, digestive capacity, sensitivity to dietary factors (promoters and inhibitors of iron absorption) when compared to humans (Aragon, Ortiz & Pachon, 2012), and thus their use is limited because the accuracy of extrapolation to man (Wienk, Marx, & Beynen, 1999). But for evaluating bioavailability

or relative biological values of iron from different sources with respect to ferrous sulfate control, hemoglobin-repletion bioassay has been used widely in recent studies (Swain, Newman & Hunt, 2003; Rohner *et al*., 2007; Weber *et al*., 2010; Martino *et al*., 2011; Aragon, Ortiz & Pachon, 2012; Lucia *et al*., 2013). Moreover, the comparison of *in vitro*, animal and clinical determinations of iron bioavailability study done by Forbes *et al*. (1989) have showed that the rat model hemoglobin depletion-repletion method serve as the most reliable predictor of iron bioavailability in the human although *in vitro* methods are promising screening techniques.

The biological availability of iron from other diets have been measured by the rat bioassay (Weber *et al*., 2010; Martino *et al*., 2011; Lucia *et al*., 2013) and only single study previously carried out by Urga *et al*. (1998) in Ethiopian diet prepared from teff to show the effect of commonly processed forms of teff (*Injera* and *Kitta*) using rat hemoglobin repletion assay method for iron bioavailability.

2.3. Teff (Eragrostisis tef (Zucc) Trotter)

Teff is one of the major and indigenous cereal crops in Ethiopia, where it is believed to have originated. Teff belongs to the family *Poaceae* and has the smallest size of all grains in the world (about 100-150 teff grains equal the size of 1 wheat kernel). The name 'teff' is derived from the Amharic téf, which means lost - a reference to the grain's small size (Vinning & McMahon, 2006; Saturni *et al*., 2010; Temesgen, 2013).

Unusual thing about teff is that, although the literature makes frequent reference to its being an important staple food in Ethiopia, the grain's low yield and highly labor intensive method of production result in it being quite expensive; but usually staple foods are rarely expensive

(Vinning & McMahon, 2006). Moreover, because of the grain's small size, teff poses production challenges; any gaps in the mechanical harvesting system will result in lost grain; and contamination with soil is a serious problem since it is virtually impossible to separate the soil from the grain (Vinning & McMahon, 2006).

There are few different varieties of tef that vary in color from light to dark. According to Ethiopian Health and Nutrition Research Institute (EHNRI, 1997), the various types of injera produced from the different varieties of teff do not have significant variation in their calorie, moisture, protein, carbohydrate but with respect to iron content the red teff has higher than the white (Vinning & McMahon, 2006) as in other darker colored cereal grains, red varieties of teff contained higher levels of iron which has been proposed to be due to higher levels of pigmented material, such as tannins and polyphenols (Umeta & Parker, 1996; Alaunyte, 2013).

Teff is a self-pollinated, warm season grass and its production occurs in a wide range of environments, from drought-stressed country to water-logged land. Teff prefers altitudes between 1700 and 2200 meters and relatively dry conditions (450–550 millimeters a year); its preferred temperature is 10–27°C with 12 hours of sunlight (Vinning & McMahon, 2006). Ethiopia remains the centre of production; *teff* has the largest share of area (22.6%, 2.7 million hectares) under cereal cultivation and third (after maize and sorghum) in terms of grain production (16%, 34.9 million quintals) in Ethiopia, providing grain yield about 12.93 quintals per hectare (CSAE, 2012). Its production has been increasing at approximately 11% per year, with high latent demand resulting in price increases (ATA, FDRE-MOA & EIAR, 2013).

Nutritionally, teff is the most valuable grain in Ethiopia, which is considered an excellent source of fiber, iron, and calcium than other cereal grains (Umeta *et al*., 2005; ATA, FDRE-MoA, & EIAR, 2013). Recently, there is a growing interest in teff grain utilization because of nutritional merits (whole grain) and free of the protein gluten that make teff an increasingly important dietary component for individuals who suffer from gluten intolerance or Celiac Disease (Boka *et al*., 2013).

Celiac Disease (CD) is a genetically based autoimmune condition that results in permanent sensitivity to the protein gluten (Alaunyte, 2013). It is characterized by chronic inflammation of the intestinal mucosa, atrophy of intestinal villi and several clinical manifestations. Its prevalence has increased in recent years. Gluten is a mixture of storage proteins (prolamins and glutenins with a high content of proline and glutamine domains) that is contained in several cereals (wheat, rye, barley and derivatives). These protein domains are resistant to degradation by gastric, pancreatic and proteases in the human intestinal brush border membrane, this results in an accumulation of relatively large peptide fragments and gluten free-diet remains the cornerstone treatment for CD patients (Saturni *et al*., 2010). Teff is a gluten-free cereal lacking gluten homologues; it can be used as a substitute for wheat in food applications where exclusion of gluten is required (Alaunyte, 2013).

Teff is ground into flour and primarily used to make a fermented, sour dough type, flat bread called *injera* (Boka *et al*., 2013; Temesgen, 2013). *Injera* is a thin fermented Ethiopian traditional bread made from teff flour, water and starter (ersho), which is a fluid saved from previously fermented dough. Teff is the most popular grain for making *injera*, although other grains such as sorghum, maize, barley, and wheat are sometimes used (Ashagrie & Abate, 2012).

Teff is generally accepted that the grain is highly nutritious although there is some debate about the precise nutritional value of it (Vinning & McMahon, 2006). Regarding the iron content, some authors have estimated to be high and others reflect this high level is attributed to the dust and dirt that cling to the grain (Vinning & McMahon, 2006). As per the study done by Umeta *et al*. (2005), to evaluate mineral contents of 36 foods consumed in rural Ethiopia have shown tef *enjera* was the best source of bioavailable iron of all foods analyzed based on its high iron content and favorable phytate to iron molar ratio. Moreover, the prevalence of IDA has been reported to be lower in some regions of Ethiopia, which has been attributed to teff forming a staple part of the diet (Bokhari *et al*., 2012). Whereas, other studies revealed that iron content of teff vary between geographical regions possibly as a result of soil contamination (Saturni *et al*., 2010; Bokhari *et al*., 2012). With respect to soil contamination, teff is extremely exposed and complete removal of the extrinsic iron from teff is not possible (Baye *et al*., 2013b).

2.4. Intrinsic and Extrinsic to Food Iron Sources

Iron sources can be broadly classified as intrinsic and extrinsic to food (Harvey *et al*., 2000), and they are briefly reviewed below.

2.4.1. Intrinsic to Food Iron Sources (Dietary Iron Sources)

Dietary iron presents in food in two forms as heme and non-heme iron. Haem iron comes from animal foods and is absorbed relatively well, its absorption being largely independent of other constituents in the diet (Carpenter & Mahoney, 1992; Hallberg & Hulthen, 2000; Harvey *et al*., 2000). Animal products contain both heme and non-heme iron, heme iron is that derived from hemoglobin and myoglobin. Plant materials contain only non-heme iron,

the absorption of non-haem iron from the common pool, whether originally intrinsic or extrinsic to food, is subject to the same interactions with other constituents of the diet, as well as to endogenous factors of the host such as the nature of gastric secretions, transit time, rate of erythropoiesis and the size of existing iron stores (Harvey *et al*., 2000). Heme iron is considered to be nutritionally important as it is higher in bioavailability (>15%) than non-heme iron (Kalpalathika *et al*., 1991). However, heat treatment/cooking decreases the amount of heme-iron concentration in food, while non-heme iron increased (Clark *et al*., 1997; Kongkachuichai *et al*., 2002).

All iron consumed in the diet is either haem or non-haem and is absorbed from two distinct pools in the lumen of the gastrointestinal tract, largely in the small intestine. The amount of iron absorbed from a meal is determined by the content of heme and nonheme iron among other factors (Hallberg & Hulthen, 2000; Harvey *et al*., 2000).

Red meat is more supplied with blood vessels and contains more myoglobin and hemoglobin than light meat. Therefore, it would be expected to have higher concentrations of iron. The studies by Carpenter and Mahoney (1992) and Carpenter and Clark (1995) reported that heme iron content in raw beef and chicken muscles was approximately 61% and 25% respectively. Similarly, the study by Kongkachuichai *et al*., (2002) showed that both pork and chicken liver are not only the richest sources of non-heme iron but also contain the highest percentage of non-heme iron (80%) due to the storage site of iron.

2.4.2. Extrinsic to Food Iron Sources (Non-Food Sources of Iron)

A significant proportion of the iron in the diets of developing countries is extrinsic to food and usually referred to as contaminant iron (Harvey *et al*., 2000). Sources of iron extrinsic to food include: contamination of foods from soil, dust and water; metal fragments from milling; that leached from iron or steel pots during processing, storage or cooking; and from the practice of geophagia. These contaminant forms of iron are largely ferric hydroxide and ferric oxide, but they are likely to vary widely in terms of solubility and affinity for reaction with other compounds (Harvey *et al*., 2000). Thus, iron extrinsic to food can be consumed either through unintentional entrapment during food processing or deliberately via a practice of geophagy.

Regular and deliberate eating of non-food substances is called pica; geophagy is a type of pica, characterized by craving and consumption of soil, clay or soft stone (Kawai *et al*., 2009; Njiru, Elchalal & Paltiel, 2011). Geophagy among pregnant women is common in many cultures and in some African countries, higher prevalence has been observed. In Nigeria, the most populous country in Africa, the prevalence of geophagy was estimated to be 50% (Njiru, Elchalal & Paltiel, 2011); in Kenya 73% of the pregnant women eat soil regularly and their estimated median daily ingestion was 41.5 g (Geissler *et al*., 1999) and in Ghana 46%, 42% in Namibia, to 28% in Tanzania (Kawai *et al*., 2009).

It is postulated that geophagy is practiced due to micronutrient deficiencies, cultural influences, and gastrointestinal upsets. Despite their potential to supply micronutrients, soils interfere with bioavailability of micronutrients leading to an increased risk of anemia and can

also act as a pathway for ingestion of geohelminths and heavy metals, putting woman and fetus at risk (Kawai *et al*., 2009; Njiru, Elchalal & Paltiel, 2011).

An earlier study on the Ethiopian foods cooked in iron, aluminum and clay pots, have shown that there was more crude iron in all foods cooked in iron pots - around twice as much iron in meat and vegetables, and 1.5 times as much iron in legumes- than in food cooked in the other two types of pots (Adish *et al*., 1999). Moreover, soil contamination of cereals in Ethiopia occurs mainly during traditional threshing under the hooves of cattle for grains like teff (Baye *et al*., 2013b). This traditional threshing method was found to increase iron contents up to 20 times higher in teff than other cereals which were attributed to soil contamination (Abebe *et al*., 2007).

According to Soil surveys made in different parts of Ethiopian, the concentration of available iron in soil samples in flat plain areas of Abaya Chamo Lake Basin ranged from 6.41 - 40.25 ppm (Ayele *et al*., 2014) and similar study in north Tigray by Abayu (2012) has shown the highest iron in soil was found to be 42.93 ppm. In both cases the level of iron in soils have shown to be higher than the suggested critical limit of 4.5ppm iron in soil (Ayele *et al*., 2014). Available iron level in soil was negatively correlated with soil pH and clay content; but positively correlated to slit and sand content of the soil (Ayele *et al*., 2014).

Study done by Burayu (2006), shown that in Eastern Shoa zone including sample collection site, there are three types of soils with their local names: *Ganbore* constitute high sand proportion, while *Koticha* soils composed of high clay content and *Carii* soil types are in between the two. Black or *Koticha* soil type is predominant in sample collection site

according to the study by Ayele (2013). The data in textural class with corresponding local names are presented in Table 2.1.

Table 2.1: Soil textural class and its local names around Eastern Shoa Zone (Burayu, 2006)

Proportion (%)			Textural Class	Color of Soil	Local Name
Sand	Slit	Clay			
53	36	12	Sand-Loam	Red	*Ganboree*
46	30	24	Loam	Brown	*Carrii*
28	24	48	Clay	Black	*Koticha*

The impact of contaminant iron on iron status is controversial (Harvey *et al*., 2000). Studies conducted by Seim *et al*. (2013) and Hooda *et al*. (2002) have shown that soil has a high iron concentrations compared to iron-rich foods, but this iron was not bioavailable. However, other studies using simulated gastric acid solubility have reported contaminant iron was 20% soluble (Icard-Verniere *et al*., 2013) and even very high acid solubility was reported by Ayele (2013). Regarding dialysability, earlier studies by Prabhavathi and Rao (1981), Smith *et al*. (2000), Icard-Verniere *et al*. (2013) and Abrahams *et al*. (2006) have shown that contaminant iron had a non-negligible nutritional interest with maximum potentially bioavailable or bioacessible fraction of $< 2\%$, 2.9%, 4% and 4.1% respectively.

Chapter Three

Materials & Methods

3. MATERIALS AND METHODS

3.1. Study Site, Sample Collection and Preparation

Red Teff (Eragrostisis tef (Zucc) Trotter) samples were collected from Adulala, Zekuwala Abo woreda, East Shoa zone, Oromia region which is one of the sites under Debre Zeit Agricultural Research Center (Tadele, 2011), and geographically proximal to Addis Ababa, being only 93km away (Figure 3.1).

The harvested teff was purchased from a volunteer farmer on January 15, 2014 and placed in polyethylene bag by taking preventive measures to avoid any possible contamination and this harvested teff was threshed in the Center for Food Science and Nutrition laboratory manually, by wearing sterile surgical gloves with precautionary measures to prevent any adventitious contamination and the resulting teff grain was sifted and placed in polyethylene bag and was labeled as 'Lab Threshed (LT)' and with date of threshing. Teff grain, which was the same variety and grown under the same condition (Annex I) as the lab threshed, that was threshed traditionally (Field threshed) was collected applying all the preventive measures to avoid contamination as stated above and was sifted to separate foreign matter in the same way as in the LT and was placed in polyethylene bag and was labeled as 'Field Threshed (FT)'.

