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Determination of iron fractions of laboratory and field threshed *teff* flour, fermented dough and *injera*: implications to iron bioavailability

By

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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 SUPERVISORS	SIGNATURES	DATE
Dr. Cherinet Abuye	_____	_____
Dr. Kaleab Baye	_____	_____

DEDICATION

This work is dedicated to both of my family who I love most in this world more than anything

To: Ambaw Ayele (My father)

Adina Gedamu (My mother)

Bethlehem Muluneh (My wife)

Mikeal Ashenafi and Amanda Ashenafi (My children)

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Finally I would like thank my examiners for their willingness to review my work.

List of Abbreviations and acronyms

LTT- Laboratory Threshed Teff

FTT- Field Threshed Teff

LT- Laboratory Threshed

FT- Field Threshed

FLTT- Fermented Laboratory Threshed Teff

FFTT- Fermented Field Threshed Teff

ILTT- Injera Laboratory Threshed Teff

IFTT- Injera Field Threshed Teff

AAS- Atomic Absorption Spectroscopy

ml- Milliliter

ppm- Parts Per Million

Kg- Kilogram

l- Litre

g- Gram

DM- Dry matter

FAO- Food and agriculture organization

WHO- World Health Organization

GAE- Gallic acid equivalent

TAE- Tannic acid equivalent

IP6- Inositol hexa-phosphate

Eh- reduction potential

PPO- Polyphenol Oxidase

Fe- Iron

Fe^{2+}- Ferrous iron

Fe^{3+}- Ferric iron

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Abstract

Background-Iron deficiency is prevalent in Ethiopia despite high dietary iron intake. The contribution of iron from extrinsic sources from soil contamination during threshing is likely to be high. However, the extent of contamination and bioavailability of the contaminant iron remains unknown.

Objective-To evaluate whether there is difference in iron fractions of field and laboratories threshed teff and investigate how fermentation affects the mobility of the different iron fractions and mineral absorption inhibitors.

Methods-Teff variety grown under the same conditions but threshed differently (Laboratory and field threshed) was collected and characterized for their proximate composition and mineral absorption inhibitors. Total iron analysis and iron fractionation into five fractions using sequential extraction scheme were performed. The effect of fermentation and baking on the mobility of iron fractions was also evaluated.

Results-Field threshed teff had ~37% more iron content than that of laboratory threshed teff. Threshing significantly contributed to the exchangeable, reducible and residual fractions. Fermentation plays a great role in mobilizing iron fractions of both laboratory and field threshed teff. In both lab and field threshed teff samples, fermentation significantly increased the exchangeable and decreased the residual fraction. Furthermore, fermentation lead to significant decreases in phytate, iron-binding polyphenols and tannin ($P<0.05$.) in both laboratory and field threshed teff-injera. No significant differences in proximate composition between lab and field threshed teff, except for higher ash content in the field threshed teff, was observed.

Conclusion- Contaminant iron, mainly due to threshing, adds up to the total iron content of field threshed teff. Fermentation degraded mineral absorption inhibitors and mobilized iron fractions into more mobile and bioavailable fraction i.e. exchangeable fraction. Contaminant iron is likely to contribute to the intake of bioavailable iron.

Key words: Sequential extraction, threshing, fermentation, soil, contamination, fractionation, bioavailability

Chapter one

Introduction

1. Introduction

1.1 Prevalence of iron deficiency

Iron deficiency is one of the leading risk factors for disability and death worldwide, affecting an estimated 2 billion people (Zimmermann & Hurrell, 2007). Nutritional iron deficiency arises when physiological requirements cannot be met by iron absorption from diet. Dietary iron bioavailability is low in populations consuming monotonous plant-based diets (Zimmermann, Chaouki, & Hurrell, 2005). The high prevalence of iron deficiency in the developing world has substantial health and economic costs (Horton & Ross, 2003), including poor pregnancy outcome, impaired school performance, and decreased productivity (Lozoff, Beard, Connor, Felt, Georgieff, & Schallert, 2006; Zimmermann & Hurrell, 2007).

This is attributed to the low mineral content of plant based foods, as well as to their high chelating agent contents like phytic acid which strongly chelate metal ions in the gastrointestinal tract (Cheryan & Rackis, 1980). Although iron deficiency can occur at any age, women of reproductive age, pregnant women and young children are particularly vulnerable to develop iron deficiency due to increased nutritional demand. During pregnancy, the need for iron increases significantly. The reason for this is that an extra need of iron for the development of the fetus (Khan & Rehman, 2005).

1.2 Consequences of iron deficiency

Risk for iron deficiency is a function of iron loss, iron intake, iron absorption, and physiological demand. Women of child bearing are especially at high risk for iron deficiency. Iron deficiency can lead not only to anemia but also to decreased work capacity, abnormal neuro-transmitter function and altered immunological and inflammatory defenses (Ross, 2002).

Iron deficiency at any time in life may disrupt metabolic processes and subsequently change cognitive and behavioral functioning (Murray-Kolb & Beard, 2007).

Important subclinical and clinical consequences of iron deficiency are impaired physical work performance, developmental delay, cognitive impairment, and adverse pregnancy outcomes (Zimmermann & Hurrell, 2007).

The bulk of experimental and epidemiological evidence in humans suggests that functional consequences of iron deficiency (related both to anemia and low serum iron) occur only when iron deficiency is of a severity sufficient to cause a measurable decrease in hemoglobin concentration (Baker, 2000). Among the earliest functions to be affected are those associated with the brain enzymes involved in cognition and behavior and a number of immune mechanisms important for protection against infection (Zimmermann & Hurrell, 2007). The adverse effects of iron deficiency on cognition appear to be irreversible(Murray-Kolb & Beard, 2007). Furthermore, iron deficiency is physically and intellectually disabling at all ages.

Therefore the economic and social consequences of iron deficiency anaemia, as yet un-quantified, are thought to be enormous including a significant drain on health care, education resources and labor productivity, and reduced physical and mental capacity of large segments of the population(Ania, Suman, Fairbanks, & MELTON III, 1994; Baker, 2000; Viteri, Ali, & Tujague, 1999).

Although the most important determinant factor of iron deficiency anaemia in most developing countries is low dietary intake, poor bioavailability of dietary iron, intestinal parasites, especially hookworm (a parasitic worm which attach itself in the intestinal wall) infestation are reported to be a major cause. Other causes include malaria and congenital hemolytic diseases (Ania, Suman, Fairbanks, & MELTON III, 1994; Dallman, 1998).

The etiology of anaemia in Ethiopia is not well established and the information available is limited in representativeness of the country. Various researchers came up with different conclusions despite the problem being among the ten top morbidities reported by most health institutions in the country (J. Haidar, 2010). Some studies in the past have documented the problem as being rare in Ethiopia and attributed this to the consumption of "*teff* ", a cereal which has high iron content. However at least part of the iron in teff may be attributed to soil contamination (Wolde-Gabriel, Gebru, & Fisseha) while others have concluded the issue as is a mild to moderate public health problem (Umeta, Jemal, Demissie, Girma, & Gonfa, 2008).

Although the magnitude of Iron Deficiency Anemia in Ethiopia has not yet been well documented nationwide, the limited data available on the prevalence rate of IDA among pregnant and lactating women in the rural communities, reported a prevalence rate of 18.7% (Getahun, Urga, Genebo, & Nigatu, 2001; J. Haidar, 2010).

Ethiopia has a wide range of agro-climatic conditions which permits, a wide variety of foods (fruit, dairy etc). However, the diet remains monotonous resulting in low intake of minerals and vitamins. Furthermore, bioavailability of much of the iron in the average Ethiopian diet is restricted, presumably affecting the iron status of the community (Getahun, Urga, Genebo, & Nigatu, 2001).

1.3 Bioavailability of dietary iron

There are two forms of iron in the diet, heme and non-heme iron. These two differ in their mechanism of absorption. Heme iron is a constituent of hemoglobin, cytochrome and myoglobin and non-heme is iron found in plant based diets. The absorption of these two kinds of iron are influenced by the form and content of iron, the presence of modifying dietary factors (such as inhibitors and enhancers of iron absorption) and host factors including iron status.

Heme-iron constitutes a relatively minor part of iron intake. Even in diets with high meat content it accounts for only 10-15% of the total iron intake. Diets in developing countries usually contain negligible amounts of heme iron. Non-heme iron is thus the main source of dietary iron.

Iron can be found in three chemical forms in foods of plant and animal origin. These are iron oxides, salt (inorganic and organic) and organic heme-iron complex. Some of the iron present in the diet may be in a chemical form that is poorly or not at all absorbable (i.e. oxide). It may be present as a very stable complex or as iron compounds with a low solubility in the gastrointestinal contents.

1.4 Intrinsic and extrinsic iron

Intrinsic iron is an iron source from the food matrix itself and represents the actual iron content of the food matrix. On the other hand extrinsic iron can be from external contamination that may result during post harvest operation and food processing. For example, extreme soil contamination is observed in teff (Hofvander, 1968).

In soil, iron is commonly found in the form of oxides and hydroxides of low bioavailability. In developing countries, dietary iron intake can be overestimated because foods are often contaminated with iron from soil residue or with dust that has settled on the surface during processing such as air drying (Geerligs, Brabin, & Omari, 2003) .

1.5 Statement of the problem

It is a common paradoxical finding in many developing countries that the prevalence of iron deficiency is high despite a high dietary intake of iron. This is also true in populations with no excessive losses of iron (L. Hallberg & Björn-Rasmussen, 1981).

The study on the potential impact of geophagia on the bioavailability of iron shows that eating soil can decrease potentially bioavailable iron due to sorption in the GI tract. This means that eating soil may have negative impact on iron bioavailability (Hooda, Henry, Seyoum, Armstrong, & Fowler, 2002).

Moreover bioavailability of extrinsic iron remains understudied. Consumption of extrinsic iron from cooking utensils which are made of iron pots showed improvement in iron availability especially for meat and vegetables more than in legumes. In a community study, children fed foods cooked in iron pots had higher concentrations of hemoglobin than those fed foods cooked in aluminum pots (Adish, Esrey, Gyorkos, Jean-Baptiste, & Rojhani, 1999).

Grefeuille et al., 2011 stated that fermentation mainly due to the acidification can greatly increase the amount of bioaccessible iron. Thus, the combined effect of contamination and fermentation resulted in enhanced levels of iron potentially accessible for absorption. However, whether extrinsic iron from soil contaminated teff is likely to be bioavailable, and how it is affected by processing such as fermentation remains unknown (Greffeuille, Polycarpe Kayodé, Icard-Vernière, Gnimadi, Rochette, & Mouquet-Rivier, 2011).

1.6 Objective

1.6.1 General objective

To fractionate iron in field threshed and laboratory threshed teff flours, fermented dough and baked injera, and to evaluate its mobility.

1.6.2 Specific objectives:

-Determine the extent of iron contamination in teff grains

-Evaluate whether intrinsic and extrinsic iron differ in iron fractions

-Evaluate how food processing such as fermentation and baking affect intrinsic/extrinsic iron

-Understand the implication of different fractions of intrinsic and extrinsic iron on its bioavailability.

Chapter two

Literature review

2. Literature Review

2.1 Iron

Iron is involved in many vital functions in the human body. First, iron is important for oxygen transport. Further, iron is essential to brain function and development and severe iron deficiency can cause retarded mental development, which may be irreversible (Walker, Wachs, Meeks Gardner, Lozoff, Wasserman, Pollitt, et al., 2007).

Dietary iron is present in foods in two main forms; haem iron only in foods of animal origin (high amounts in liver and red meat) and non-haem iron in both animal and plant foods, mostly in the ferric state. Haem iron and non-haem iron are absorbed through different mechanisms.

Haem iron is transported into the enterocyte by the haem receptor, while non-haem iron uses the divalent metal transporter (DMT1), which means that dietary ferric iron (Fe^{3+}) must be reduced to ferrous iron (Fe^{2+}) before uptake (Ganz, 2007).

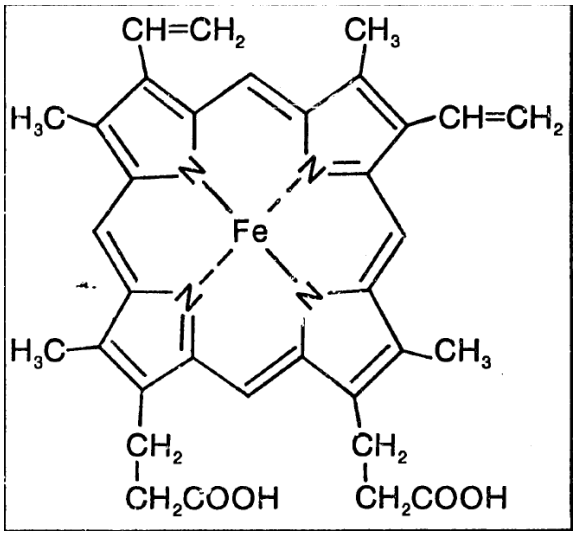

Figure 2.1 Iron porphyrin complex (**Source:** American Cereal Chemist Association 1981)

Absorption of non-haem iron can be enhanced or inhibited by various dietary components and thus depends on the meal composition. The absorption of haem iron is much higher than the

absorption of non-haem iron; about 25% for haem iron and less than 10% for non-haem iron. Iron absorption is also influenced by total iron content in the diet (lower iron content increases absorption efficiency), and by iron status and physiological state of the individual (low iron stores and pregnancy increases absorption efficiency). Milk has low iron content and the absorption is relatively poor. Older studies suggested that calcium in milk had a negative effect on iron absorption, but more recent studies have suggested that calcium supplements did not decrease absorption (Mølgaard, Kæstel, & Michaelsen, 2005).

Some studies have suggested that cow's milk can induce occult intestinal bleeding in young infants which may contribute to the negative effect of milk on iron status (Ziegler, Jiang, Romero, Vinco, Frantz, & Nelson, 1999).

However, it seems like the process involved in drying milk eliminates this effect, so that milk products based on powdered milk do not cause bleeding (Ziegler, Fomon, Nelson, Rebouche, Edwards, Rogers, et al., 1990).

In the human body, iron is present in all cells and has several vital functions:

- In the form of hemoglobin carries oxygen to the tissues from the lungs
- As myoglobin, facilitates oxygen use in the muscles
- In the form of cytochromes, transports electrons within the cells and
- Is an integral part of enzyme reactions in various tissues

Symptoms of iron deficiency are in most cases the result of the associated anaemia and may include fatigue, rapid heart rate, palpitations and rapid breathing on exertion. An extreme overdose of iron has a negative effect on health. The lethal dose is approximately 180-300 mg/kg (Schümann, Ettle, Szegner, Elsenhans, & Solomons, 2007) body weight, but symptoms of iron

overdose can appear at levels in the range of 20-60 mg/kg body weight. The recommended daily intake is about 8 mg/day for men 18 mg/day for women (19-50 year) (Whitney, Whitney, & Rolfes, 2010).

2.2 Tef grains [*Eragrostis tef* (Zucc.) Trotter]

Tef is one of the ancient grains of the world finding resurgence in the modern diet. It originated in Ethiopia as a wild forage grass and was eventually cultivated in the highlands of Ethiopia. Seeds of tef have been found in a brick of the Dassur Egyptian Pyramid built in 3359 B.C (Tadesse, 1975). Today tef straw is still used to make adobe in Ethiopia and it is cultivated for its hay in Kenya and Australia. In the U.S. tef crops are being grown in Idaho (Stallknecht, Gilbertson, & Eckhoff, 1993).

Tef is fine stemmed, tufted annual grass characterized by a large crown, many shoots, and a shallow diverse root system. The plants germinate quickly and are adapted to environments ranging from drought stress to water logged soil conditions. It is a reliable low risk crop.

Tef is adapted to a wide range of climate and agricultural conditions. It can be grown from sea level up to 2800meter of altitude, under different temperatures, soil conditions, and rainfall. Tef grains are very small: 1000 grains weigh 0.3-0.4 gram. Their color varies from white to dark brown, with the lighter the color being the more expensive (S. Ketema, 1997). For example, white tef grows only in the highlands and will thrive only under this environmental conditions, and then only through a great deal of effort. Particularly in poor households tef is often mixed with wheat because of the latter's lower price (Ginbot & Farrant, 2011).

This food not only provides energy and protein but is also the major source of zinc, iron, calcium and phosphorus. It contains 11% protein, 80% complex carbohydrate and 3% fat (Rouk & Mengesha, 1963). It is an excellent source of essential amino acids, especially lysine, the amino

acid that is most often deficient in grain foods (Jansen, DiMaio, & Hause, 1962). Tef is also an excellent source of fiber and iron, and has many times the amount of calcium, potassium and other essential minerals found in an equal amount of other grains (Seyfu Ketema, 1997). When tef is used to make injera, a short fermentation process allows the yeast to generate more vitamins.

Tef is nearly gluten-free, and is gaining popularity in the whole food and health food industry in the U.S. as an alternative grain for persons with gluten sensitivity. Tef may also have applications for persons with Celiac Disease (Stallknecht, Gilbertson, & Eckhoff, 1993)

Figure 2.2: Picture showing teff grass and grains

2.3 Mineral absorption inhibitors

Mineral absorption inhibitors are those ligands that chelate iron to form insoluble complexes or complexes of very high affinity, so that iron is not released from the chelate for absorption. Examples include tannins, phytate, and oxalates.

2.3.1 Phytate

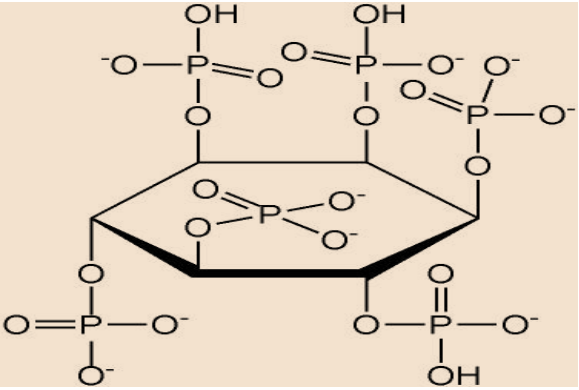

Figure 2.3 Structural formula of Phytate, myo-inositol hexakisphoshate

Phytate, myo-inositol hexakisphoshate, is the salt of phytic acid and is widely distributed in all seeds and possibly all cells of plants. It serves as a storage of phosphorous and minerals and accounts for 60-90% of the phosphorous in the plant. Besides phytate, other inositol phosphates are present in the seeds, however to a much lower extent (Zhou & Erdman Jr, 1995).