Teff Flours were prepared from both samples using stainless steel laboratory miller (FW100, Ohaus, Bejing, China) in a condition that did not add any adventitious contamination and finally the flours of LT and FT teff were kept packed in dry polyethylene bag and stored at cool and dry place for the subsequent study.

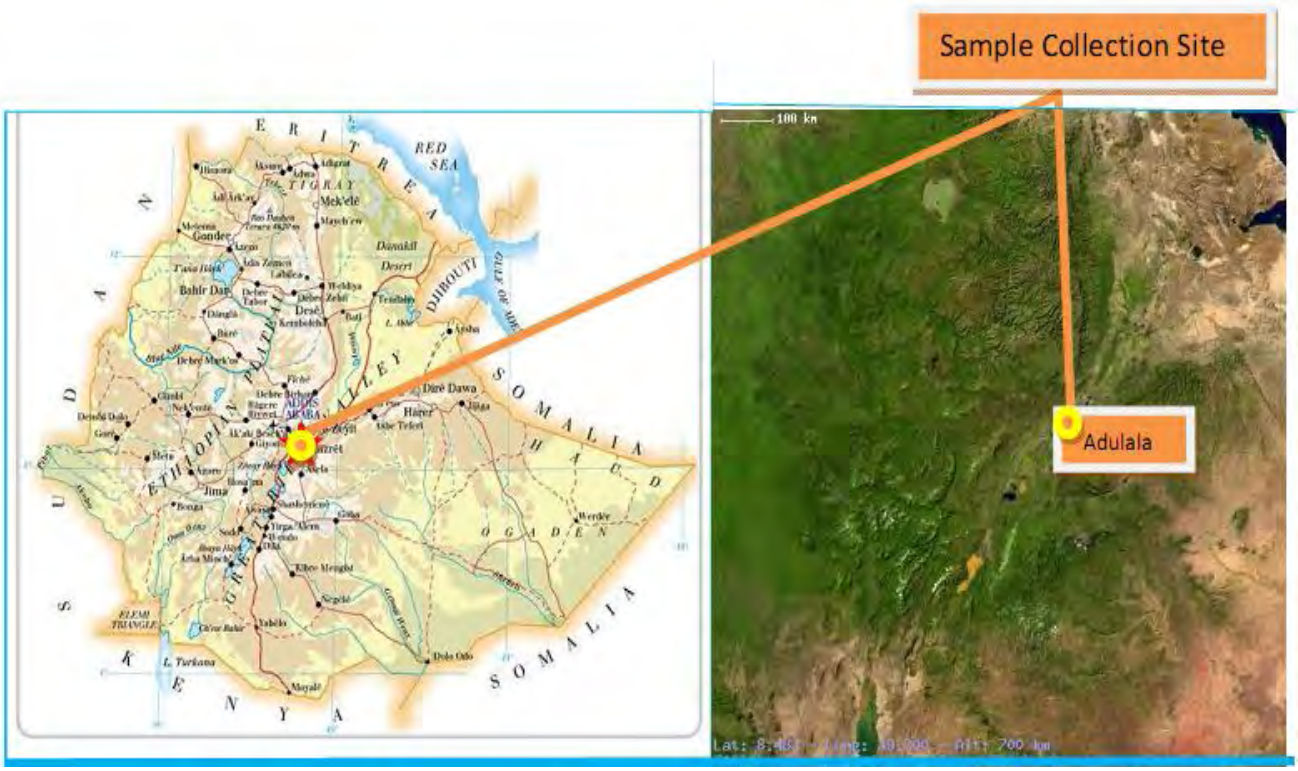


Figure 3.1: Map of Ethiopia showing the sample collection site

The subsequent experimental analysis was conducted in Addis Ababa at the Center for Food Science and Nutrition research laboratory for sample preparation and compositional analysis and partly in the Ethiopian Public Health Institute (EPHI) for animal study and iron determination. Addis Ababa is a high-land located at an altitude of 2,300 meters above sea level, with an average annual rainfall of 1,800 mm and a temperature of 14 to 21°C.

3.2. Experimental Design

The experimental design was completely randomized controlled design, and study was carried out in two phases as shown in Figure 3.2:

Phase I: Compositional analysis of dry matter, proximate, and mineral content.

Phase II: Evaluating iron bioavailability using rat hemoglobin repletion bioassay.

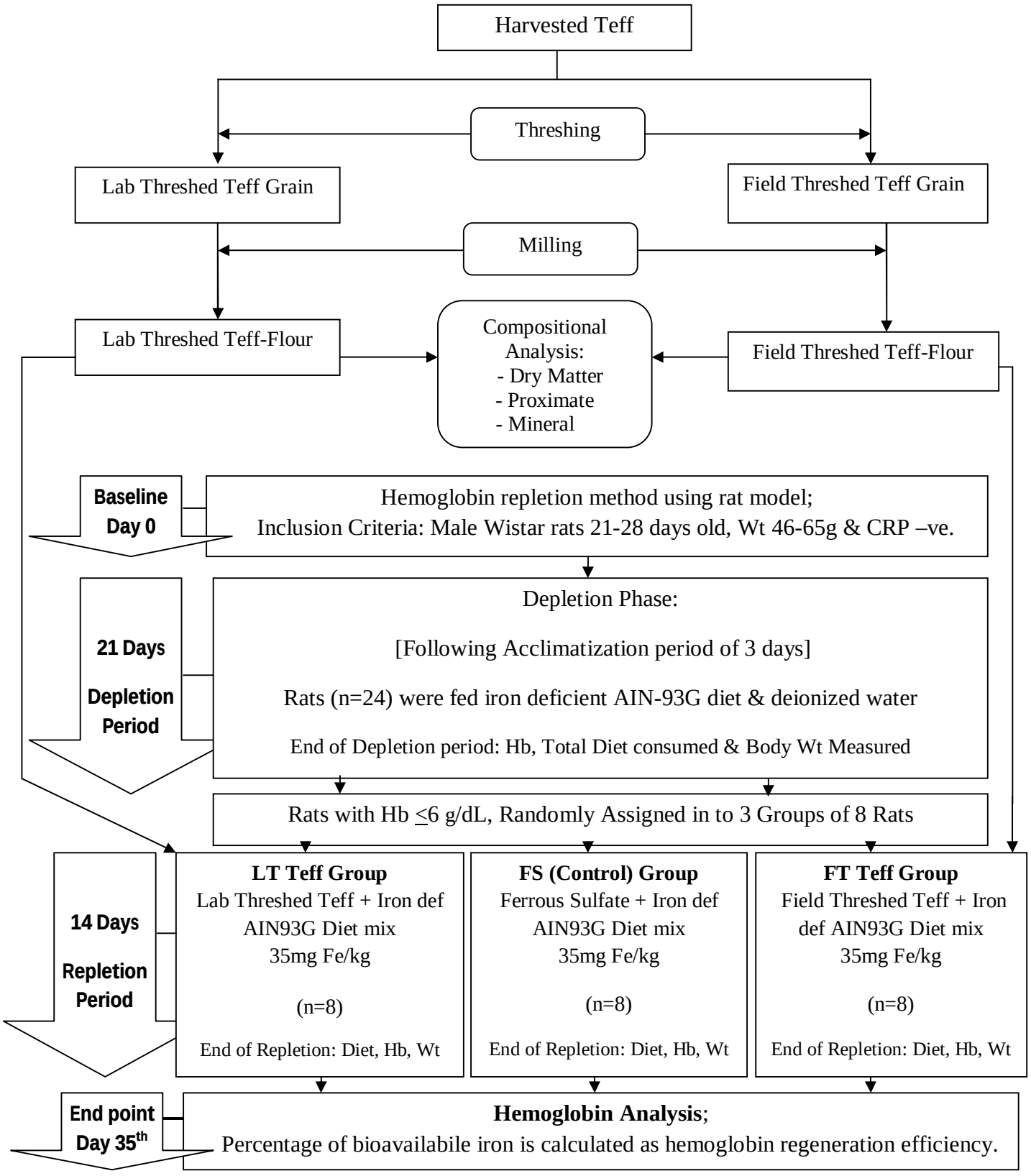


Figure 3.2: Experimental design from teff harvesting to animal study

3.3. Analytical Methods of Dry Matter, Proximate and Mineral Content

Dry matter content and proximate composition determination (crude protein, fat, ash content) were conducted as per the AOAC (2000) using official methods. Determination of iron content was done using atomic absorption spectrophotometer method after preparing series of working standard solutions by diluting metal stock solution with deionized water to set calibration curve (Osborne & Voogt, 1978).

3.3.1. Dry Matter (DM) 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 crucible (W1) was determined. 5g samples was weighed in dry crucible (W2) and dried at 105°C for 3 hour and after cooling to room temperature in desiccators it was again weighed (W3). The moisture content was determined by using Equation 1.

$$\text{Moisture Content in \%} = \frac{W2 - W3}{W2 - W1} * 100 \qquad \text{Eq. 1}$$

3.3.2. Proximate Composition Determination

Proximate chemical analysis of two samples: laboratory threshed and field threshed teff samples was determined according to AOAC (2000).

3.3.2.1. Determination of Crude Protein

Protein content was determined according to AOAC (2000) using the official method 979.09.

Digestion: About 0.5 gm of fresh samples in triplicate was added to a Tecator tube and 6 ml of acid mixture (5 parts of concentrated ortho-phosphoric acid and 100 parts of concentrated sulfuric acid) was added and mixed again on to this mixture 3.5 ml of 30% hydrogen peroxide was added step by step. As soon as the violet reaction had ceased, the tubes were shaken and placed back to the rack. Three grams of catalyst mixture (ground 0.5 gm of selenium metal with 100 gm potassium sulfate) was added in to each tube and allowed to stand for about 10 minutes before digestion. When the temperature of the digester attained 370°C, the tubes lowered in to the digester. The digestion was continued until appearance of clear solution (at about 4 hours). The tubes in the rack were cooled in a fume hood: 25 ml of de-ionized water was added, and shaken to avoid precipitation of sulfate in the solution.

Distillation and titration: the digested and diluted sample solution was distilled using boric acid and the distillate was titrated using 0.1N hydrochloric acid until reddish color appeared (Persson, 2008).

The crude protein was determined as follow:

$$\text{Nitrogen \%} = \frac{(VHCl\ (1) * NHCl)}{Wo} * 14 * 100 \qquad \text{Eq. 2}$$

$$\text{\% Protein} = 6.25 * \text{\% Nitrogen} \qquad \text{Eq. 3}$$

Where: V is volume of HCl in Litter consumed to the end point of titration, N is normality of HCl (0.1N is usually used), Wo is sample weight on dry matter basis and 14 is molecular weight of nitrogen. The % of nitrogen is converted to % of protein by using the following conversion factor based on the assumption that proteins contain 16% nitrogen.

(% Protein = 6.25 * % Nitrogen for both teff samples; laboratory and field threshed).

3.3.2.2. Determination of Crude Fat

The crude fat was extracted according to AOAC (2000) Official method 4.5.01.

About 2 gm of teff flour was extracted with 50ml diethyl ether at least for 4 hours in a soxhlet extractor (SZC-D Fat Determinator, China). The solvent was then evaporated and the extracted fat was dried in an oven and cooled in a desiccators. The crude fat was determined as follow:

$$\text{Crude Fat \% Wt} = \frac{W2 - W1}{W} * 100 \qquad \text{Eq. 4}$$

Where: W1 = Weight of extraction flask (gm)

W2 = Weight of extraction flask plus dried crude fat (gm)

W = Weight of sample (gm)

3.3.2.3. 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 were placed in a muffle furnace for 30 minutes at 550°C. The dishes were cooled in desiccators (with granular silica gel) for about 30 minutes at room temperature and were weighed (M1). About 2.5 gm of fresh sample was weighed in a dish (M2). The dishes were placed on a hot plate under a fume-hood and the temperature was slowly increased until smoking ceases and samples become thoroughly charred. The dishes with the samples were placed inside a muffle furnace (CARBOLITE, England) at 550°C for 5 hours and then cooled in desiccators for 1 hour. The ash was clean and white in appearance. When cooled to room temperature, each dish with ash was reweighed (M3).

$$\text{Total Ash \%} = \frac{M3 - M1}{M2 - M1} * 100 \qquad \text{Eq. 5}$$

Where: (M2 – M1) is sample mass in grams on dry base and (M3 – M1) mass of ash in grams.

3.3.2.4. Determination of Crude Fiber

Digestion: A 1 g of sample (W3) was measured and placed into a 660 ml beaker where 200 ml of 1.25% sulfuric acid was added, boiled gently for 30 minutes putting watch glass on top of the beaker. Hot distilled water was applied to top up to keep constant the level of the solution. 20 ml of 28% KOH was measured and added and boiled gently for another 30 minutes, with stirring.

Filtration: The bottom of a sintered glass crucible was covered with 10mm sand layer and wetted with distilled water. The heated solution was poured on the crucible and filtered using high performance vacuum pump. The wall of the beaker was rinsed with hot distilled water several times where it was transferred to the crucible and filtered.

Washing: The residue on the crucible was washed with 1% sulfuric acid and filtered, and again by hot distilled water and filtered. Washing will continue and this time by 1% NaOH and filtered. The washing step was repeated again and finally the residue was washed with water free acetone.

Drying and combustion: Drying was done by placing the crucible in a drying oven for 2 hours at 130°C and cooled for 30 min in desiccators, and then weighed (W1). The crucible was cooled in desiccators and weighed (W2).

$$\text{Crude Fiber g/100g} = \frac{W1-W2}{W3} * 100 \qquad \text{Eq. 6}$$

Where: W1= weight of crucible with sample after drying

W2= weight of crucible with sample after ashing

W3= fresh sample weight.

3.3.3. Determination of Iron Contents

Iron content was determined by using atomic absorption spectrophotometer according to the method by Osborne and Voogt (1978).

Ash was obtained from dry ashing of teff flours. The ash was wetted completely with 5 ml of 6N HCl, and dried on a low temperature hot plate. About 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 in to a 50 ml graduated flask using a filter paper (whatman 45, 125mm). About 5ml of 3N HCl was 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 volumetric flask was also prepared for flam atomic absorption spectroscopy (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 iron using the prepared standard solution was prepared.