In cereals, phytate is located up to 80% in the aleurone layer, but is also found in the germ, while the endosperm is almost free of phytate (Zhou & Erdman Jr, 1995).

Moreover, phytate refers to phytic acid (*myo*-inositol hexaphosphate), made up of an inositol ring with six phosphate ester groups, and its associated salts: magnesium, calcium, or potassium phytate. It is noteworthy that *myo*-inositol phosphates with less than five phosphate groups (i.e., IP-1 to IP-4) do not have a negative effect on zinc absorption (Zhou & Erdman Jr, 1995) whereas *myo*-inositol phosphates with less than three phosphate groups do not inhibit iron absorption (Sandberg, Brune, Carlsson, Hallberg, Skoglund, & Rossander-Hulthén, 1999). The content of phytate in cereals varies from 0.06% to 2.22% (Reddy, 2002).

The phytate content of cereals is highly influenced by environmental conditions (climate, soil, and irrigation), fertilizer applications, and stage of maturation. In cereals, legumes, and oleaginous seeds: the highest levels are reached in mature seeds (Reddy, 2002).

Sources of phytate

The main sources of phytate in the daily diet are cereals and legumes, but also oil seeds and nuts (Jacela, DeRouchey, Tokach, Goodband, Nelssen, Renter, et al., 2012). Cereals are rich in phytate containing approximately 1% phytic acid on dry matter (DM) basis (from 0.06 to 2.2%). Up to 7.2% and 8.7% (DM) basis phytate contents are found in wheat and rice bran, respectively.

In legume seeds phytate predominately occurs in the protein bodies of the endosperm, ranging from 0.2 -2.9% (DM) basis.

In oil seeds such as sunflower seeds, soybeans, sesame seeds, linseeds and rape seeds the phytic acid content ranges from 1- 5.4% (DM) basis. In soy concentrates a content of 10.7% (DM) basis has been reported.

In nuts, the forth group of food rich in phytate, such as hazelnuts, walnuts, almonds and cashew nuts, the phytic acid content varies from 0.1 – 9.4% the highest in almonds. There is a huge variation of phytate content in different raw and unprocessed foods, depending on botanical varieties, various climate condition and different stages of seed.

Phytic acid and nutrient bioavailability

Phytic acid chelate metal ions, especially zinc, iron, and calcium, but not copper (Egli, Davidsson, Zeder, Walczyk, & Hurrell, 2004), forming insoluble complexes that cannot be digested or absorbed in the gastrointestinal tract of humans because of the absence of intestinal phytase enzymes (Iqbal, Lewis, & Cooper, 1994).

Phytate also complexes endogenously secreted minerals such as zinc (Hansen, Sandström, Jensen, & Sørensen, 1997) and calcium (Morris & Ellis, 1985), making them unavailable for re-absorption into the body. The inhibitory effect of phytate on mineral absorption has been

confirmed by *in vivo* radioactive and stable isotope studies, in which fractional absorption of iron, zinc, and calcium has been reported to be significantly lower from diets with a high content of phytate than from diets with lower phytate content (Egli, Davidsson, Zeder, Walczyk, & Hurrell, 2004).

Phytic acid molecule contains 12 dissociable hydrogen atoms. Depending on the pH of the solution different phytic acid different phytic acid anions may be formed having different degree of protonation (Lasztity, 1991). This property of phytic acid also affects the availability of macro nutrients like protein and that of enzymes. The terminal amino groups like lysyl, histidyl and arginyl can be positively charged at a low pH below the isoelectric point of proteins. Any of these groups can directly form a complex with a negatively charged phytate anion (Thompson & Serraino, 1986).

One phytate anion can interact with two charged groups of protein in the normal steric condition. According to the number of positively charged groups and conformational conditions, the protein molecule can bind more phytate anions at the same time. Also at intermediate pH values, the lysyl and arginyl groups are only positively charged, so in this case a slight possibility of electrostatic interaction between these groups and phytate anions. The interaction between phytic acid and protein is reduced when the pH is very high. It should also be noted that the ternary complexes can be formed when the polyvalent cations are present. In this case the cation forms a bond between the phytate anion and a negatively charged group of the protein. (Bindra, Gibson, & Thompson, 1986) showed that ternary complexes of protein, phytic acid and carbohydrate might form; complexes that affect the rate of digestibility. In addition to being a chelator of important minerals, phytic acid also inhibits the enzymes that are needed to digest our food, namely pepsin, amylase and trypsin.

2.3.2 Polyphenols

Polyphenols constitute one of the most numerous and ubiquitous groups of plant metabolites and are an integral part of both human and animal diets. Ranging from simple phenolic molecules to highly polymerized compounds with molecular weights of greater than 30,000 Da, the occurrence of this complex group of substances in plant foods is extremely variable.

Natural polyphenols can range from simple molecules, such as phenolic acids, to highly polymerized compounds, such as tannins (Bravo, 1998).

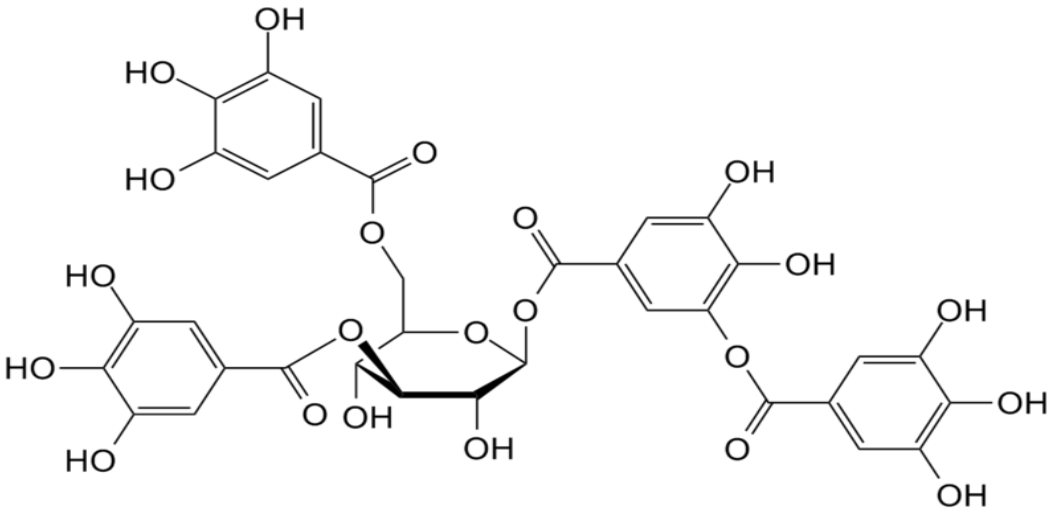

Figure 2.4 Structure of condensed tannin

They occur primarily in conjugated form, with one or more sugar residues linked to hydroxyl groups, although direct linkages of the sugar unit to an aromatic carbon atom also exist. The associated sugars can be present as monosaccharides, disaccharides, or even as oligosaccharides (Bravo, 1998). Glucose is the most common sugar residue, although galactose, rhamnose, xylose, and arabinose are also found, as well as glucuronic and galacturonic acids and many others

(Bravo, 1998). Associations with other compounds, such as carboxylic and organic acids, amines, and lipids, and linkages with other phenols are also common (Bravo, 1998).

Source of polyphenols

Polyphenols are common constituents of foods of plant origin; they comprise a wide variety of molecules that have a polyphenol structure (i.e. several hydroxyl groups on aromatic rings), but also molecules with one phenol ring, such as phenolic acids and phenolic alcohols.

Polyphenols are divided into several classes according to the number of phenol rings that they contain and to the structural elements that bind these rings to one another. The main groups of polyphenols are: flavonoids, phenolic acids, phenolic alcohols, stilbenes and lignans.

Influence of Polyphenols on Bioavailability of Minerals

Polyphenols can form complexes with metal cations through their carboxylic and hydroxylic groups, and thus interfere with the intestinal absorption of minerals. Numerous experiments in both humans and animals have shown that polyphenols strongly inhibit iron absorption. This action has been attributed to the galloyl and catechol groups of polyphenolic compounds (M. Brune, Rossander-Hultén, Hallberg, Gleerup, & Sandberg, 1992).

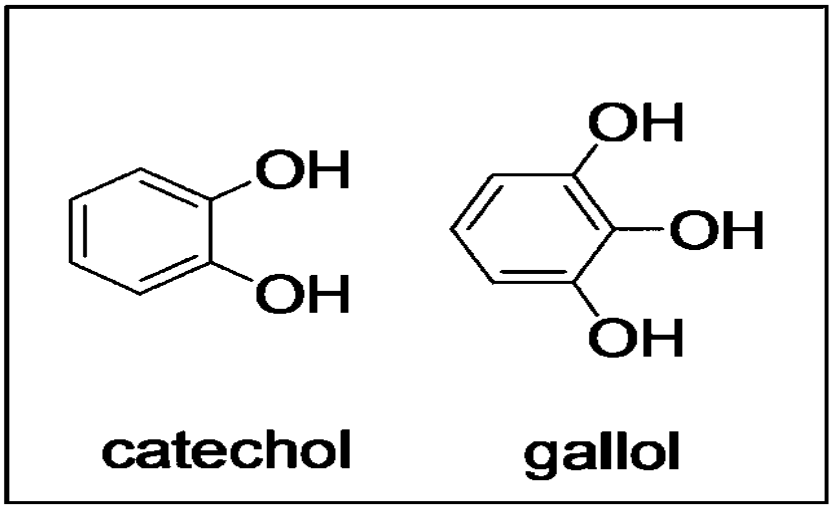

Figure 2.5 Di-hydroxy benzene (Catechol) and tri-hydroxy benzene (Gallyol)

Phenolic compounds have been identified as potent inhibitors of iron absorption (A. Brune, 1998; Gillooly, Bothwell, Torrance, MacPhail, Derman, Bezwoda, et al., 1983; L Hallberg & Rossander, 1982; Moore & Disler, 1983), presumably by forming insoluble complexes with iron ions in the gastro-intestinal lumen and thereby making the iron unavailable for absorption. The phenolic compounds embrace a very large and heterogeneous group of compounds.

Phenolic compounds bearing catechol groups and galloyl groups with at least two adjacent hydroxyls may have marked iron-binding properties, whereas compounds with other hydroxylation patterns can be expected to bind iron to a lesser extent. Results reported (A. Brune, 1998) indicate that galloyl (tri-hydroxy benzene) phenolic group is the structure mainly responsible for inhibition of iron absorption by phenolic compounds in foods. Chlorogenic acid, with a catechol group, interfered with iron absorption, though to a lesser extent (M. BRUNE, HALLBERG, & SKÅNBERG, 1991).

Polyphenoles and their anti-oxidant property

Until recently, polyphenoles were only known for their negative effects caused by the ability of certain polyphenols to bind and precipitate macromolecules, such as dietary protein, carbohydrate, and digestive enzymes, thereby reducing food digestibility. Recent interest, however, in food phenolics has increased greatly because of the antioxidant and free radical-scavenging abilities associated with some phenolics and their potential positive effects on human health.

Polyphenols are the most abundant antioxidants in our diet and are widespread constituents of fruits, vegetables, cereals, olive, dry legumes, chocolate and beverages, such as tea, coffee and wine. The main factor responsible for the delayed research on polyphenols is the variety and the complexity of their chemical structure.

As antioxidants, polyphenols may protect cell constituents against oxidative damage and, therefore, limit the risk of various degenerative diseases associated to oxidative stress (Hertog, Kromhout, Aravanis, Blackburn, Buzina, Fidanza, et al., 1995). Experimental studies, in fact, strongly support a role of polyphenols in the prevention of cardiovascular disease, cancer, osteoporosis, diabetes mellitus and neurodegenerative disease (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). In particular, it has been shown that the consumption of polyphenols limits the development of atheromatous lesions, inhibiting the oxidation of low density lipoprotein (Weinbrenner, Fito, de la Torre, Saez, Rijken, Tormos, et al., 2004), which is considered a key mechanism in the endothelial lesions occurring in atherosclerosis.

However, emerging findings suggest a variety of potential mechanisms of action of polyphenols in preventing disease, which may be independent of their conventional antioxidant activities. Furthermore, prooxidant effects of polyphenols have been described (Hoelzl, Bichler, Ferk, Simic, Nersesyan, Elbling, et al., 2005), having opposite effects on basic cell physiological processes: if as antioxidants they improve cell survival, as pro-oxidant they may induce apoptosis and block cell proliferation (Lambert, Hong, Yang, Liao, & Yang, 2005). Polyphenols do have many health impacts basically due to their anti-oxidant property. However, they are also potential inhibitors of mineral iron. Thus dietary polyphenols have to be degraded using processing methods like fermentation for vulnerable groups like child bearing women and children.

Polyphenols Anti-Oxidant Mechanism of action

Both radical scavenging pathways (Hanasaki, Ogawa, & Fukui, 1994) and iron chelating mechanisms (Perron & Brumaghim, 2009; Zhao, Dodin, Yuan, Chen, Zheng, Jia, et al., 2005) have been attributed to the antioxidant ability of these compounds. Recently, research has shown that for several polyphenols with catechol or gallol substituents (Fig. 2.6), the iron-chelating mechanism of antioxidant activity is especially efficient at protecting plasmid DNA from damage

by the Fenton reaction (reaction 1). (Perron & Brumaghim, 2009; Perron, Hodges, Jenkins, & Brumaghim, 2008).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} \, {}^{\bullet}OH + OH^{-} \quad \text{…………………………….. (1)}$$

Two pathways for this DNA protective effect are proposed: polyphenols bind to Fe^{2+} (Fig. 2A), preventing it from reacting with H_2O_2 in the Fenton reaction, or oxidation of the Fe^{2+}- polyphenol complex occurs in the presence of O_2 to generate a Fe^{3+} complex that cannot participate in the Fenton reaction. Stability constants of Fe^{3+} with catecholate complexes are extremely high, for monocatecholate coordination and for tris (catecholate) coordination(Avdeef, Sofen, Bregante, & Raymond, 1978). Stability constants for Fe^{2+} catecholates are somewhat lower for Fe^{2+}-mono(catecholate) (Clarke & Martell, 1992). Although fewer binding constants for iron gallate complexes have been reported, stability constants for the Fe^{2+} and Fe^{3+} complexes of gallic acid (GA) and *n*-propyl gallate (PrEGA) are lower. Because polyphenol ligands strongly stabilize Fe^{3+} over Fe^{2+}, when catechol and gallol compounds bind Fe^{2+} under aerobic conditions, Fe^{2+}-polyphenol complexes are rapidly oxidized in the presence of dissolved O_2 in solution to give Fe^{3+}- polyphenol complexes, a process commonly referred to as auto oxidation (Fig. 2.6B and

2.6C) (Chvátalová, Slaninova, Březinová, & Slanina, 2008)

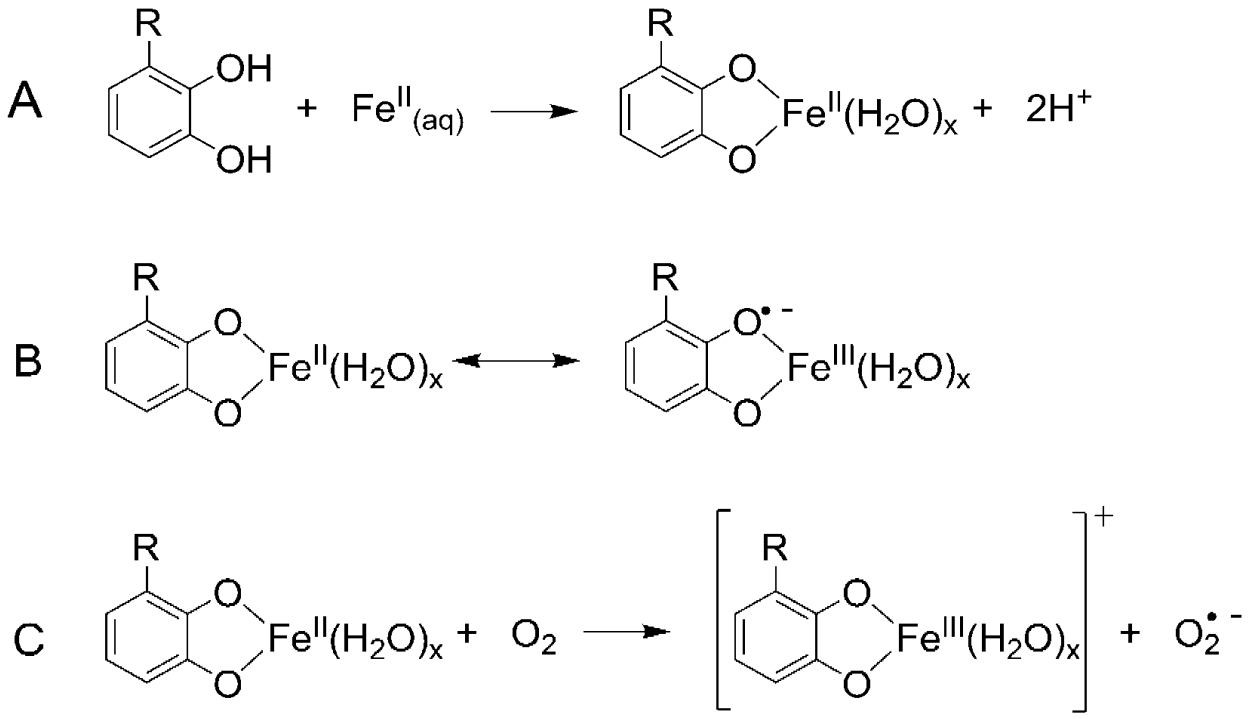

Figure 2.6 (A) Chelation of Fe^{2+} by catecholate (R = H) or gallate (R = OH) ligands; (B and C) proposed reactions of Fe^{2+}-polyphenol complexes.

Although aqueous Fe^{2+} oxidizes slowly when O_2 is present, polyphenols bound to Fe^{2+} lower the reduction potential of iron significantly, (Hider, Liu, & Khodr, 2001) enhancing the rate of iron oxidation. (Momić, Savić, & Vasić, 2009).