The sample concentration was analyzed using FAAS (Spectr AA 6800, Shimadzu, Japan) by aspirating deionized 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

$$\text{Metal Content (mg/100g)} = \frac{[(a - b)*V]}{10*W} \qquad \text{Eq. 7}$$

Where:

W = Weight (g) of sample

V = 50ml = Volume of extract

a = Concentration (μg/ml) of sample solution

b = Concentration (μg/ml) of blank solution

3.4. Hemoglobin Repletion Method Using Rat Model

3.4.1. Study Animals and Screening

A total of twenty four male Wistar rats (*Rattus novergicus*, albinus variety, Rodent class) weaned at 21 - 28 days, with initial weight ranging from 45 – 65 grams, were obtained from the Ethiopian Public Health Institute (EPHI) and were housed individually in plastic cages at room temperature with 12 hrs light and dark cycles. For inclusion at baseline, rats were screened for weight, sex, and C-reactive protein (CRP). Qualitative CRP test (Linear chemicals SL, Lot: 24108, Barcelona, Spain) was used and CRP negative result indicated the absence of infection/inflammation. This was done to prevent the inclusion of infected animals which may interfere with the subsequent animal study since the use of disease free animals often lead to a substantial reduction in the number of study animals needed for any given experiment as indicated by Weber *et al*. (2010) and Gudissa *et al*. (2011).

3.4.2. Animals Handling and Ethical Consideration

The study protocol and animals handling was conducted in compliance with the National Research Council of National Academies Guide for the care and use of laboratory animals (2011). During the whole study period, the rats received *ad libitum* deionized water and rations. All rats were handled in a way to reduce stress at blood collection. Blood samples were taken at three stages: at baseline, at the end of depletion, and repletion periods by means of a small incision at the end of the rats' tail which is posing little stress and is convenient even for frequent blood sampling that can be used for longitudinal studies as indicated by Fluttert, Dalm and Oitzl (2000). After blood specimen collection at the end of repletion phase, all rats were sacrificed under light diethyl ether anesthesia.

Ethical approval for the study was granted by the Ethical Committee of the College of Natural Sciences of Addis Ababa University in its meeting held on May 12, 2014 (Minutes: RERC/009/2014; with Reference No: CNSDO/678/06/14) (Annex II).

3.4.3. Experimental Design of Hemoglobin Repletion Method

A rat hemoglobin repletion assay (AOAC Method 973.31) was used to determine the relative bioavailability of iron according to AOAC (1997). The experiment had two phases, the depletion phase, where rats were fed iron deficient AIN 93G diet for 3-4 weeks followed by a repletion phase where rats were fed treatment iron containing diets whose bioavailability determined relative to ferrous sulfate (control) group. Before the study begun, rats were acclimatized for 3 days in order to get them acquainted with the new environment of individualized cage and diet as indicated by Miyada *et al*. (2011).

3.4.3.1. Experimental Diets Composition and Preparation

Diets were prepared based on the nutritional need of animals using AIN-93G composition, according to Reeves, Nielsen and Fahey (1993). The composition of the diets is presented in Table 3.2. Ingredients were weighed and mixed using plastic tools, followed by mixing in a stainless steel mixer (IKA®, Eurostar Overhead Stirrer, Germany) at low speed for 15 minutes as indicated by Lucia *et al*. (2013). Vitamins and minerals content of the mixes among groups were made similar because variation in the levels of vitamin B2, B12, folate and vitamin A affect hemoglobin levels (Brabin *et al*., 2001; Ouedraogo *et al*., 2010). Iron absorption enhancer (Vitamin C) and minerals that can compete with iron absorption like, zinc, and calcium (Zimmermann & Hurrell, 2007; Abbaspour *et al*., 2014) and other micronutrients were also adjusted by adding vitamin mix (AIN-93G VX; Harlan Teklad,

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 37g and 33.5g of soybean oil per Kg to the LT and FT containing diets, respectively; 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 formulation of pellet (Reeves *et al*., 1993).

Table 3.2: Composition of Experimental Diets (g/Kg mixture)

Components	**Depletion Period**	**Repletion Period**		
	Fe def AIN 93G	**LT+ AIN 93G**	**FT+ AIN 93G**	**$FeSO_4.7H_2O$+AIN 93G**
Total Iron (mg)	-	35	35	35
Total Lipid (Soy bean oil)	70	86*	82*	70
Total Protein	200	150.3	149	200
Total Carbohydrate by difference	629.5	643.2	653.7	629.5
Iron-free Mineral Mix	35	35	35	35
Vitamin Mix	10	10	10	10
Total Energy (Kcal/Kg)	**3,948**	**3,948**	**3,948**	**3,948**

Fe def: iron deficient, AIN-93G: American Institute of Nutrition formula for growing rats, LT: lab threshed, FT: field threshed, Kcal: Kilocalories. *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 stainless steel bowl and the formed clumps forced to pass through a hollow stainless steel tube to have a uniform cylindrical shape (diameter of 9mm); the extruded strip might have rough surface which could be removed by rolling the strips of diet on a smooth wooden slab on bench top and then the strip cut in to shorter length (about 6cm) with stainless steel cutting knife (Figure 3.3) and finally placed in an oven to dry at 50°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 pellets formulations: clump formation mixing powder diet with deionized water in stainless steel bowel (left), rolling the strips on a smooth surface slab (center) and the strips cut in to shorter length pellets to be dried in an oven (right)

3.4.3.2. Depletion Phase

According to the hemoglobin depletion-repletion method, to induce iron deficiency anemia, the rats were fed iron deficient AIN-93G diet (Dytes Inc., USA), recommended for rats in a growing phase utilizing an iron free mineral mix and deionized water *ad libitium* for three to four weeks, until they become anemic, with hemoglobin level lower than 6 g/dL and this level allow us to use the rat depletion-repletion methods (Forbs *et al*., 1989; Weber *et al*., 2010). Dietary intake by individual rat was recorded daily and body weight measured every three days. At the end of depletion period, rats were weighed and blood was collected by tail incision, with precautions taken to avoid hemodilution.

After placing rats in restrainer, through incision at the terminal portion of the tail of each rat, blood was dripped on glass slide and immediately taken up by microcuvates of HemoCue (Hb 301, Lot No: 1408389, Angelholm, Sweden) and these microcuvates were wiped and

placed in to the Hemocue machine (Hemocue Hb 301, Sweden) to take reading of hemoglobin measurement from whole blood in duplicate (Figure 3.4). HemoCue method is easy for operation and has high sensitivity and specificity relative to the gold standard Hemoglobin assessment method, direct cyanmethaemoglobin method (Sari *et al*., 2001). Rats with a hemoglobin level of < 6 g/dL were considered depleted and were then randomly assigned in to 3 groups of 8 rats with similar hemoglobin values among the three groups $p<0.05$.



Figure 3.4: Rat in individualized plastic cage (left), Rat in restrainer for blood collection via tail incision (center) and Hemoglobin reading using Hemocue machine (right).

3.4.3.3. Repletion Phase

Following induction of Fe-deficiency anemia, the randomized eight rats in three groups were provided with 35 mg intrinsic iron per Kg of isocaloric diet; this iron was supplied from three different iron sources: (1) Iron from Lab threshed teff (intrinsic) mixed to Fe-free AIN-93G diet; (2) Iron from Field threshed teff (intrinsic + extrinsic) mixed to Fe-free AIN-93G diet on the basis of its intrinsic iron level; and (3) Iron from Ferrous Sulfate Hepta-hydrate ($FeSO_4.7H_2O$) mixed to Fe-free AIN-93G diet as a control.

The amount of total iron of the treatment groups was set considering the level of iron intrinsic to teff. The rats were fed with the experimental diets and deionized water *ad libitum*

for 14 days. The treatment diet was provided in a sufficient amount, since dietary restriction in rats were shown to be associated with decreases in total leukocyte, segmented neutrophil, lymphocyte, platelet counts and serum biochemistry including decrease in total protein and albumin (Hubert *et al*., 2000). The amount of diet fed by the rats was recorded daily. At the end of repletion period, rats were weighed, and blood was collected by tail incision and carried out in similar way as described under section 3.4.3.2.

3.4.3.4. Data Quality Management

HemoCue machine calibration was done after prior calibration of HMX Hematology analyzer (Beckman Coultar, California, USA) through known standards of hemoglobin levels (high, normal and low levels) using method agreement for clinical measurements as indicated by Bland and Altman (1986) (Annex III). Blood specimen collection was made in duplicate and if the two readings showed wide variations, a third measurement was carried out to avoid extreme values arising from operational errors and hemodilution with extracellular fluids (Rohner *et al*., 2007). Treatment diets were prepared in identical process of pellet formation (Figure 3.5) with respect to diameters of pellets, drying temperature and drying time as indicated by Green and Turner (1974) in the previous section.



Figure 3.5: HemoCue machine calibration at Betezata Hospital Diagnostic Laboratory (left), uniform pellets formed from Fe deficient AIN-93G diet (center) and from Teff-AIN-93G diet mix (right).

3.4.3.5. Iron bioavailability measurements based on rat hemoglobin repletion assay

Based on hemoglobin readings at the end of the depletion phase (initial Hb) and repletion phase (final Hb), iron bioavailability and utilization was estimated by calculating percentage bioavailability as hemoglobin regeneration efficiency (HRE). HRE is a measure of dietary iron incorporated into hemoglobin (Onabanjo *et al*., 2008). The calculation of hemoglobin iron (Hb-Fe, mg) was based on the assumption that 6.7% of rat's body weight is blood and hemoglobin contains 3.35 mg of iron per gram of blood (Miyada *et al*., 2011; Lucia *et al*., 2013). The relative biological value was determined by dividing the individual HRE values of test iron sources by the mean HRE of ferrous sulfate control group (Urga *et al*., 1998). The following formula were used (Equation 8-13):

i. Iron Consumption

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 formula below:

$$\text{Fe Consumption} = \frac{[\text{total diet consumption during repletion period (g)} * \text{Iron in diet (mg/1000 g)}]}{1000} \quad \text{Eq. 8}$$

The results of the Hb concentrations and iron consumption were used to estimate the Hb-Fe pool (mg), using the above mentioned assumptions:

$$\text{Hb-Fe pool (mg)} = \frac{[(\text{Hb Fe final} - \text{Hb Fe initial}) * 100]}{\text{Fe intake (mg)}} \quad \text{Eq. 9}$$

ii. Hemoglobin Regeneration Efficiency (HRE):

$$\% \text{ HRE} = \frac{[(\text{Hb Fe pool final} - \text{Hb Fe pool initial}) * 100]}{\text{Fe intake (mg)}} \quad \text{Eq. 10}$$

Where:

$$\text{Hb Fe (Initial)} = \frac{[\text{Initial weight (g)} * \text{Initial Hb (g /dL)} * 6.7 * 0.335]}{1000} \quad \text{Eq. 11}$$

$$\text{Hb Fe (Final)} = \frac{[\text{Final weight (g)} * \text{Final Hb (g /dL)} * 6.7 * 0.335]}{1000} \quad \text{Eq. 12}$$

iii. Relative Biological Value (RBV):

$$\text{RBV} = \frac{\text{\% HRE test group} * 100}{\text{\% HRE Ferrous Sulfate Control group}} \quad \text{Eq. 13}$$

Relative bioavailability was determined using the mean ratio of test response to the standard, since this study used a single iron concentration among the treatment groups to measure responses (Littell, Lewis, & Henry, 1995). For the bioassays containing a series of concentrations ($\geq$3), either the slope ratio or the parallel lines assay method would be used based the on parameters indicated in the European Pharmacopoeia (2005) and US Pharmacopeia (2010).

3.5. Statistical Analysis

Descriptive statistics were used and the results were presented as mean and standard deviation. Three groups (FS, LT & FT) were compared for hemoglobin levels, hemoglobin regeneration efficiency, weight, iron consumption and other animal parameters using one way analysis of variance (ANOVA) and statistical differences among means were tested by the Duncan's *post-hoc* test, considering ferrous sulfate group as a control group and a significance level of 5% ($p < 0.05$). Independent samples t-test was used to compare proximate and total iron contents between LT and FT. Data was analyzed using the software Statistical Package for the Social Sciences (SPSS), version twenty.

Chapter Four

Results and Discussions

4. RESULTS AND DISCUSSION

Two samples of teff were collected; both harvested from the same plot of land, but one threshed traditionally in the field and the other threshed manually in the laboratory. Both samples were from the same variety (red teff) and were grown under similar condition. The flours were analyzed for their proximate composition, total iron and *in vivo* bioavailability was evaluated using hemoglobin repletion bioassay in rats and the bioassay results were compared to the standard ferrous sulfate (control group).

4.1. Proximate Composition of Teff Flours

Protein, fat and total carbohydrate contents of laboratory threshed and field threshed teff flours were comparable ($p>0.05$) (Table 4.1). However, field threshed teff had 45.5 % greater ash content than the lab threshed one ($P<0.05$). This is expected as soil contamination during traditional threshing adds up mineral content, which has also been previously reported (Ambaw, 2013). However, the level of contamination in the present sample was shown to be higher than the value reported by Ambaw (2013).

The proximate composition (dry matter basis) particularly protein and fat contents of the red teff samples analyzed in this study agrees with previous reports by Ambaw (2013), and the Ethiopian food composition table (ENRHI, 1997).