This phenomenon has been reported for many Fe^{2+} complexes in addition to catecholates and gallates (Stumm & Lee, 1961).

2.4 Mineral absorption enhancers

Enhancers of iron absorption bind iron securely to maintain the stability of the bond and the solubility of the complex through the gastrointestinal tract. Thus, enhancers are those ligands that form soluble chelates with iron (especially ferric iron) and prevent its precipitation, allowing the release of iron for absorption by the mucosal cell. Amino acids, the "meat factor," ascorbic acid, and citric acid are such enhancers.

Some of mineral absorption enhancers are discussed below.

2.4.1 Organic acids

Among the various organic acids, ascorbic acid is the most efficient absorption enhancer of zinc and non-heme iron (R. F. Hurrell, Lynch, Bothwell, Cori, Glahn, Hertrampf, et al., 2004). Several mechanisms for enhancing properties of ascorbic acid exists, these are: promotion of acidic conditions in the stomach and intestine providing optimal conditions for iron and zinc absorption; chelation of ferric iron and zinc to maintain it in a stable and soluble complex at higher pH and thereby preventing formation of insoluble complexes with phytate and, or polyphenols; and finally reduction of ferric iron to its ferrous form prevents the precipitation of iron as ferric hydroxide (R. F. Hurrell, et al., 2004). However, to promote iron absorption in meals containing low to medium amounts of inhibitors, ascorbic acid (AA): Fe molar ratio of 2:1 is needed, whereas ratios as high as 4:1 may be needed for those with high contents of inhibitors (R. F. Hurrell, et al., 2004).

Among the various organic acids, ascorbic acid is the most efficient absorption enhancer of zinc and non-heme iron (R. F. Hurrell, et al., 2004).

Other organic acids such as citric acid, lactic acid, etc., have also been shown to enhance absorption, but for them to be effective, their molar ratio to iron needs to be very high (>100) (Teucher, Olivares, & Cori, 2004).

2.4.2 Animal Tissue

Besides contributing highly available heme iron, animal tissue in the diet produces a twofold to fourfold increase in the absorption of nonheme iron. This phenomena applies to a variety of meat products, but eggs, milk and cheese either have no effect or are somewhat inhibitory to iron absorption (J. D. Cook & Monsen, 1976).

2.5 Methods for assessing iron bioavailability

The definition of "iron bioavailability" can be expressed as "the part of an orally administered iron dose that reaches the blood circulation and by that can be used for physiological functions". According to this definition, an optimal bioavailability is dependent on an optimal digestion and solubility, an optimal transport over the mucosal layer into the circulation, and finally an optimal incorporation into target organs.

Table 2.1 Source of bioavilability of dietary iron **(Source:** American Association of Cereal Chemists 1981**)**

Source	Bioavailability	Examples
Food of animal origin		
Heme	High	Hemoglobin, myoglobin
Nonheme	High	Muscle tissue, liver, fish
	Low	Eggs, purified ferritin
Food of vegetable origin		
Nonheme	Moderate	Wheat, maize, beans
	Low	Rice, spinach
Fortification iron		
Nonheme	High	Ferrous salts (sulfate, fumarate)
	Moderate	Ferric orthophosphate
	Low	Sodium iron pyrophosphate
Contamination Iron		
Nonheme	High	Beer fermented in iron drums
	Low	Ferric hydroxide, ferric oxide

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

However, all techniques and methods have limitations and advantages, and the balance between these differs depending on the context they are used in.

Some of iron bioavailability measurement methods are discussed below:

2.5.1 The chemical balance method

The introduction of radioisotopes made it possible to label single food items biosynthetically with radio iron. Studies with labeled foods have shown that absorption from individual foods differs markedly. These differences in bioavailability are apparently related to differences in solubility and dissociation of the chemically uncharacterized iron compounds in foods. It was found early on that one food could interact with the absorption of iron from another food (56).

2.5.2 Extrinsic Tag Method

In recent years some unexpected observations provided an important breakthrough and led to the development of the extrinsic tag method and to the introduction of the pool concept in food iron absorption (J. D. Cook, Brown, & Valberg, 1964; Leif Hallberg, 1981). When single foods biosynthetically labeled with radio iron (intrinsic tracer) were carefully mixed with a trace amount of iron salt labeled with another radio iron isotope (extrinsic tracer), the observation was made that the absorption of the two tracers, from such doubly labeled foods, was almost identical. The magnitude of the absorption was different from different foods and in different subjects, but the absorption from the extrinsic and intrinsic tracers was the same in each subject. Based on these findings, the concept of a common non-heme iron pool was introduced. This concept assumes that the non-heme iron compounds in different foods in a meal can be uniformly labeled by an extrinsic inorganic radio iron tracer and that the absorption of non-heme iron takes place from this common pool (Leif Hallberg, 1981).

Heme iron cannot be labeled by an extrinsic inorganic tracer. The main heme iron sources in the diet are hemoglobin and to a lesser extent myoglobin. The similarity in structure of the heme moiety of these two compounds made it reasonable to assume that they are absorbed in the same way, and hence the absorption of hemoglobin biosynthetically labeled with radio iron gives a true

measure of the absorption of all heme iron from a meal (J. Cook, Layrisse, Martinez-Torres, Walker, Monsen, & Finch, 1972).

Heme iron is absorbed as an iron-poryphyrin complex directly into the mucosal cells and this is apparently an entirly different pathway from non-heme iron mucosal entry (J. Cook, Layrisse, Martinez-Torres, Walker, Monsen, & Finch, 1972; WREBY, Suttle, & FORD III, 1970).

Thus, heme iron and non-heme iron are absorbed from separate pools of iron in the gastrointestinal tract. Use of two different radio iron isotopes enables these two pools to be independently and simultaneously labeled with biosynthetically radio iron-labeled hemoglobin and with an extrinsic inorganic radio iron tracer (Erik Björn-Rasmussen, Hallberg, Isaksson, & Arvidsson, 1974; L Hallberg, Björn-Rasmussen, Howard, & Rossander, 1979).

For example, the non-heme iron pool has been labeled with intrinsic and extrinsic tracers under different experimental conditions, and the results were compared. The two tracers were absorbed to the same extent, even when, for instance, inorganic iron in various amounts, ascorbic acid, or desferrioxamine were added to the diet (J. Cook, Layrisse, Martinez-Torres, Walker, Monsen, & Finch, 1972; Monsen, Hallberg, Layrisse, Hegsted, Cook, Mertz, et al., 1978).

The extrinsic and intrinsic tracers gave identical plasma radioactivity curves for several hours when giving doubly labeled foods (Erik Björn-Rasmussen, Hallberg, & Walker, 1973).

The two tracers were absorbed to the same extent when a doubly labeled food was given alone and when the food item formed part of a composite meal. It is probable that the reason for the identical absorption of the extrinsic tracer and the native iron, intrinsic tracer, is a complete and rapid isotopic exchange within the common pool of non-heme iron. There are a few known exceptions in which this isotopic exchange is incomplete: (a) unmilled, unpolished, in which it is likely that the outer dense aleurone layer of the rice grain impairs the diffusion of iron (Erik Björn-Rasmussen, Hallberg, & Walker, 1973); (b) ferrtin and (c) some poorly soluble iron

compounds used to fortify foods, such as most forms of reduced iron (E Björn-Rasmussen, Hallberg, & Rossander, 1977) and ferric orthophosphate (J. D. Cook, Minnich, Moore, Rasmussen, Bradley, & Finch, 1973).

All of these studies verify that under most conditions the absorption of both heme and non-heme iron can be determined simultaneously in the same meal. The absorption of non-heme iron draws the most interest, because it forms the main part of the dietary iron intake and because its absorption is much more varied due to the marked effect of both dietary factors and the iron status of the subjects. A "one pool-one radioisotope" model is then applied.

Moreover, it has been shown that the non-heme iron absorption can be reliably measured from freely chosen meals, provided that the exact intake of foods is recorded and the radio iron is administered in some bulky component. This method is of great importance in field studies (Rossander, Hallberg, & Björn-Rasmussen, 1979).

2.5.3 Mathematical models

Over the years there have been many attempts to develop mathematical models for estimating iron absorption and bioavailability (Anand & Seshadri, 1995; Bhargava, Bouis, & Scrimshaw, 2001; Leif Hallberg & Hulthén, 2000). The complexity of the many dietary factors and their interactions has made this challenging. The first model described for estimating iron absorption from different meals required the five factors, total iron, non-heme iron, heme iron, ascorbic acid, and meat (including fish and poultry). From this information any meal could then be classified as having high, medium, or low bioavailability (Monsen, et al., 1978). A more extensive algorithm for calculating absorption and bioavailability of dietary iron has been validated and published (Leif Hallberg & Hulthén, 2000). The strength of this algorithm lies in all the known dietary factors, both enhancing and inhibiting, and their interactions that are taken into account, as well

as the possibility of converting the algorithm to any iron status (Leif Hallberg, Hultén, & Gramatkovski, 1997). However, as a result of this complexity, the accuracy of the algorithm is dependent on total information of all dietary factors influencing iron absorption (Fairweather-Tait, Lynch, Hotz, Hurrell, Abrahamse, Beebe, et al., 2005).

2.5.4 Solubility and dialyzability

Solubility is the first step in covering the bioavailability definition mentioned above, and thus a necessity for iron absorbability.