Table 4.1: Proximate Composition of Laboratory and Field Threshed Teff Flours

Parameters Analyzed	Teff Flour	
	Lab Threshed	Field Threshed
Dry Matter (g/100g)	83.5±0.22[a]	86.7±0.12[b]
Fat (% DM)	3.0± 0.08[a]	2.9±0 .15[a]
Protein (% DM)	10.55±0.14[a]	10.3±0.14[a]
Ash (g/100g DM)	1.8±0.16[a]	3.3±0.31[b]
Total Fiber (% DM)	2.59±0.03[a]	2.65±0.05[a]
Total Carbohydrate (% DM)	65.56±0.56[a]	67.55± 1.44[a]

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

4.2. Total Iron Content in Teff Flours

The total iron content of the FT teff flour (29.44 ± 2.38 mg/100g) was about 4 times higher than that of LT flour (6.65 ± 0.05 mg/100g) ($p<0.05$), as presented in Table 4.2. The difference in the total iron content was due to the significant contribution of contaminant iron from soil introduced during the traditional threshing because this study blocked the effect of other unit operation that were likely to add non-food iron sources like milling (Icard-Verniere *et al*., 2013) and sifting by conducting these processes in the same way for both teff grains. The standard ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) was analyzed for iron content and found to be 17.83±0.87 % w/w, which was comparable to its theoretical content reported by

Hurrell (2002). It is used as a standard such that relative bioavailability (RBV) could be assessed for the experimental sources of iron (Boling *et al*., 1998).

Table 4.2: Total Iron Content of Lab and Field Threshed Teff Flours

Sample Analyzed	Type of Threshing	Total Iron (mg/100g DM)
Teff Flour	Lab Threshed	6.65 ± 0.05^{a}
	Field Threshed	29.44 ± 2.38^{b}

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

The traditional process of threshing grains under the hooves of cattle extremely contaminate the grain with soil iron (Alaunyte, 2013; Baye *et al*., 2013b) and was reported increase the teff iron concentration to aberrant levels (>150mg/100g) (Abebe *et al*., 2007). This contamination occur mainly due to contact with surface soil (Ambaw, 2013) and according to Atengo (2005), the exchangeable cations and the contents of potentially available micronutrients are higher in the surface soils than in the sub-surface soils, a phenomenon partly attributed to the cycling of nutrients by the plants.

According to limited soil surveys made in Ethiopia, the concentration of iron in soil samples ranged from 6.41 - 40.25 ppm (Ayele *et al*., 2014) and the concentration reported by Abayu (2012) was 42.93 ppm.

There has been much discussion and conflicting evidences about the iron content of teff. Table 4.3 presents the findings from several previous studies and shows that large variations exist in the magnitude of iron content in teff, ranging from 5.9mg/100g iron in clean red teff seeds (Almgard, 1963), to ≥150mg/100g in red teff flour purchased from local market (ENRHI, 1997; Abebe *et al*., 2007), to 430mg/100g in teff injera from market (Umeta *et al*., 2005). Teff seeds and flour should have a similar nutritional composition as teff grain gives a 99% return in flour since it is not decorticated like other grains (Alaunyte, 2013). However, the higher iron content of injera could be due to additional contamination during processing that may come from the utensils, the water used and effect of microbial fermentation.

Table 4.3: Iron content of red teff from previous studies

		Iron Content mg/100g	
Study	**Sample Preparation Condition**	**Mean**	**Range**
Mengesha, 1966	Uncontaminated, washed seed	19.3	11.6 - 24.1
Hofvander, 1968	Acid-washed sample	24.4	20.0 - 24.8
Besrat, 1980	Acid-washed & cleaned	6	4.5 - 7.8
Areda *et al*., 1993	Acid and Deionised Water Washed sample	6.9	3.0 - 12.3
Abebe *et al*., 2007	Teff grains Purchased from local market	150	__
Almgard, 1963	Cleaned Red Teff seeds	5.9	__
Umeta *et al*., 2005	Fermented and unfermented *injera* from market	430	__
ENRHI, 1997	Purchased from local market, flour	150	__
Present study	Manually Threshed (Lab Threshed), flour	6.65	6.6 - 6.7*
Present study	Field Threshed (under hooves of oxen), flour	29.44	27.04 -1.82*

*Average Deviation from the Mean

Soil contamination is not unique to teff (Baye *et al*., 2013b); however, the very small size of teff grains leads to contacts with soil over a large area and thus explain the relatively extreme contamination of this grain. Further confirming that at least part of the iron is from extrinsic sources are studies that showed reduction in iron content of teff after acid/water washing (Areda *et al*., 1993; Baye *et al*., 2013b) and it is very difficult to rid completely of soil contamination (Umeta *et al*., 2005). Although the variation in the methods used to analyze iron vary across studies, much of the variation is likely due to variation in the extent of extrinsic contamination.

4. 3. Baseline Characteristics of the Experimental Rats

The rats (21-28 days) were placed on depletion phase for 21-28 days, and they were 45-59 days old at the start of the repletion phase (baseline). The fact that initial hemoglobin, body weight, age and CRP status were not statistically different among the three groups illustrates that the randomization and homogeneity among groups were effective (Table 4.4). 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 4.4: Rats characteristics at baseline (End of depletion phase)

	Red teff		
Indices	**Lab Threshed**	**Field Threshed**	**FS Control**
Initial Hemoglobin (g/dL)	5.73 ± 0.17^{a}	5.72 ± 0.18^{a}	5.6 ± 0.21^{a}
Age (Days)	52.25 ± 3.62^{a}	51.00 ± 3.2^{a}	52.5 ± 3.96^{a}
Weight Initial (g)	147.13 ± 10.90^{a}	160.25 ± 25.39^{a}	146.50 ± 14.2^{a}
CRP Status (Qualitative test)	Negative	Negative	Negative

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

4.4. Food Intake and Body Weight during the Repletion Period

There were no statistically significant differences in weight gain and feed efficiency ratio (FER) among treatment groups indicating isocaloric formulation of treatment diets. However, the total food and iron intake in ferrous sulfate group was significantly higher than the lab threshed teff group ($P<0.05$) 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) (Table 4.5).

Table 4.5: Data from food consumption, body weight and iron intake at the end of 14 days of repletion period

Indices	Red teff		FS Control
	Lab Threshed	Field Threshed	
Total food intake (g)	234.25 ± 13.44^{a}	242.0 ± 14.85^{ab}	255.75 ± 20.75^{b}
Iron intake (mg)	8.20 ± 0.47^{a}	8.47 ± 0.52^{ab}	8.95 ± 0.73^{b}
Weight Gain (g)	47.88 ± 4.7^{a}	49.38 ± 3.06^{a}	51.38 ± 4.47^{a}
Feed Efficiency Ratio	0.20 ± 0.01^{a}	0.21 ± 0.01^{a}	0.20 ± 0.01^{a}

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

The FER values of the lab, field threshed teff flours and ferrous sulfate was similar to values reported elsewhere (Urga *et al*., 1998; Martino *et al*., 2011).

4.5. Hematologic Indices and Iron Bioavailability

The change in hemoglobin and the hemoglobin regeneration efficiency of rats consuming diets containing lab threshed, field threshed and ferrous sulfate enriched AIN-93G diets all supplying 35mg/kg iron were described in Table 4.6.

Table 4.6: Hemoglobin Repletion of Rats Receiving 35mg Iron per Kg of Diet from LT, FT and FS groups

Indices	Red Teff		FS Control
	Lab Threshed	Field Threshed	
Final Hemoglobin (g/dL)	10.16 ± 0.8[a]	11.63 ± 0.54[b]	13.4 ± 1.28[c]
Hemoglobin Gain (g/dL)	4.25 ± 0.46 [a]	5.79 ± 0.60 [b]	7.53 ± 1.01[c]
Initial Hemoglobin Iron (mg)	1.96± 0.25	2.12± 0.44	1.93±0.29
Final Hemoglobin Iron (mg)	4.46± 0.61	5.48± 0.78	5.98±0.96
Hb Regeneration Efficiency (HRE)	30.41±3.04 [a]	39.74±4.0 [b]	44.96±5.01[b]

Values are mean ± standard deviations and different superscripts within 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 (7.53 ± 1.01), followed by the field threshed (5.79 ± 0.60) and the lab threshed (4.25 ± 0.46) teff groups. The final hemoglobin concentration of rats in the lab threshed teff group (10.16 ± 0.8) was similar to earlier findings obtained for teff injera (10.45 ± 0.63) by Urga *et al*. (1998). The final hemoglobin concentration in the ferrous sulfate group (13.4 ± 1.28) was also in line with values (14 ± 0.21) reported by Dostal *et al*. (2012) for rats fed 37.47 ± 1.8 mg ferrous sulfate/kg diet. This study elucidated that 45% 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 lab threshed teff diet group, but the difference was not statistically significant when compared to the field threshed teff group.

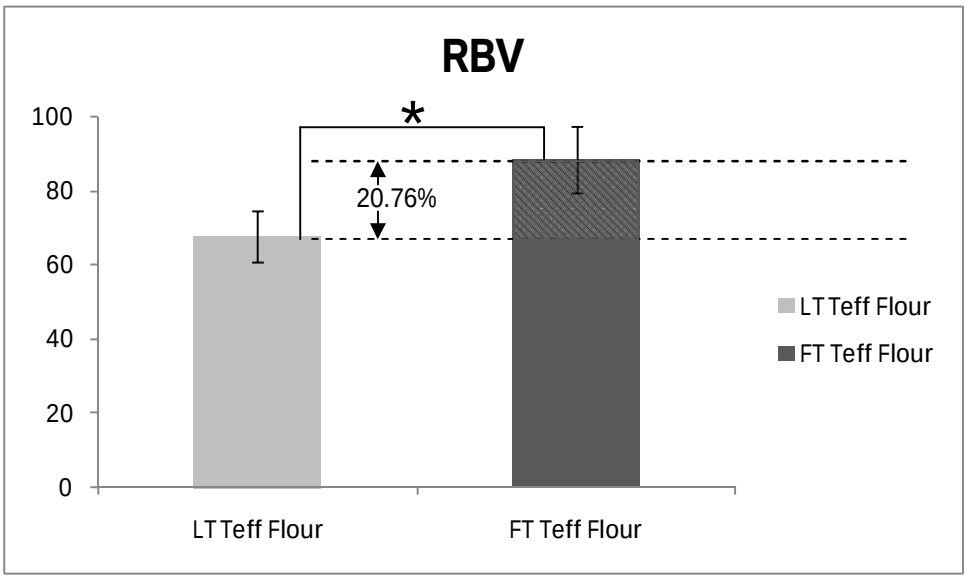


Figure 4.1: Relative Biological values (RBV) among treatment groups receiving equivalent amount of iron from LT, and FT sources. *Contribution of contaminant iron.

The Relative Biological Values (RBV) for the lab and field threshed teff flours were 67.64±6.76% and 88.4±8.9%, respectively ($p<0.05$) (figure 4.1.). The RBV of field threshed teff agrees reasonably well with the previous report of 94.76% for teff injera (Urga *et al*., 1998). This suggests that a significant proportion of the soil iron was bioavailable.

Total intrinsic iron intake of the rats during the hemoglobin repletion period was not statistically different ($p>0.05$) in the LT and FT group, which was 8.2±0.47mg and 8.47±0.52mg respectively; however, the FT group had 29 mg of additional extrinsic iron, which contributed to a 9.33% and 20.76% increase in HRE and RBV, respectively.

The impact of contaminant iron on iron status has remained controversial (Harvey *et al*., 2000). According to the previous *in vitro* studies, some authors (Smith *et al*., 2000; Icard-Verniere *et al*., 2013) have shown that a small proportion of the extrinsic iron can be potentially bioavailable, whereas others reported its role as negligible (Hooda *et al*., 2002;

Seim *et al*., 2013). However, the authors proposed that *in vivo* studies are needed to better assess the impact of contaminant iron. A recent study conducted by Ambaw (2013), fractionated iron in laboratory and field threshed teff samples and revealed that acid soluble fraction of lab threshed teff flour was higher and accounted to 44% of the total iron whereas 27.6% was exchangeable. In field threshed teff flour, the exchangeable fraction and acid soluble fraction was significantly higher than that of the lab threshed, showing the contaminant iron from soil can be exchangeable and then thus bioavailable. This is in support of the present finding showing significantly higher RBV due to contamination in FT teff flour. The study by Thums, Farago and Thornton (2008) reported that metals present in soil in a non-residue (exchangeable) phase would be potentially available for uptake into the human system once soil has been ingested.

On the contrary, using an *in vitro* digestion/Caco-2 cell model, Seim *et al*. (2013) reported that the iron from soil was not bioavailable, this report was also supported by an earlier study (Hooda *et al*., 2002). There was evidence from animal studies to indicate that ingested soil may remain bound to the mucosal layer in the intestine for many hours, even days, after it has been ingested (Gonzalez *et al*., 2004), which suggests that bioavailability of iron in soil samples cannot be studied in isolation and its interaction need to be considered and hence *in vivo* study would likely be the best means to determine extrinsic iron bioavailability. In addition, the Caco-2 system is limited to measuring the entrance of iron from a food digestate into the mucosal cell, whereas iron bioavailability also involves serosal transfer which is the movement of iron from the mucosal epithelial cell interior to circulation (Beiseigel *et al*., 2007).

It is documented that there are two phases of iron transfer, once iron is introduced in to the intestinal lumen, there is a rapid phase (within a few minutes) in which large amount of the given dose of iron appear in the circulation and then there is a slower phase (6-24 h) in which some iron, which was initially retained in the epithelial cells, is transferred to the circulation (McKie & Simpson, 2002; Roughead, Zito, & Hunt, 2005; Suttle, 2010).

The present *in vivo* study clearly illustrates that extrinsic iron can lead to hemoglobin repletion in iron deficient rats and is thus an important source of bioavailable iron. Considering that adult humans require 1 mg of iron/day to replenish their loss (Abbaspour *et al*., 2014), the seemingly low proportion of bioavailable iron from soil cannot be neglected, especially in extreme cases of contamination like in teff. This is especially true considering that iron is found in abundant concentration in soils. For example, Hooda *et al*. (2002) performed analysis of five soil samples from Uganda, Tanzania, Turkey and India, and reported an iron content range of 1483–9400 mg/100g. Similarly Smith *et al*. (2000) reported high concentrations was found on Ugandan geophagic soils around 1580 - 8160 mg/100g and Abrahams *et al*. (2006), reported soils form south Asia had a median 9600 mg iron/100g. Again, the study done by Seim *et al*. (2013) observed a range of 94-2640 mg/100g iron with a median of 350 mg/100g though relatively the last study reported lower iron concentrations in earth samples than other studies, the concentrations observed were still in orders of higher magnitude than foods considered to be iron-rich. The varying iron concentrations observed may be attributable to differences in iron content due to geographical context (Saturni *et al*., 2010; Bokhari *et al*., 2012) and iron level in soil was shown to correlate negatively with soil pH and clay content, and positively with sand content of the soil (Ayele *et al*., 2014).

Increased surface area was positively associated with the extent of *in vivo* bioavailability, since reducing particle size increases solubility at a given pH and time (Swain, Newman & Hunt, 2003). The study done by Arredond *et al*. (2006) showed that reducing the particle size of hydrogen-reduced iron from 45μm to 8μm increased the relative iron bioavailability of a fortified bread from 31.1% to 68.2%. Hence, particle size of the soil should also be noted since it is likely that some soil constituents are not completely attacked by the simulated gastric acid mixture in *in vitro* studies. According to the study by Abrahams *et al*. (2006), soil properties related to particle size may explain the low recoveries of some minerals determined from a soil; in their study, soil sample was disaggregated in a porcelain mortar with a pestle, sieved through a 2mm nylon mesh, and the < 2mm fine earth fraction retained; and up on *in vitro* physiologically based extraction test procedure, that simulate digestion in stomach and small intestine, determined that the bioaccessibility of iron from London soil sample was 4.1%. Immigrant Asian geophagist pregnant women consumed up to 65gm soil per day and this amount was estimated to contribute 118.6% of the daily iron requirement by adolescent women.

The particle size of teff flours in the present study was 125-250μm (Annex IV), which is by far less than the size (<2mm) used in the study by Abrahams *et al*. (2006), that might resulted in provision of more surface area for the contaminant soil in teff to be attacked by the gastric acid to enhance both its solubility and thereby its bioavaliability. Therefore, the measurements of iron bioavailability in soils are likely to be an underestimate of bioavailability when studied without consideration of soil particle size.

4.6. Bioavailability of Iron from Teff in Comparison to Other Iron Sources

Iron fortification of staple foods is used to combat iron deficiency anemia (Hurrell, 2002) and is considered to be a cost-effective strategy for reducing the problem of iron deficiency (Allen *et al*., 2006). The iron compound selected for food fortification should preferably be the one with the highest relative biological value (RBV), of low cost, and is not supposed to cause sensory changes to the food vehicle (Hurrell, 2002). Elemental iron powders now account for most of the iron used for food fortification (Hunt, 2001; Hurrell, 2002), and among them Electrolytic Iron Powders (EIP) are often used to fortify foods with iron (Swain *et al*., 2013). Sodium iron EDTA (NaFeEDTA) is recommended as the most suitable iron-fortifying agent for developing countries as it provides highly bioavailable and nonreactive iron (Lysionek *et al*., 2001). The other widely applied form of fortificant include iron phosphate compound (Hurrell, 2002).

The RBV of these fortificants obtained using the rat hemoglobin repletion method as in this study (Swain *et al*., 2013) are compared to the present results in Table 4.7.

Lab threshed teff with 35mg/kg level intrinsic iron showed similar RBV to EIP at 12mg/kg and FePO4 at 20mg/kg. Similarly, field threshed teff flour at 35mg/kg intrinsic iron but with additional 28mg of extrinsic soil iron had comparable RBV to 20mg/kg NaFeEDTA and 20mg/kg of EIP. The recommended levels of iron in fortification; 60 mg Fe/kg for electrolytic iron, 30mg Fe/kg for FeSO4, and 20 mgFe/kg NaFeEDTA (Huang *et al*., 2009).

Table 4.7: Relative Biological Value (RBV) of anemic rats fed diets fortified with graded levels iron in comparison to the RBV of iron in teff

Iron Source	Supplemental Iron Level (mg Fe/kg Diet)	*RBV %	Reference	Statistical Assay Method Used
Electrolytic Iron Poweder (EIP)	12 mg/kg	64.5	Swain *et al*., 2013	Slope Ratio
Electrolytic Iron Poweder (EIP)	12 mg/kg	66±2	Forbes *et al*., 1989	Slope Ratio
Electrolytic Iron Poweder (EIP)	20 mg/kg	86±6	Forbes *et al*., 1989	Slope Ratio
NaFeEDTA	20mg/kg	86	Lysionek *et al*., 2001	Mean Ratio
$FePO_4$	20 mg/kg	61±4	Forbes *et al*., 1989	Slope Ratio
Teff Injera (Fermented teff product)	35 mg/kg	94.76±0.16	Urga *et al*., 1998	Mean Ratio
Lab Threshed Teff Flour	35 mg/kg	67.64±6.7	This Study	Mean Ratio
Field Threshed Teff Flour	35 mg/kg	88.40±8.9	This Study	Mean Ratio

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

Regular consumption of staple foods naturally rich in iron could help individuals with increased nutritional needs to achieve dietary intakes (Bokhari *et al*., 2012). Gebre-Medhin *et al*. (1976) showed that regular consumption of teff was associated with prevention of iron deficiency. A human study conducted by Bokhari *et al*. (2012) showed that consumption of 30% teff-enriched wheat breads helped pregnant women maintain serum iron levels.

4.7. Consumption Trend of Teff in Ethiopia and Iron Status Paradox

According to previous studies, among developing countries only Ethiopia had a low prevalence of iron deficiency anemia (Wick, 2002). There was evidence suggesting that lower level of iron deficiency anemia was correlated with teff consumption (Wick, 2002; Saturni *et al*., 2010). An earlier study by Gebre-Medhin *et al*. (1976) also revealed that women who regularly consume teff products had a normal level of hematological parameters and did not suffer from either iron deficiency or anemia.

On the other hand, recent studies (Umeta *et al*., 2008; Haidar & Pobocik, 2009; Haidar, 2010) revealed moderate form of iron deficiency anemia become a problem in Ethiopia. This may partly be attributed to a decrease in consumption of teff overtime (Baye, 2014) as the share of teff in household consumption has declined since 1999/2000; findings from the analyses of a nationally representative household consumption and income survey conducted in 2004/05, suggested that teff's contribution to energy (calorie) intake in Ethiopia is only 11% (Berhane *et al*., 2011) but its production has been increasing at approximately 11% per year, with high latent demand resulting in price increases (ATA, FDRE-MOA & EIAR, 2013). These findings on consumption challenge the assumptions that teff is a major staple in Ethiopia that is based solely on production figures (Baye, 2014). Although teff is increasingly becoming expensive to the point of limiting its consumption (Berhane *et al*. 2011; Baye, 2014), the present finding further illustrates its nutritional benefit pointing that its consumption need to be promoted along with every possible intervention mechanisms making it affordable by different segment of the population.

4.8. Limitation of the Study

This study was unable to see the effect of fermented teff product, injera, as one experimental treatment group for the iron bioavailability study that was because of the expensiveness of iron deficient AIN-93G standard diet required for the depletion phase. Determining iron bioavailability from teff flour in rats partly solves this limitation, unlike humans; rats are naturally equipped with endogenous intestinal phytase (Pallauf & Rimbach, 1997).

Humans contain no or only negligible amounts of endogenous phytase in the stomach and small intestine, therefore depends mainly on dietary phytase or microbial phytase (Pallauf & Rimbach, 1997; Lopez *et al*., 2000). Phytase activity in human small intestine was found at low values 30 times less than that in rat tissue and 1000-fold lower than alkaline phosphatase in the same tissue (Iqbal, Lewis & Cooper, 1994).

Micronutrient deficient diet in rats led to increased intestinal phytate degradation (Lopez *et al*., 2000) and similarly, Siqueira *et al*. (2001) revealed that dietary phytate level has no effect on the iron status in undernourished rats. Lopez *et al*. (2000) also have shown that dietary phytic acid intake significantly induced intestinal phytase level in rats. Apart from phytase, rats also have the ability to synthesize vitamin C by themselves (Hemila, 1992; Valpuesta & Botella, 2004; Djurasevic *et al*., 2008), which is the known enhancer of iron absorption (Zimmermann & Hurrell, 2007; Abbaspour *et al*., 2014).

Rats have shown differences in digestive capacity and sensitivity to dietary factors (promoters and inhibitors of iron absorption) when compared to humans (Aragon, Ortiz & Pachon, 2012). This limits their use in accuracy of extrapolation to human (Wienk, Marx, & Beynen, 1999). However, for evaluating bioavailability or relative biological values of

iron from different sources including commonly used iron fortificants, with respect to ferrous sulfate control, hemoglobin-repletion bioassay in rats have been widely used in recent studies (Swain, Newman & Hunt, 2003; Rohner *et al*., 2007; Weber *et al*., 2010; Martino *et al*., 2011; Aragon, Ortiz & Pachon, 2012; Lucia *et al*., 2013; Swain *et al*., 2013). Moreover, the comparison of *in vitro*, animal, and human determinations of iron bioavailability study done by Forbes *et al*. (1989) have showed that the rat model hemoglobin depletion-repletion method could serve as the most reliable predictor of iron bioavailability in the human.

Chapter Five

Conclusions & Recommendations

5. CONCLUSIONS AND RECOMMENDATIONS

Total iron content of FT teff flour (29.44 $\pm$ 2.38 mg/100g) was fourfold greater than that of the LT teff flour (6.65 $\pm$ 0.05). In comparison to the ferrous sulfate control group, the efficiency of converting iron from field threshed teff flour in to hemoglobin was 88.4 % and 67.64 % of iron in lab threshed teff. This suggests that teff is a good source of bioavailable iron. Furthermore, additional soil iron contamination resulting from the traditional threshing of the grain contributes to the pool of bioavailable iron. The present *in vivo* study clearly illustrates that extrinsic iron can lead to hemoglobin repletion in iron deficient rats and is thus an important source of bioavailable iron.

This study therefore recommends that the country's future fortification plan needs to consider not only the contribution of intrinsic but also that of extrinsic iron on iron status. This clearly complicates the estimation of the fortificant level to be used as the level of grain contamination by soil is extremely variable. However, further studies are needed with various soil types collected from different geographical locations.

Although soil contamination is shown to improve iron status in rats, there are potential hazards that need to be considered. Soil is associated with helminthiasis caused by the ingestion of parasitic worms (geohelmints) (Luoba *et al*., 2005) although the process of baking *teff* flour into *injera* may be effective in destroying any deleterious organisms unless thermo-stable. The other potential hazard as elucidated by Abrahams *et al*. (2006) that soils may contain elevated concentrations of potentially harmful heavy metals or small concentration but upon frequent soil ingestion with meal may potentially expose subjects to deleterious amounts of these heavy metals including lead irrespective of individuals' age and pregnancy status.

REFERENCES

Abebe, Y., Bogale, A., Hambidge, K.M., Stoecker, B.J.,Bailey, K. & Gibson, R.S. (2007). Phytate, zinc, iron and calcium content of selected raw and prepared foods consumed in rural Sidama, Southern Ethiopia, and implications for bioavailability. *Journal of Food Composition and Analysis,* 20, 161–168.

Abrahams, P.W., Follansbee, M.H., Hunt, A., Smith, B. & Wragg, J. (2006). Iron nutrition and possible lead toxicity: an appraisal of geophagy undertaken by pregnant women of UK Asian communities. *Appl Geochem*, 21, 98-108.

Adish, A.A., Esrey, S.A., Gyorkos, T.W., Jean-Baptiste, J., & Rojhani, A. (1999). Effect of consumption of food cooked in iron pots on iron status and growth of young children: a randomized trial. *Lancet,* 353, 712-716.

Agriculture Transformation Agency of Ethiopia (ATA), Ministry of Agriculture (MoA) and Ethiopian Institute of Agriculture Research (EIAR). (2013). *Working Strategy for Strengthening Ethiopian's Tef Value Chain. Vision, Systemic Challenges, and Prioritized Interventions.* Page 8-12. Accessed on July 13, 2014 from: http://www.ata.gov.et/wp-content/uploads/National-Tef-Working-Strategy_July2013.pdf

Alaunyte, I. (2013). *Development of Nutrient-Rich Teff Bread and Its Effects on Iron Status and Exercise Performance in Female Runners.* PhD Dessertation, Manchester Metropolitan University. Retrieved on December 12, 2014, from http://www.e-space.mmu.ac.uk/e-space/bitstream/2173/313142/1/THESIS-%20definitive%20copy.pdf

Allen, K.J., Gurrin, L.C., Constantine, C.C., Osborne, N.J., Delatycki, M.B., Amanda J. & Nicoll, A.J. (2008). Iron Overload Related Disease in *HFE* Hereditary Hemochromatosis. *N Engl J Med*, 358, 221–30.

Allen, L., Benoist, B.D., Dary, O., & Hurrell, R. (Editors). (2006). *Guidelines on food fortifcation with micronutrients. World Health Organization and Food and Agriculture Organization of the United Nations*. Accessed on April 28, 2014 from: http://www.who.int/nutrition/publications /guide_food_fortification_micronutrients.pdf

Ambaw, A. (2013). *Determination of iron fractions of laboratory and field threshed teff flour, fermented past and injera: Implications to iron bioavailability* (Master's Thesis). Addis Ababa University, Center for Food Science and Nutrition.

Andrews, N.C. (2008). Forging a field: the golden age of iron biology. *Blood*, 112, 219-230. doi:10.1182/blood-2007-12-077388

AOAC – Association of Official Analytical Chemists. (1997). *Official methods of analysis of the Association of Official Analytical Chemists. Method 973.31 Bioavailability of Iron: Rat Hemoglobin Bioassay*. 16th ed., 3rd Revised, AOAC International, Gaithersgurg, Maryland. Chapter 45, Page 62-63.

Aragon, I.J., Ortiz, D., & Pachon, H. (2012). Comparison between in vitro and in vivo methods to screen iron bioavailability, *CyTA - Journal of Food*, 10(2), 103-111. DOI: 10.1080/19476337.2011.596283

Arogundade, L.A. (2006). Functional characterization of teff (Eragostics tef) protein concentrate: Influence of altered chemical environment on its gelation, foaming, and water hydration properties. *Food Hydrocolloid*, 20, 831-838.