In order to simulate the gastrointestinal environment a commonly used procedure is to measure the solubility in dilute hydrochloric acid (HCl) (Forbes, Arnaud, Chichester, Cook, Harrison, Hurrell, et al., 1989). To further mimic the iron absorption taking place in vivo, methods also introducing the aspect of dialyzability have been used (D. D. Miller, Schricker, Rasmussen, & Van Campen, 1981). The principle of the dialyzability method is that following an in vitro enzymatic digestion of a test meal the dialyzable iron passes over a dialysis membrane, which then can be quantified by spectrophotometry(R. Hurrell, Lynch, Trinidad, Dassenko, & Cook, 1988).

2.5.5 Caco-2 cells

A commonly used *in vitro* model is the uptake of iron by Caco-2 cells. Although these cells are derived from human colon adenocarcinoma, they exhibit many features of small intestinal cells, including the influence from dietary factors on iron absorption (Han, Failla, Hill, Morris, & Smith, 1995; Han, Failla, Hill, Morris, & Smith Jr, 1994). One indicator of the iron uptake in Caco-2 cells is the ferritin formation in the cells (Glahn, Lee, Yeung, Goldman, & Miller, 1998). Another approach to study Caco-2 uptake is to use radioactive iron isotopes (Han, Failla, Hill, Morris, & Smith Jr, 1994). The Caco-2 cell model has in some studies proved to be a rapid

screening procedure of the potential maximum bioavailability for iron compounds (Lund, Fairweather-Tait, Wharf, & Johnson, 2001).

2.6 Effect of fermentation on iron availability

Fermentation can be defined as the metabolic process by which carbohydrates are oxidized, releasing energy in the absence of external electron acceptor. The electron acceptors are organic compounds produced directly by the decomposition of carbohydrates.

According to (Cheftel & Cheftel, 1976), it can be defined as a desirable process of biochemical changes caused by microorganisms and their enzymes on the products of primary processing. In addition, fermentation provides a natural way to reduce the volume of the material to be transported, to destroy undesirable components, to enhance the nutritive value and appearance of the food, to reduce the energy required for cooking and to make a safer product (Nout & Motarjemi, 1997).

Fermentation makes the foods easier to digest and the nutrients easier to assimilate and also it retains enzymes but also generate vitamins, and other nutrients that are usually destroyed by food processing (Yousif, Dawyndt, Abriouel, Wijaya, Schillinger, Vancanneyt, et al., 2005). Fermentation has been used for several thousand of years as an effective and low cost means to preserve the quality and safety of foods. Animal and plant tissues subjected to the action of microorganisms and/or enzymes which caused desirable biochemical changes and significant modification of food quality.

Food fermentation is an important technique in the developing countries where the lack of resources limits the use of recent techniques such as vitamin enrichment of foods and the use of energy and capital intensive processes for food preservation.

The natural fermentation is a widespread practice among the world's technology transformation of cereals, vegetables and nuts. All are from fermentation pyruvate hub of metabolism. According to (Simango, 1997), the conversion of monosaccharide's (glucose and fructose) and disaccharides (lactose and maltose) to pyruvic acid mainly follows the path of glycolysis.

A large number of microorganisms are involved in fermentation process. Some of them produce amylases that could act on starch flour used for the preparation of boiled foods and thus reduce their ability to bind water. However, the route then followed by pyruvic acid depends on the type of microorganism involved in the process of fermentation and metabolism: the lactic acid bacteria convert pyruvic acid into lactic acid by a hydrogenation reaction; on the other hand, yeast convert it into ethanol by the reaction of decarboxylation. Basically there are two types of lactic fermentation (not clear).

The homolactic fermentation where glucose is converted primarily into lactic acid and heterolactic fermentation where, in addition to lactic acid, there is the production of other compounds that are: CO_2, acetic acid, ethanol and glycerol. The products formed during fermentation are the results of activities of enzymes secreted by the carbohydrate substrates, the homo and heterofermentative bacteria involved in fermentation and microflora present in cereal grains during their production.

Natural fermentation also provides optimum pH conditions for enzymatic degradation of phytic acid which is present in cereals and grains in the form of complexes with polyvalent cations such as iron, zinc, calcium, magnesium and proteins. Such a reduction in phytate may increase the amount of soluble iron, zinc and calcium several folds (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003). (Mohamed, Amro, Mashier, & Elfadil, 2007) have shown through their result that fermentation of millet grains for 12 and 24 hour could reduce the mineral absorption inhibitor (phytate and tannin).

(Reale, Konietzny, Coppola, Sorrentino, & Greiner, 2007) found that phytate degradation during cereal dough fermentation was positively correlated with endogenous plant phytase activity and also that when heat was applied to the endogenous cereal phytates after lactic acid fermentation it resulted in the inactivity of phytase.

In an in vitro study (Liang, Han, Nout, & Hamer, 2008) found that by acidifying phytic-acid-rich whole wheat dough via sourdough fermentation or by adding lactic acid to the dough, resulted in a large phytate breakdown (around 70% compared to 40% control). They concluded that a slight drop in pH is sufficient to reduce phytate content of whole meal flour and magnesium bioavailability was improved.

(Sanz-Penella, Tamayo-Ramos, Sanz, & Haros, 2009) observed that breads with fermented bifidobacterium bacterial strains had lower levels of IP6 and lesser amounts of IP3. They suggested that bifidobacterium (a beneficial bacterium that lives in the colon) as a safe means to reduce phytic acid content in fibre rich products intended for human consumption, because of the bacteria phytate-degrading enzyme production.

Fermentation is one of the most economic and effective method for reducing the content of mineral absorption inhibitors. Studies have shown that both spontaneous fermentations as well as fermentation with starter culture significantly reduce the content of phytic acid in millet (Bilgiçli, Elgün, & Türker, 2006; Elyas, El Tinay, Yousif, & Elsheikh, 2002; Sharma & Kapoor, 1996). One study found starter culture fermentations were to be more effective than spontaneous fermentation (Bilgiçli, Elgün, & Türker, 2006). Similarly, as a result of lactic acid fermentation, the protein digestibility can be elevated (Antony & Chandra, 1998; Ranjit, Taylor, & Kung Jr, 2002) and the tannin content may be reduced in some cereals, leading to increased absorption of iron (KHETARPAUL & Chauhan, 1989; Onyango, Noetzold, Ziems, Hofmann, Bley, & Henle, 2005).

2.7 Geophagia and iron bioavailability

Geophagia, the deliberate ingestion of soil, has been practiced for centuries (Simon, 1998). It occurs throughout the world and is found in all age groups in both sexes across all socio-economic, racial and cultural boundaries (Halsted, 1968; Reid, 1992). Nevertheless, the practice is most common among certain groups – especially young children, childbearing women and those with poor socio-economic background in developing countries (D. Danford & Huber, 1982; Simon, 1998). In modern human societies, the practice of geophagia represents a complex eating behavior with an obscure aetiology, including physiological and psychological reasons, and cultural, religious and medicinal beliefs.

In Africa for example, childbearing women eat clays probably as a mineral nutrient supplement (Hunter, 1973) and soil ingestion has also been used as a remedy for diarrhoea and intestinal parasites (Vermeer & Ferrell, 1985). Ingestion of certain types of soils may, therefore, provide medicinal benefits for general gastrointestinal ailments. However, geophagia (D. E. Danford, 1982) has been associated with a range of health/medical problems, including iron deficiency anaemia, hypoklaemia, parasitic infections, mechanical bowel disorder and perforation and nutritional dwarfism.

Nutrient, particularly iron, deficiency is a common occurrence in geophagic individuals (D. E. Danford, 1982). This has led to suggestions that iron deficiency causes geophagia (Lanzkowsky, 1959). It appears that geophagia may be common in certain population groups, which are at greatest risk of iron deficiency (e.g. poorly nourished, pregnant women). The occurrence of iron or another mineral nutrient deficiency in geophagic individuals, therefore, seems to be an association rather than a causal relationship (Simon, 1998).

Geophagia has also been perceived as a means of supplementing essential mineral nutrients, particularly in subsistence communities. This view, however, stems largely either from total

elemental contents of the soils or from their partial extractions (Smith, Rawlins, Cordeiro, Hutchins, Tiberindwa, Sserunjogi, et al., 2000). Based on total elemental contents and acidified ammonium oxalate extractions predicted dietary/nutritional benefits of geophagic soils collected from the USA, China and Zimbabwe. However, strong acid-soluble (total) nutrients are unlikely to be available for absorption into the body, as a large fraction of the total nutrient content will not be soluble in environments like the gastrointestinal tract (Alloway, 1990), and adsorption onto ingested soil can remove food bioavailable nutrients from the intestine (Hooda, Henry, Seyoum, Armstrong, & Fowler, 2002). Mineral nutrients released in the stomach (pH 2) may be re-adsorbed through cation exchange and adsorption as the soil enters the intestine (pH 7–10), since the retention of nutrient-ions by these processes tends to increase with pH. This was further supported by a physiologically-based extraction test (pH range 2–7) used by (Smith, et al., 2000), which showed that only a small fraction of the total contents of elements (e.g. Mg, Fe, Zn) present in the geophagic soils were potentially available for absorption in the intestine.