Arredondo, M., Salvat, V., Pizarro, F., & Olivares, M. (2006). Smaller iron particle size improves bioavailability of hydrogen-reduced iron–fortified bread. *Nutr Res*, 2, 235-239.

Ashagrie, Z., & Abate, D. (2012). Improvement of Injera Shelf Life Through The Use of Chemical Preservatives. *AJFAND*, 12(5), 6409-6423.

Association of Official Analytic Chemists. (1990). *Bioavailability of iron Rat hemoglobin repletion bioassay: In Official Methods of Analysis of the Association of Official Analytic Chemists*, [Helrich, K. Editor] Arlington, VA Association of Official Analytic Chemists Inc, Page 1098-1099.

Baye, K. (2014). *Teff: nutrient composition and health benefits*. IFPRI Ethiopian Strategy Support Program Working Paper 67. Addis Ababa: International Food Policy Research Institute. Page 1-20. Retrieved on December 20, 2014, from http://www.ifpri.org/sites /default/files/publications/esspwp67.pdf

Baye, K., Guyot, J.P., Icard-Verniere, C., Mouquet-Rivier, C. (2012). Nutrient intakes from complementary foods consumed by young children (aged 12–23 months) from North Wollo, northern Ethiopia: the need for agro-ecologically adapted interventions. *Public Health Nutrition*, First View, 1–10. doi:10.1017/S1368980012005277.

Baye, K., Mouquet-Rivier, C., Icard-Vernière, C., Picq, C., & Guyot, J. (2013b). Changes in mineral absorption inhibitors consequent to fermentation of Ethiopian *injera*: implications for predicted iron bioavailability and bioaccessibility. *International Journal of Food Science and Technology*, DOI: 10.1111/ijfs.12295

Baye, K., Mouquet-Rivier, C., Icard-Vernière, C., Rochette, I., Guyot, J. (2013a). Influence of flour blend composition on fermentation kinetics and phytate hydrolysis of sourdough used to make injera. *Food Chemistry*, 138, 430–436.

Beard, J. J. (2003). Iron deficiency alters brain development and functioning. *J Nutr*, 133(Suppl), 1468S–1472S.

Beiseigel, J.M., Hunt, J.R., Glahn, R.P., Welch, R.M., Menkir, A., & Maziya-Dixon, B.B. (2007). Iron bioavailability from maize and beans: a comparison of human measurements with Caco-2 cell and algorithm predictions. *Am J Clin Nutr*, 86(2), 388-396.

Benoist, B., McLean, E., Egll, I., & Cogswell, M. (2008). *Worldwide prevalence of anemia 1993–2005: WHO global data base on anemia*. Geneva: World Health Organization.

Berhane, G., Paulos, Z., Tafere, K., & Tamru, S. (2011). *Food grain Consumption and Calorie Intake Patterns in Ethiopia*. Development Strategy and Governance Division, International Food Policy Research Institute, Ethiopia Strategy Support Program II, Ethiopia. Retrieved Sept., 4, 2013, from http://www.ifpri.org/sites/default/files/publications/esspwp23.pdf

Berhane, G., Paulos, Z., Tafere, K., & Tamiru, S. (2011). *Food grain Consumption and Calorie Intake Patterns in Ethiopia. IFPRI Ethiopia Strategy Support Program II (ESSP II) Working Paper 23*. Addis Ababa: International Food Policy Research Institute. Retrieved on December 20, 2014, from http://www.ifpri.org/sites/ default/files/ publications/esspwp23.pdf

Bhaskaram, P. (2001). Immunobiology of mild micronutrient deficiencies. *Br J Nutr*, 85 (Suppl 2), S75-80.

Bland, J.M., & Altman, D.G. (1986). Statistical methods for assessing agreement between two methods of clinical measurements. *Lancet*, 1, 307-310.

Bloom, P.R., & Inskeep, W.P. (1986). Factors affecting bicarbonate chemistry and iron chlorosis in soils. *J Plant Nutr*, 9(3-7), 215-228.

Boka, B., Woldegiorgis, A.Z., & Haki, G.D. (2013). Antioxidant Properties of Ethiopian Traditional Bread (Injera) as Affected by Processing Techniques and Tef Grain (Eragrostis tef (Zucc.)) Varieties. *Canadian Chemical Transactions*, 1(1), 7-24.

Bokhari, F., Derbyshire, E., Li, W., Brennan, C.S., & Stojceska, V. (2012). A study to establish whether food-based approaches can improve serum iron levels in child-bearing aged women. *J Hum Nutr Diet,* 25(1), 95-100.

Boling, S.D., Edwards, H.M., Emmert, J.L., Biehl, R.R., & Baker, D.H. (1998). *Poultry Science,* 77, 1388–1392.

Bovell-Benjamin, A., Viteri, F.E., & Allen, L.H. (2000). Iron absorption from ferrous bisglycinate and ferric trisglycinate in whole maize is regulated by iron status. *Am J Clin Nutr*, 71(6), 1563-1569.

Burayu, W. (2006). *Conservation Tillage Options For Sustainable Crop Production Systems in the Semi-Arid and Sub-Humid Oromiya*. A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (Tropical Agriculture) Graduate School, Kasetsart University. ISBN 974-16-1118-8

Carpenter, C.E. & Clark, E. (1995). Evaluation of methods used in meat iron analysis and iron content of raw and cooked meats. *J Agric Food Chem*, 43, 1824–1827.

Carpenter, C.E., & Mahoney, A.W. (1992). Contributions of heme and nonheme iron to human nutrition. *Crit Rev Food Sci*, 31, 333–367.

Cary, E.E. Grunes, D.L. Dallyn, S.L., Pearson, G.A., Peck, N.H., Hulme, R.S. (1994). Plant Fe, Al and Cr concentrations in vegetables as influenced by soil inclusion. *J Food Qual*, 17(6), 467−476. DOI: 10.1111/j.1745-4557.1994.tb00167.x

Central Statstical Agency of Ethiopia (CSAE). (2012). *Agricultural Sample Survey 2011 / 2012*: Report on Area and Production of Crops. Addis Ababa. Retrieved on July 29, 2014, from http://213.55.92.105/nada3/index.php/ ddibrowser/309/download/2119

Clark, E.M., Mahoney, A.W., & Carpenter, C.E. (1997). Heme and total iron in ready-to-eat chicken. *J Agric Food Chem*, 45, 124–126.

Clark, H.E., Coats, M.E., Eva, J.K., Ford, D.J., Miliner, C.K., O´Dorogue, P.N., Scott, P.P., & Ward, R.J. (1997). Dietary standards for laboratory animals: Report of laboratory animals Centre Diets Advisory Committee. *Lab Animals Report*, 11, 1-4.

Cook, J.D. (1999). Defining optimal body iron. *Proceedings of the Nutrition Society*, 58, 489–495.

Cook, J.D., Boy, E., Flowers, C., & Daroca, M.C. (2005). The influence of high-altitude living on body iron. *Blood*, 106, 1441-1446

Cook, J.D., Flowers, C.H., & Skikne, B.S. (2003). The quantitative assessment of body iron. *Blood*, 101, 3359-3364.

Costenbader, K.H., Chibnik, L.B., & Schur, P.H. (2007). Discordance between erythrocyte sedimentation rate and C-reactive protein measurements: clinical significance. *Clin Exp Reheumatol*, 25(5), 746-749.

De-Silva, A., Atukorala, S., Weerasinghe, I., Ahluwalia, N. (2003). Iron supplementation improves iron status and reduces morbidity in children with or without upper respiratory tract infections: a randomized controlled study in Colombo, Sri Lanka. *Am J Clin Nutr*, 77, 234–41.

Djurasevic, S.F., Djordjevic, J., Drenca, T., Jasnic, N., & Cvijic, G. (2008). Influence of vitamin C supplementation on the oxidative status of rat liver. *Arch Biol Sci, Belgrade*, 60 (2), 169-173. DOI:10.2298/ABS0802169D

Dostal, A., Chassard, C., Hilty, F.M., Zimmermann, M.B., Jaeggi, T., Rossi, S., & Lacroix, C. (2012). Iron depletion and repletion with ferrous sulfate or electrolytic iron modifies the composition and metabolic activity of the gut microbiota in Rats. *J Nutr*, 142, 271-277.

EHNRI. (1997). *Food Composition Table for use in Ethiopia Part III*. Ethiopian Health and Nutrition Research Institute, Addis Ababa. Page 1- 91.

Ethiopia Central Statistical Agency and ICF International. (2012). *2011 Ethiopia Demographic and Health Survey (EDHS): Key Findings*. Calverton, Maryland, USA: CSA and ICF International.

European Pharmacopoeia. (2005). *Statistical analysis*. Retrieved on December 12, 2014, from,http://lib.njutcm.edu.cn/yaodian/ep/EP5.0/05_general_texts/5.3.__statistical_analysis_of_results_of_biological_assays_and_tests/5.3.%20Statistical%20analysis%20of%20results%20of%20biological%20assays%20and%20tests.pdf

Fairweather-Tait, S., Fox, T., Wharf, S.G., & Eagles, J. (1995). The bioavailability of iron in different weaning foods and the enhancing effects of a fruit drink containing ascorbic acid. *Pediatric Research*, 37(4), 389-394

FAO/WHO. (2004). *Expert Consultation on Human Vitamin and Mineral Requirements, Vitamin and mineral requirements in human nutrition: Report of joint FAO/WHO expert consolation*. Retrieved December 10, 2014, from http://nrc.ajums.ac.ir/_nrc/documents/WHO%20Expert%20Consultation%20on%20Human%20Vitamin%20and%20Mineral%20Requirements.pdf

Ferruzza, S., Rossi, C., Scarino, M.L., & Sambuy, Y. (2012). A protocol for differentiation of human intestinal Caco-2 cells in asymmetric serum-containing medium. *Toxicology in Vitro, doi:10.1016/j.tiv.2012.01.008*. Retrieved on June 30, 2014, from http://www.researchgate.net/profile/Yula_Sambuy/publication/224832445_A_protocol_for_differentiation_of_human_intestinal_Caco-2_cells_in_asymmetric_serumcontaining_ medium /file/d912f505abc 8b7779a.pdf

Fidler, M.C. (2003). *Optimizing the Absorption of Fortification Iron* (Doctoral dissertation). A dissertation submitted to the Swiss Federal Institute of Technology Zurich for the degree of Doctor of Natural Sciences. pp 25-27. (Diss ETH No. 15113). Retrieved on December 28, 2014 from http://e-collection.library.ethz.ch/eserv/eth:26487/eth-26487-02.pdf

Fluttert, M., Dalm, S., & Oitzl, M.S. (2000). A refined method for sequential blood sampling by tail incision in rats. *Laboratory Animals,* 34, 372-378.

Fogh, J., Fogh, J.M., & Orfeo, T. (1977). One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst,* 59**,** 221–226.

Forbes, A.L., Adam, C.E., Arnaud, R.F., Chichester, C.O., Cook, J.D., Harrison, B.N., Hurrell, R.F., Khan, S.G., Morris, E.R., Tanner, J.T. & Whittaker, P. (1989). Comparison of in vitro, animal and clinical determinations of iron bioavailability: International Nutritional Anemia Consultative Group – Task force report on bioavailability. *Am J Clin Nutr*, 49, 225-238.

Frontela, C., Ros, G., & Martínez, C. (2011). Phytic acid content and "*in vitro*" iron, calcium and zinc bioavailability in bakery products: The effect of processing. *Journal of Cereal Science*, 54, 173-179

Galan, P., Cherouvrier, F., Zohoun, I., Zohoun, T., Chauliac, M. & Hercberg, S. (1990). Iron absorption from typical West African meals containing contaminant Fe. *British Journal of Nutrition*, 64, 541–546.

Gautam, C.S., Saha, L., Sekhri, K., & Saha, P.K. (2008). Iron deficiency in pregnancy and the rationality of iron supplements prescribed during pregnancy. *Medscape J Med*, 10, 283.

Gautam, S., Platel, K., Srinivasan, K. (2010). Higher Bioaccessibility of Iron and Zinc from Food Grains in the Presence of Garlic and Onion. *J Agric Food Chem*, 58, 8426–8429.

Gebre-Medhin, M., Killander, A., Vahlquist, B. & Wuhib, E. (1976). Rarity of anaemia in pregnancy in Ethiopia. *Scandinavian Journal of Haematology*, Vol. 16(3), 168-175.

Geissler, P.W., Prince, R.J., Levene, M., Poda, C., Beckerleg, S.E., Mutemi W., & Shulman, C.E. (1999). Perceptions of soil-eating and anaemia among pregnant women on the Kenyan coast. *Social Science & Medicine***,** 48(8), 1069–1079. doi:10.1016/S0277-9536(98)00409-2

Gera, T., Sachdev, H.P.S., & Nestel, P. (2007). Effect of combining multiple micronutrients with iron supplementation on Hb response in children: systematic review of randomized controlled trials. *Public Health Nutrition*, 12(6), 756–773.

Gibson, R.S., Bailey, K.B., Gibbs, M., & Ferguson, E.L. (2010). A review of phytate, iron, zinc, and calcium concentrations in plant-based complementary foods used in low-income countries and implications for bioavailability. *Food Nutr Bull*, 31(Suppl 2), S134-46.

Gonzalez, R., de Medina, F.S., Martinez-Augustin, O., Nieto, A., Galvez, J., Risco, S., & Zarzuelo, A. (2004). Anti-inflammatory effect of diosmectite in hapten-induced colitis in the rat. *Br J Pharmacol*, 141,951–960.

Green R.S. & Turner, J.C. (1974). The Manufacture of Small Quantities of Rat Food Pellets in the Laboratory. *Laboratory Animals*, 8,131-132.

Greffeuille, V., Mouquet-Rivier, C., Icard-Vernière, C., Ouattara, L., Avallone, S., Hounhouigan, J., Kayodé, P., Amoussa, W., & Fatoumata, B.H. (2010). *Traditional recipes of millet-, sorghum- and maize-based dishes and related sauces frequently consumed by young children in Burkina Faso and Benin*. INSTAPA, European Union's Seventh Framework Program, ISBN: 978-90-8585-903-3.

Gudissa, T., Mazengia, H., Alemu, S., & Nigussie, H. (2011). Prevalence of gastrointestinal parasites of laboratory animals at Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa. *Journal of Infectious Diseases and Immunity*, 3(1), 1-5.

Haidar, J. (2010). Prevalence of Anaemia, Deficiencies of Iron and Folic Acid and Their Determinants in Ethiopian Women. *J Health Popul Nutr*, 28(4), 359–368.

Haidar, J.A., & Pobocik, R.S. (2009). Iron deficiency anemia is not a rare problem among women of reproductive ages in Ethiopia: a community based cross sectional study. *BMC Blood Disorders*, 9, 7. doi:10.1186/1471-2326-9-7.

Hall, A., Bobrow, E., Brooker, S., Jukes, M., Nokes, K., Lambo, J., *et al*. (2000). Anemia in school children in eight countries in Africa and Asia. *Public Health Nutrition,* 4(3), 749-756.

Hallberg, L., & Hulthén, L. (2000). Prediction of dietary iron absorption: an algorithm for calculating absorption and bioavailability of dietary iron. *Am J Clin Nutr*, 71, 1147–60.

Harvey, P.W., Dexter, P.B. & Darnton-Hill, I. (2000). The impact of consuming iron from non-food sources on iron status indeveloping countries. *Public Health Nutrition*, 3, 375–383.

Hemila, H. (1992). Vitamin C and Plasma Cholesterol. *Critical Reviews in Food Science and Nutrition*, 32(l):33-57

Hooda, P., Henry, C., Seyoum, T., Armstrong, L. and Fowler, M.B. (2002). The potential impact of geophagia on the bioavailability of iron, zinc and calcium in human nutrition. *Environmental Geochemistry and Health*, 24 (4), 305-319.

Huang, J, Sun, J., Li, W., Wang, L., Wang, A., Huo J. *et al*. (2009). Efficacy of Different Iron Fortificants in Wheat Flour in Controlling Iron Deficiency. *Biomedical and Environmental Sciences,* 22, 118-121.

Hubert, M., Laroque, P, Gillet, J., & Keenan, K.P. (2000). The Effects of Diet, *ad Libitum* Feeding, and Moderate and Severe Dietary Restriction on Body Weight, Survival, Clinical Pathology Parameters, and Cause of Death in Control Sprague-Dawley Rats. *Toxicol Sci*, 58 (1), 195-207. doi: 10.1093/toxsci/58.1.195

Hunt, J.R. (2001). How important is dietary iron bioavailability? *Am J Clin Nutr*, 73(1), 3-4.

Hurrell, R. & Egli, I. (2010). Iron bioavailability and dietary reference values. *American Journal of Clinical Nutrition*, 91, 1461S–1467S.

Hurrell, R. F. (2002). Fortification: Overcoming Technical and Practical Barriers. *J Nutr*, 132, 806S-812S.

Hurrell, R.F. (2004). Phytic acid degradation as a means of improving iron absorption. *Int J Vitam Nutr Res*, 74(6), 445-52.

Iannotti, L.L., Tielsch J.M., Black, M.M., & Black, R.E. (2006). Iron supplementation in early childhood: health benefits and risks. *Am J Clin Nutr*, 84, 1261–76.

Icard-Vernière, C., Hama, F., Guyot J.P., Picq, C., Diawara, B., & Mouquet-Rivier, C. (2013). Iron contamination during in-field milling of millet and sorghum. *J Agric Food Chem*, 30, 61(43), 10377-83. doi: 10.1021/jf402612k.

Imran, M., Arshad1, M., Khalid, A., Kanwal, S. & Crowley, D.E. (2014). Perspectives of Rhizosphere Microflora for Improving Zn Bioavailability and Acquisition by Higher Plants. *Int J Agric Biol*, 16, 653–662

Iqbal, T.H., Lewis, K.O., & Cooper, B.T. (1994). Phytase activity in the human and rat small intestine. *Gut*, 35, 1233-1236. doi: 10.1136/gut.35.9.1233

Ismail, M. (1999). The use of Caco-2 cells as an in vitro method to study bioavailability of iron. *Mal J Nutr*, 5, 31-45.

Kalantar-Zadeh, K., Block, G., McAllister, C.J., Humphreys, M.H., & Kopple, J.D. (2004). Appetite and inflammation, nutrition, anemia, and clinical outcome in hemodialysis patients. *Am J Clin Nutr*, 80, 299–307.

Kalpalathika, P.V., Clark, E.M., & Mahoney, A.W. (1991). Heme iron content in selected ready-to-serve-beef products. *J Agric Food Chem*, 39, 1091–1093.

Karunaratne, A.J., Amerasinghe, P.H., Ramanujam, V.M.S., Sandstead, H.H., & Perera, P.J.A. (2008). Zinc, iron and phytic acid levels of some popular foods comsumed by rural children in Sri Lanka. *Journal of food composition and analysis,* 22(6), 481-488.

Kawai, K., Saathoff, E., Antelman, G., Msamanga, G., & Fawzi, W.W. (2009). Geophagy (Soil-eating) in Relation to Anemia and Helminth Infection among HIV–Infected Pregnant Women in Tanzania. *Am J Trop Med Hyg*, 80(1), 36-43.

Khan, M.S., Sohail, M., Atif Ali, A., Akhtar, N., Khan, H., & Rasool, F. (2014). Symptoms-Based Evaluation of Iron Deficiency Anemia in Students of Bahawalpur Correlated with their Eating Habits. *Trop J Pharm Res,* 13(5), 769.

Kongkachuichai, R., Napatthalung, P., & Charoensiri, R. (2002). *Heme and Nonheme Iron Content of Animal Products Commonly Consumed in Thailand*. Accessed on August 15,

2014 from http://www.thaiscience.info/Article%20for%20ThaiScience/Article/1/Ts-1%20heme%20and%20nonheme%20iron%20content%20of%20animal%20products%20commonly%20consumed%20in%20thailand.pdf

Leenhardt, F., Levrat-Verny, M.A., Chanliaud, E. & Remesy, C. (2005). Moderate decrease of pH by sourdough fermentation issufficient to reduce phytate content of whole wheat flour through endogenous phytase activity. *Journal of Agricultural and Food Chemistry*, 53, 98–102.

Lestienne, I., P.Besancon, B. Caporiccio, V. Lullien-Pellerin & Treche, S. (2005). Iron and zinc in vitro availability in pearl millet flours (Pennisetum glaucum) with varying phytate, tannin, and fiber contents. *J Agricult Food Chem*, 53, 3240-3247.

Lopez, H.W., Vallery, F., Levrat-Verny, M., Coudray, C., Demigne, C., & Remesy, C. (2000). Dietary Phytic Acid and Wheat Bran Enhance Mucosal Phytase Activity in Rat Small Intestine. *J Nutr*, 130, 2020–2025.

Lucia, C.M.D., Toste, M.G.V., Silveira, C.M.M., Bordalo, L.A., Roddrigues, F.C., Ana, H.M.P. *et al.* (2013). Iron bioavailability in Wistar rats fed with fortified rice by Ultra Rice® technology with or without addition of yacon flour (Smallanthus sonchifolius). *Archivos Latinoamericanos De Nutrición,* 13(1), 64-73.

Luoba, A.I., Geissler, P.W., Estambale, B., Ouma, J.H., Alusala, D., Ayah, R., Mwaniki, D., Magnussen, P., Friis, H. (2005). Earth-eating and reinfection with intestinal helminthes among pregnant and lactating women in western Kenya. *Trop Med Int Health*, 10(3), 220–227.

Lynch, S. (2005). The precision of in vitro methods and algorithms for predicting the bioavailability of dietary iron. *Int J Vitam Nutr Res,* 75(6), 436-45.

Lynch, S. (2012). The rationale for selecting and standardizing iron status indicators. In World Health Organization. *Report: Priorities in the assessment of vitamin A and iron status in populations.* Panama City, Panama, Geneva, World Health Organization. Page 55-62. Retrieved June 15, 2014, from http://www.who.int/nutrition/publications/micronutrients/background_paper3_report_assessment_vitAandIron_status.pdf

Lysionek, A., Zubillaga, M., Salgueiro, J., Caro, R., Ettlin, E., & Boccio, J. (2001). Bioavaliability Studies of a New Iron Source by Means of the Prophylactic-Preventive Method in Rats. *Biological Trace Element Research*, 84(1-3), 123-128.

Ma, G., Li,Y., Jin, Y., Zhai, F., Kok, F.J., & Yang, X. (2007). Phytate intake and molar ratios of phytate to zinc, iron and calcium in the diets of people in China. *European Journal of Clinical Nutrition,* 61 (3), 368-374.

McKie, A.T., & Simpson, R.J. (2002). *Basolateral Transport of iron in Mammalian Intestine: From Physiology to Molecules*. In: Templeton, D.M. Editor. Molecular circulation of iron transport. CRC Press. Marcel Dekker, Inc. 270 Madison Avenue, New York, NY 100016. Page 197-199.

McLean, E., Cogswell, M., Egli, I., Wojdyla, D., & Benoist, B. (2008). World wide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993–2005. *Public Health Nutrition,* 1-11. Doi:10.1017/S1368980008002401.

Mei, Z., Cogswell, M.E., Parvanta, I., Lynch, S., Beard J.L., Stoltzfus, R.J., & Grummer-Strawn, L.M. (2005). Hemoglobin and Ferritin are Currently the Most Efficient Indicators of Population Response to Iron Interventions: an Analysis of Nine Randomized Controlled Trials. *J Nutr*, 135, 1974 – 1980.

Miyada, T., Nakajima, A., & Ebihara K. (2011). Iron bound to pectin is utilized by rats. *British Journal of Nutrition*, 106(1), 73-78.

National Research Council of National Academies. (2011). *Guide for the Care and Use of Laboratory Animals*. (8th Ed). National Academics Press, Washington, D.C. Retrieved October 5, 2014, from http://grants.nih.gov/grants/olaw/Guide-for-the-care-and-use-of-laboratory-animals.pdf

Njiru, H., Elchalal, U., & Paltiel, O. (2011). Geophagy during pregnancy in Africa: a literature review. *Obstet Gynecol Surv*, 66(7), 452-9. doi: 10.1097/OGX.0b013e318232a034.

Onabanjo, O.O., Maziya-Dixon, B., Oguntona, C.R.B., Olayiwola, I.O., Oguntona, B.E. & Dixon, A.G.O. (2008). Iron bioavailability and utilization in rats fed cassava-based complementary diets. *Journal of Food, Agriculture & Environment*, 6 (3&4), 210-214.

Osborne, D., & Voogt, P. (1978). *The analysis of nutrients in foods*. Academic Press Inc. (London) Ltd., 24/28 Oval Road, London NW1 7DX.

Osorio, M.M., Lira, P.I.C., & Ashworth, A. (2004). Factors associated with Hb concentration in children aged 6–59 months in the State of Pernambuco, Brazil. *British Journal of Nutrition*, 91, 307–314.

Ouedraogo, H.Z., Traore, T., Ze`ba, A.N., Dramaix-Wilmet, M., Hennart, P., & Donnen, P. (2010). Effect of an improved local ingredient-based complementary food fortified or not with iron and selected multiple micronutrients on Hb concentration. *Public Health Nutrition*, 13(11), 1923–1930.

Pallauf, J., & Rimbach, G. (1997). Nutritional significance of phytic acid and phytase. *Arch Tierernahr*, 50(4), 301-319.

Peijnenburg, W.J.G.M., & Jager, T. (2003). Monitoring approaches to assess bioaccessibility and bioavailability of metals: Matrix issues. *Ecotoxicology and Environmental Safety*, 56, 63–77.

Persson, J. (2008). *Handbook for Kjeldahl digestion. A recent review of the classical method with improvements developed by FOSS*. 4th Edition. FOSS, DK-3400 Hilleroed, Denmark. Page 1-84. Retrieved on December 20, 2014, from http://www.foss.cn/-/media/files/documents/ industrysolution/papers/ca/handbookforkjeldahldigestion.ashx

Prabhavathi, T. & Rao, B.S.N. (1981). Contaminant iron in foods and its bioavailability predicted by in vitro method. *Indian J Med Res*, 74, 37−41

Queiroz, V.A.V., Guimarães, P.E.O., Queiroz, L.R., Guedes, E.O., Vasconcelos, V.D.B., & Guimarães, L.J. (2011). Iron and Zinc availability in maize lines. *Ciênc Tecnol Aliment Campinas*, 31(3), 577-583.

Reddy, M.B. (2005). Algorithms to assess non-heme iron bioavailability. *Int J Vitam Nutr Res*, 75(6), 405-12.

Reeves, P.G., Nielsen, F.H., & Fahey, G.C. (1993). AIN-93 Purified Diets for Laboratory Rodents: Final Report of American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of AIN-76A Rodent Diet. *J Nutr*, 123, 1939-1951.

Rohner, F., Ernst, F.O., Arnold, M., Hilbe, M., Biebinger, R., Ehrensperger, F., *et al*. (2007). Synthesis, Characterization, and Bioavailability in Rats of Ferric Phosphate Nanoparticles. *J Nutr*, 137, 614–619.

Roughead, Z.K., Zito, C.A., & Hunt, J.R. (2005). Inhibitory effects of dietary calcium on the initial uptake and subsequent retention of heme and nonheme iron in humans: comparisons using an intestinal lavage method. *Am J Clin Nutr*, 82(3), 589-597.

Rowe, L.A., & Dodson, D.M. (2012). A knowledge-to-action approach to food fortification: Guiding principles for the design of fortification programs as a means of effectively addressing micronutrient malnutrition. *Health*, 4 (10), 904-909. doi:10.4236/health.2012.410138.