2.8 Sequential extraction scheme

Sequential extraction method is applied in foods based on the chemistry of iron, physiological and biochemical process of ingestion, digestion and absorption of mineral iron (Simpson, Sidhar, & Peters, 1992).

The mobility of metals in environmental solid samples is generally evaluated by using sequential extraction schemes (SESs). A large number of procedures have been developed in which samples are treated with a succession of reagents to liberate metals with different affinities for the matrix.

The most widely applied SES has been that proposed by Tessier (A. Tessier, P. G. C. Campbell, & M. Bisson, 1979).The analytical results of this and others schemes are usually affected by selectivity and re-adsorption problems.

The problem of the incomplete selectivity of reagents used in SESs to dissolve one particular phase arises in the additional attack to other phases (Kheboian & Bauer, 1987; W. Miller, Martens, & Zelazny, 1986)

Moreover, they may be insufficiently efficient to completely dissolve the phase. To avoid these problems, modifications in the experimental conditions, such as the extraction time, the extractant-sample ratio, the reagent concentration, the extraction temperature, the use of successive extractions with the same reagent, etc., have been proposed (Gómez-Ariza, Sánchez-Rodas, Giráldez, & Morales, 2000; Thomas, Ure, Davidson, Littlejohn, Rauret, Rubio, et al., 1994).

Sequential extraction is composed of five fractions namely exchangeable, carbonate bound (acid soluble), oxide bound (reducible), organic bound (oxidisable) and residual fractions.

2.8.1 Exchangeable iron fraction (*labile fraction*)

Metals extracted in this operation would include weakly-sorbed metal species, particularly those retained on the soil surface by relatively weak electrostatic interactions and those that can be released by ion-exchange processes.

Reagents used for this purpose are electrolytes in aqueous solution, such as salts of strong acids and bases or salts of weak acids and bases at pH 7 (Rauret, 1998).

The most popular reagent is $MgCl_2$ 1 mol/l, which combines the rather strong Mg^{2+} ion-exchange capacity with the weak complexion ability of Cl^-. This reagent does not attack organic matter, silicates or metal sulfides (Chao, 1972).

2.8.2 Acid soluble iron fraction

This fraction is sensitive to pH changes, and metal release is achieved through dissolution of a fraction of the solid material at pH close to 5.

A buffered acetic acid/sodium acetate solution is generally used. The metal fraction recovered in these conditions may be thought to have been present as co-precipitated with carbonate minerals but also as specifically sorbed to some sites of the surface of clays, organic matter and Fe oxyhydroxides (Pickering, 1986).

Extraction with acetate salts (particularly sodium acetate) has also been used frequently in soil and sediment studies. Divalent cations should, in general, be more effective than monovalent cations in removing exchangeable ions, but Na^{+} promotes replacement of ions in the interlayer exchange sites of vermiculite. Metal complexes formed with acetate ions are slightly more stable than chloro-complexes; this favors the exchange and reduces re-adsorption or precipitation of the extracted metals (Martin, Nirel, & Thomas, 1987).

2.8.3 Reducible iron fraction

Iron and manganese oxides are excellent scavengers of metals. By controlling the reduction potential (Eh) and the pH of reagents, dissolution of some or all metal-oxide phases can be achieved (Martin, Nirel, & Thomas, 1987).

The most successful reagents for evaluating the total amount of metal ion associated with these minerals contain both a reducing reagent and a ligand able to retain released ions in a soluble form, the efficiency of the reagent being determined by its reduction potential and its ability to attack the different crystalline forms of Fe and Mn oxyhydroxides (Martin, Nirel, & Thomas, 1987). This dissolution can take place in one, two or three steps, separating amorphous or crystalline Mn and Fe oxides. Hydroxylamine, oxalic acid and dithionite are the most commonly used reagents.

Hydroxylamine is a reducing reagent (Eo= -1.87V) and its ability to dissolve the different metallic oxides depends on pH, concentration, extracting time and temperature. Chao (Martin, Nirel, & Thomas, 1987) showed that:

- dissolution of Fe oxides depends on these same factors, and is favored by an increase of reagent concentration and agitation time; and
- Lowering the pH (with nitric acid, for example) favors the attack of iron oxides.
- Acetic acid (25% v/v) or 0.25 mol/l hydrochloric acid replaces nitric acid in order to avoid re-adsorption problems. In this case, the complexing properties of chloride or acetate ions are used (Farrah & Pickering, 1993).

2.8.4 Oxidisable iron fraction

Trace elements may be incorporated in many forms of organic matter including living organisms, organic coatings on inorganic particles and biotic detritus. In sediments and soils, the organic content comprises mainly complex polymeric material known as humic (plant and animal excretion and decomposition) substances and, to a lesser degree, other products, such as carbohydrates, proteins, peptides, amino acids, fats, waxes and resins (Martin, Nirel, & Thomas, 1987).

Under oxidizing conditions, this organic material tends to be degraded, leading to the release of sorbed metals. So, oxidizing reagents such as H_2O_2 ($E_{\_}$=1.77V) or NaClO ($E_{\_}$= 0.90V) are frequently used in fractionation studies in order to extract the fraction associated with organic matter. Hydrogen peroxide is generally used in dilute nitric acid solutions in order to prevent metals scavenging by the formation of Fe hydroxides at higher pH values. The oxidation process is promoted by heating for several hours (Pickering, 1986).

An important re-adsorption of released metals occurs during the extraction, and this step is followed by an extraction with a soft complexing reagent, such as NH_4OAc in nitric acid (A. Tessier, P. G. Campbell, & M. Bisson, 1979).

A major oxidation by-product during the destruction of organic matter is oxalic acid, which is able to dissolve iron oxides and cause precipitation of sparingly soluble oxalates. However, (Krishnamurti, Huang, Van Rees, Kozak, & Rostad, 1995) showed that the oxalate formed can be totally destroyed by H_2O_2 and that a secondary reaction linked to it did not modify the results.

2.8.5 Residual iron fraction

Primary and secondary minerals containing metals in the crystalline lattice constitute the bulk of this fraction. Its destruction is achieved by digestion with strong acids, such as HF, $HClO_4$, HCl and HNO_3. This fraction is also explained as the amount of associated metals in which the difference between the total concentration and the sum of the fractions of the metals extracted during the previous steps (Gleyzes, Tellier, & Astruc, 2002).

Chapter three

Materials and Methods

3. Materials and methods

3.1 Materials

3.1.1 Study site and sample collection

Red Teff (Eragrostis tef) was collected from Adulala ziquala abo woreda, 93km from Addis Ababa, and was hand threshed in the laboratory. On another day, teff grain was collected from the same place where the first sample was collected and was threshed traditionally. The reason for selecting this area was that the farmers were willing to give harvested teff which other farmers were not willing to do so due to traditional beliefs. Harvested teff grain was collected in plastic bags by taking preventive measures to avoid adventitious contamination. The preventive measures taken were wearing gloves while handling the samples, using metal-free polyetelene bags for storage. The same preventive measure was taken for the collection of traditionally threshed teff grains in the field so as to avoid any adventitious contamination after threshing.

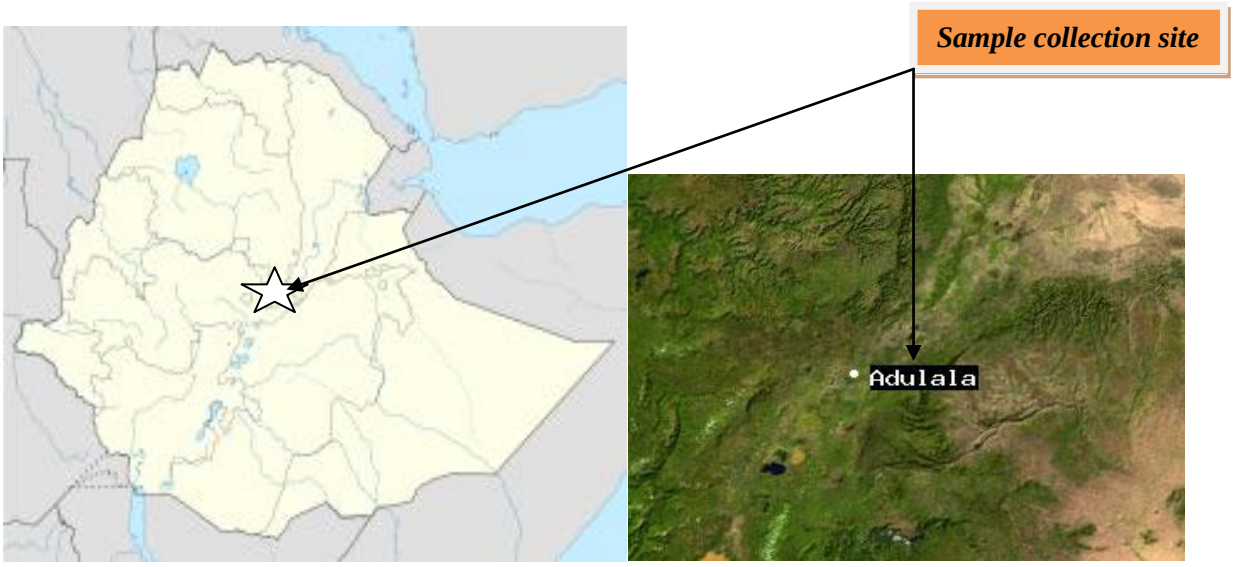

Figure 3.1: Map of Ethiopia showing the sample collection site

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Experimental analyses were carried out from January 2013-May 2013 in the Center for Food Science and Nutrition research laboratory and Ethiopian Health and Nutrition Research Institute.