Saha, P.R., Weaver, C.M., & Mason, A.C. (1994). Mineral Bioavailability in Rats from Intrinsically Labeled Whole Wheat Flour of Various Phytate Levels. *Journal of Agricultural and Food Chemistry*, 42, 2531-2535

Salas, R.E, Gamaldo, C.E., & Allen, R.P. (2010). Update in restless leg syndrome. *Curr Opin Neurol*, 23, 401-406.

Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M.L., Stammati, A., & Zucco, F. (2005). The Caco-2 Cell line as a model of the intestinal barrier: influence of cell and culture-

related factors on Caco-2 Cell functional characteristics. *Cell Biology and Toxicology*, 21, 1-26.

Sari, M., Pee, S., Martini E., Herman, S., Sugiatmi, Bloem, M.W., & Yip, R. (2001). Estimating the prevalence of anemia: a comparison of three methods. *Bulletin of World Health Organization*, 79, 506-511.

Saturni L., Gianna Ferretti, G., & Bacchetti T. (2010). The Gluten-Free Diet: Safety and Nutritional Quality. *Nutrients*, 2, 16-34. Doi: 10.3390/nu2010016

Seim, G.L., Ahn, C.I., Bodis, M.S., Luwedde, F., Miller, D.D., Hillier, S. *et al*. (2013). Bioavailability of iron in geophagic earths and clay minerals, and their effect on dietary iron absorption using an *in vitro* digestion/Caco-2 cell model. *Food Funct*, 4(8), 1263-1270. Doi: 10.1039/c3fo30380b

Siqueira, E.M.A., Arruda, S.F., De Sousa, L.M., & De Souza, E.M.T. (2001). Phytate from an alternative dietary supplement has no effect on the calcium, iron and zinc status in undernourished rats. *ALAN*, 51(3), 250-257.

Smith, B., Rawlins, B.G., Cordiero, M.J.A.R., & Hutchins, M.G. (2000). The bioaccessibility of essential and potentially toxic trace elements in tropical soils from Mukono District, Uganda. *J Geol Soc London*, 157, 884–892.

Suttle, N.F. (2010). *Mineral Nutrition of Livestock*. (4th ed.). Wallingford: CAB International, Oxfordshire OX10 8DE, UK. Retrieved December 12, 2014, from http://www.ucv.ve/fileadmin/user_upload/facultad_agronomia/Producion_Animal/Minerals_in_Animal_Nutrition.pdf

Swain, J., Kim, D., Wong, C., & Shivers, L. (2013). *Hemoglobin regeneration efficiency and relative bioavailability of an electrolytic elemental iron powder to anemic rats*. Retrived on December 15, 2015, from http://imp-india.com/wp-content/uploads/2013/03/HRE-and-RBV-of-electrolytic-iron-powder-IMP-Study-Report.pdf

Swain, J.H., Newman, S.M., & Hunt, J.R. (2003). Bioavailability of Elemental Iron Powders to Rats Is Less than Bakery-Grade Ferrous Sulfate and Predicted by Iron Solubility and Particle Surface Area. *J Nutr*, 133, 3546–3552.

Tadele, Z. (2011). *Annotated Bibliography of Tef [Eragrostis tef (Zucc.) Trotter]*. Institute of Plant Sciences, University of Bern, Switzerland. Printed at Stämpfli AG, 3001 Bern, Switzerland, September 2011. ISBN: 978-3-033-03119-7. Retrieved on December 12, 2014, from http://www.ips.unibe.ch/unibe/philnat/biology/botany/content/e6537/e546912/biblio graphy-tef.pdf

Tamanna, S., Parvin, S.,Kumar, S., Dutta, A.K., Ferdoushi, A., Siddiquee, M.A., Biswas, S.K., & Howlader, M.Z.H. (2013). Content of some minerals and their bioavailability in selected popular rice varieties from Bangladesh. *Int J Curr Microbiol App Sci*, 2 (7), 35-43.

Tegegne, A., & Bogale, A. (2005). *Ada'a – Liben Woreda Pilot Learning Site Diagnosis and Program Design*. Retrieved on December 13, 2014, from https://cgspace.cgiar.org/bitstream/handle/10568/16753/Ada.pdf?sequence=1

Temesgen, M. (2013). Nutritional Status of Ethiopian Weaning and Complementary Foods: A Review. *Open Access Scientific Reports*, 2(2), 1-9. doi:10.4172/scientifcreports.621

Thompson, B. (2007). *Food-based approaches for combating iron deficiency*. In: Kraemer, K., Zimmermann, M.B. Editors. Nutritional Anemia. Sight and Life Press. Basel, Switzerland. Page 337-358. Retrieved on September 23, 2013, from: http://www.sightandlife.org/filead min/data/ Books/Nutritional_anemia_book.pdf

Thums, C.R., Farago, M.E., & Thornton, I. (2008). Bioavailability of trace metals in brownfield soils in an urban area in the UK. *Environ Geochem Health*, 30(6), 549-63. doi: 10.1007/s10653-008-9185-6.

Tuntawiroon, M., Sritongkul, N., Pleehachinda, R., & Suwanik, R. (1998). Estimation of Available Dietary Iron, In Vitro and In Vivo Comparisons. *Thai Nuclear Medicine Newsletter*, 4 (1), 46-51.

Umeta, M., Haidar, J., Demissie, T., Akalu, G. & Ayana, G. (2008). Iron Deficiency Anaemia among Women of Reproductive Age in Nine Administrative Regions of Ethiopia. Ethiopian *J Health Dev*, 22(3), 252-258.

Umeta, M. & Parker, M.L. (1996). Microscopic studies of the major macro-components of seeds, dough and injera from tef (eragrostis tef). *An Ethiopian Journal of Science*, 19, 141-148.

Umeta, M., West, C. E., & Fufa, H. (2005). Content of Zinc, Iron, Calcium and their absorption inhibitors in foods commonly consumed in Ethiopia. *Journal of Food Composition and Analysis*, 18, 803 - 817.

Urga, K., Narasimha, H.V., Sasikala, B.V. & Vishwanatha, S. (1998). Bioavailability of Iron and Zinc from Teff in Rats. *Bull Chem Soc Ethiop,* 12(2), 95-103.

USEPA (US Environmental Protection Agency). (2003). *Ecological Soil Screening Level for Iron Interim Final*. OSWER Directive 9285.7-69. US Environmental Protection Agency Office of Solid Waste and Emergency Response 1200 Pennsylvania Avenue, N.W. Washington, DC 20460. Page 2-4. Retrieved December 12, 2014, from http://rais.ornl.gov/documents/eco-ssl_iron.pdf

US Pharmacopeial Convention. (2010). *Design and Development of Biological Assays*. Retrieved on December 12, 2014, from, http://www.ipqpubs.com/wp-content/uploads/2010/06/USP_1032.pdf

Valpuesta, V., & Botella, M.A. (2004). Biosynthesis of L-ascorbic acid in plants: new pathways for an old antioxidant. *TRENDS in Plant Science*, 9(12), 573-577.

Vinning, G., & McMahon, G. (2006). *Gluten-free Grains*. A demand-and-supply analysis of prospects for the Australian health grains industry. A report for the Rural Industries Research and Development Corporation.

Weber, T.K., Freitas, C.K., Amanco, O.M.S., & Mmorais, M.B. (2010). Effect of dietary fiber mixture on growth and intestinal iron absorption in rats recovering from iron-deficiency anemia. *British Journal of Nutrition,* 104(10), 1471-1476

Wessling-Resnick, M. (2014). Iron. In Ross, A.C., Caballero, B., Cousins, R., Tucker, K.L., Ziegler, T.R. (Eds.). *Modern Nutrition in Health and Disease*. (11th Ed). (Pp. 176-186).

Philadelphia: Lippincott Williams & Wilkins, 2001 Market Street, PA 19103. Retrieved December 1, 2014, from http://www.nutnet.ir/dl/Modern%202013.pdf

Wienk, K.J., Marx, J.J., & Beynen, A.C. (1999). The concept of iron bioavailability and its assessment. *Eur J Nutr,* 38(2), 51-75.

World Health Organization (WHO). (1992). *The Prevalence of Anemia in Women: A Tabulation of Available Information*. Geneva: World Health Organization. Retrieved December 1, 2014, from http://whqlibdoc.who.int/hq/1992/WHO_MCH_MSM_92.2.pdf

World Health Organization (WHO). (2006). *Guidelines of Food Fortification with Micronutrients*. Geneva: WHO

World Health Organization (WHO). (2011). Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity. Vitamin and Mineral Nutrition Information System. Geneva, World Health Organization, (WHO/NMH/NHD/MNM/11.1). Retrieved on December 11, 2014 from, http://www.who.int/vmnis/indicators/haemoglobin.pdf.

Yang, Z., Dewey, K.G., Lonnerdal, B., Hernell, O., Chaparro, C., Adu-Afarwuah, S., *et al*. (2008). Comparison of plasma ferritin concentration with the ratio of plasma transferrin receptor to ferritin in estimating body iron stores: Results of 4 intervention trials. *Am J Clin Nutr*, 87, 1892–8.

Zimmermann, M.B. (2006). The influence of iron status on iodine utilization and thyroid function. *Annu Rev Nutr*, 26, 367–89

Zimmermann, M.B., & Hurrel, R.F. (2007). Nutritional iron deficiency, *Lancet*, 370, 511–20

ANNEX

Annex I: Sample Collection Site, Teff Type, Growing Condition

Sample Collection Site: Adulala, Ziquala Abo Woreda, Oromia Region; 90Km from Addis Ababa

Teff Type: Red Teff

Soil Type: Clay Soil (Local name: *Koticha*)

Type of weed: Weed types are Megeresere and Bokusere

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 25kg and UREA 15kg used per 0.25 Hectar

Annex II: Approval Letter from the Ethical Committee of the College of Natural Science, Addis Ababa University

COLLEGE OF NATURAL SCIENCES
Addis Ababa University

OFFICE OF THE DEAN
የዲን ጽ/ቤት

የተፈጥሮ ሣይንስ ኮሌጅ
አዲስ አበባ ዩኒቨርሲቲ

Ref: ቁጥር: CNSDO/678/06/14
Date: ቀን: July 04, 2014

TO WHOM IT MAY CONCERN

The Ethical committee of the college of Natural Sciences in its meeting held on 12/05/2014 (Minutes: RERC/009/2014) has examined the MSc, thesis project of Ato Habtamu Guja's, entitled **"Evaluating the Bioavailability of iron form Teff using Hemoglobin Repletion Test in Rats: the Effect of Iron Contamination."** for ethical compliance The proposal is approved for implementation.

This is therefore, to kindly request your good office to allow the candidate access in accordance with established ethical requirements.

With regards,

Negussie Retta, (Professor)

Dean, College of Natural Sciences

Encl:

RERC Minutes & Letter

ሥልክ/Tel: +251-11-123-9472
ፋክስ/Fax: +251-11-123-9469
ፖ.ሣ.ቁ/POBox: 1176, Addis Ababa, Ethiopia
ኢ.ሜይል/Email: dean_cns@aau.edu.et

Please quote our reference number in your correspondence.

"Examine all things; hold fast that which is good"

Annex III: HemoCue Calibration Using Bland and Altman (1986) Method

Blood Samples with Different Levels of Hb	Hemoglobin Measurement Method		Difference
	HemoCue	Hematology Analyzer	
L1	4.2	4.3	-0.1
L2	6.5	6.4	0.1
L3	8.2	8.3	-0.1
L4	9.6	9.6	0
L5	9	9.1	-0.1
L6	6.3	6.2	0.1
L7	10.6	10.7	-0.1
N8	13.2	13.4	-0.2
H9	15.3	15.5	-0.2
H10	17.6	17.7	-0.1
Mean	**10.05**	**10.12**	**-0.07**
Stan Dev.	**4.22**	**4.28**	**0.106**

Lower Hb level (L), Normal Hb level (N) and Higher Hb level (H)

For assessing two different methods agreement by Bland and Altman (1986), measurements from different methods said to be in agreement when all points of difference lies in between Mean of the difference $\pm$ 2SD, [i.e. -0.07 $\pm$ 2 X 0.106] which is in between -0.282 & 0.142, so all points lied within range.

Annex IV: Result of Particle Size Determination of Teff Flours

Ethiopian Pharmaceuticals Manufacturing Sh. Co.

Operator: Tefera P.

Sample Type: Red Teff Flour

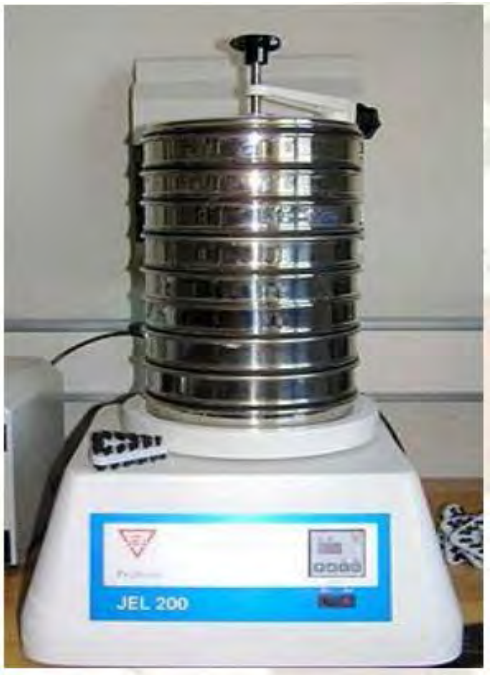
Total Sample weight: 50.5g

Sieve Size (μm)	Wt of teff flour retained in each sieves (g)	% Retained	% Cumulative Retained
2000	0.00	0	0
850	0.1	0.198413	0.198413
600	0.4	0.793651	0.992063
425	0.8	1.587302	2.579365
250	27.7	54.96032	57.53968
180	9.2	18.25397	75.79365
150	8.1	16.07143	91.86508
125	2.3	4.563492	96.42857
Receiver	1.8	3.571429	100





Sieves with different mesh sizes



Mechanical Shaker