3.1.2 Experimental design

Differences in iron fractions were determined using sequential extraction method on laboratory threshed and field threshed teff flours, fermented dough and baked injera. The iron fractions that were determined by sequential extraction method were: exchangeable, carbonate bound, oxide bound, organic bound and residual fractions.

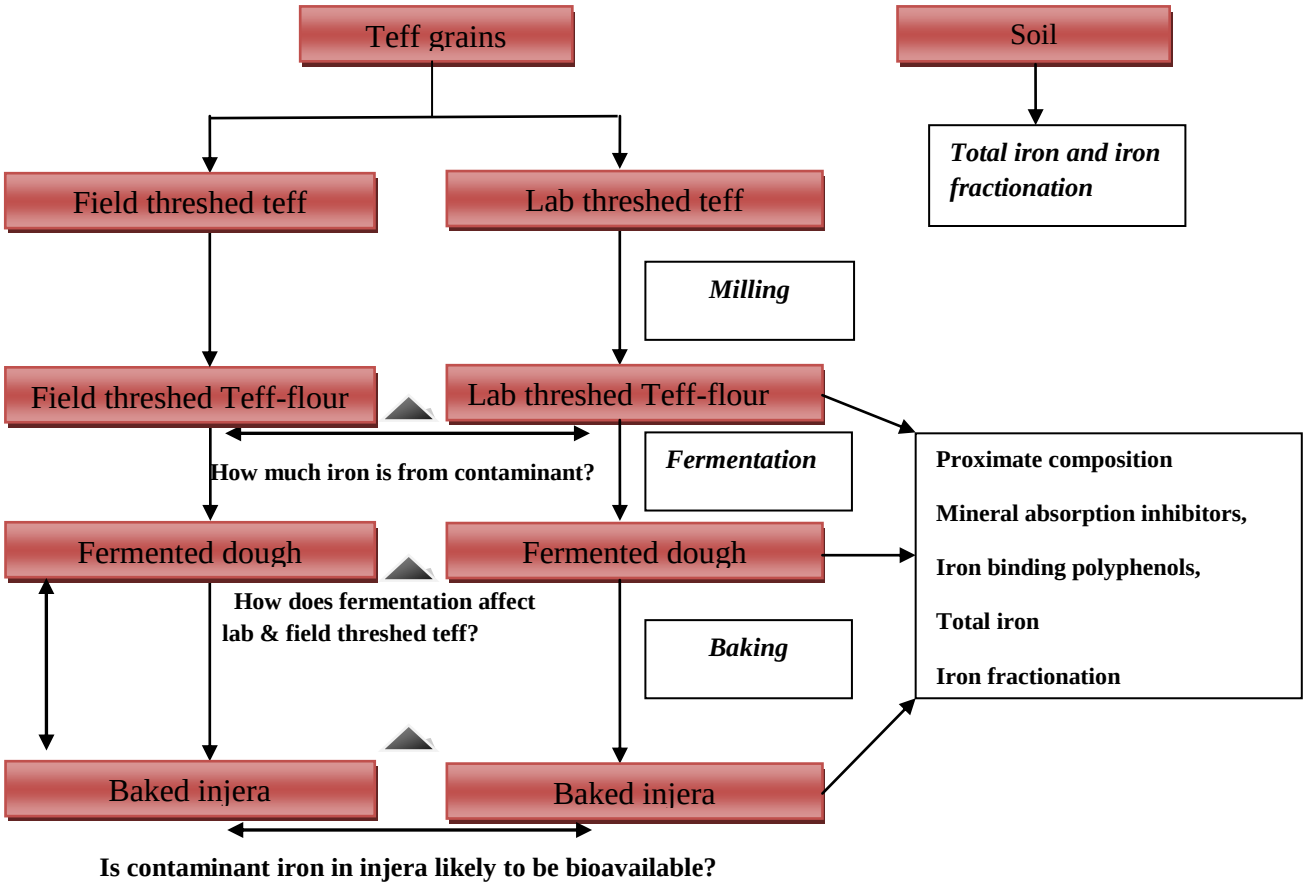

Figure 3.3 Experimental design

A total of 42 sample in triplicate (**126** samples) were tested for total iron and iron fractionation from sequential extraction of teff-injera and soil (in which teff-grain was growing) using flame atomic absorption spectroscopy (FAAS) (Varian SpectrAA-20 Plus, Varian Australia Pty., Ltd., Australia).

3.1.3 Preparation of samples

The harvested teff grains were manually threshed at the Center for Food Science and Nutrition laboratory using metal-free sterile latex gloves. Both traditionally threshed and manually threshed teff grains were winnowed in the same way to separate grains from any foreign matter.

Preparation of teff flour

Teff flour was prepared from both samples using stainless steel laboratory miller (FW 100, china) in a manner that does not add any adventitious contamination

Preparation of teff fermented dough

Teff flour was fermented, and the fermented dough was collected in triplicate. All ingredients were added accurately so as to control the fermentation kinetics and other parameters following the traditional teff dough preparation procedure.

Preparation of injera

Preparation of injera was carried out using both field threshed and laboratory threshed teff flours and baking of injera was done following the traditional injera baking process in a manner that does not add any extrinsic iron to the sample.

Both teff dough and baked injera were lyophilized (Madras 600 097, India) at Center for Food Science and Nutrition laboratory before further analytical investigations.

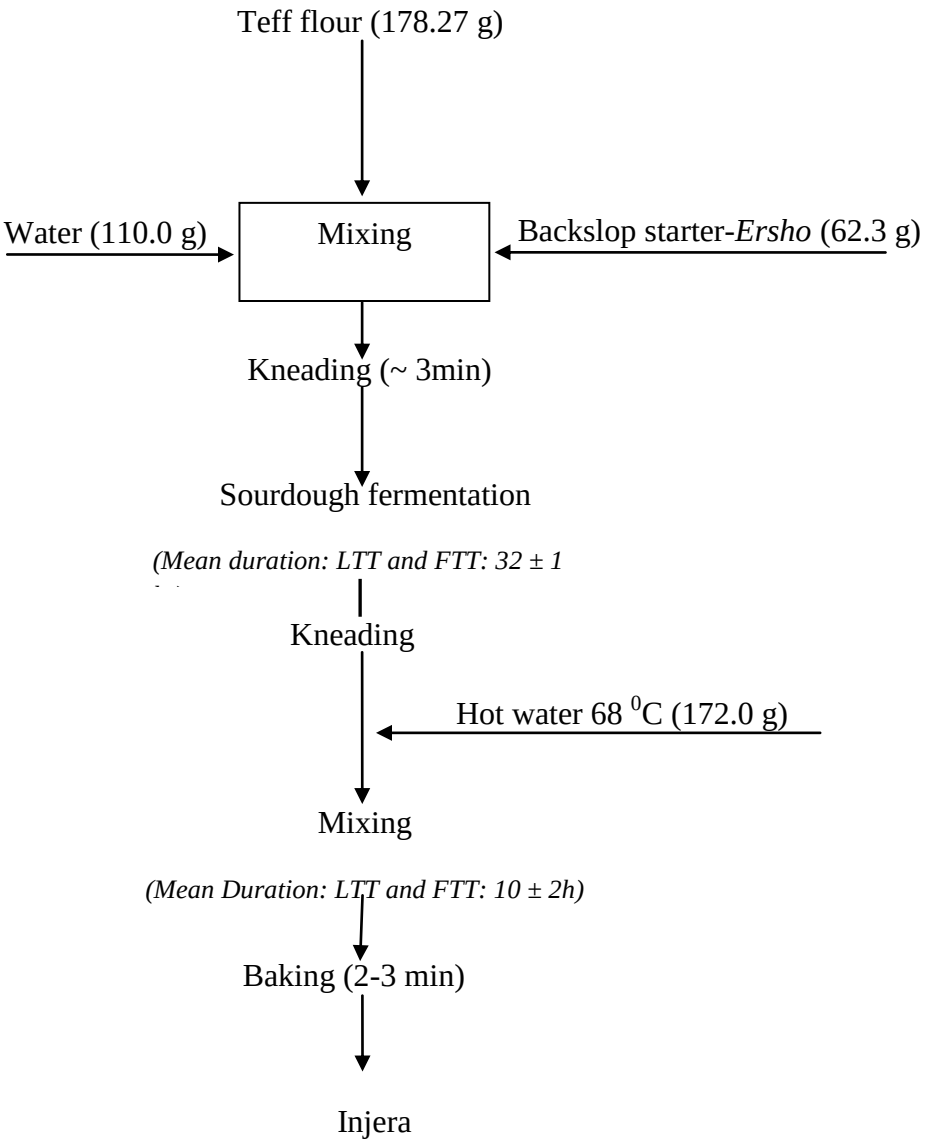

Figure: 3.2 Description of *injera* processing (Adapted from Baye et al., 2012)

3.2 Analytical methods

3.2.1 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^{o}C for 1 hour and placed in desiccators to cool. The weight of the crucible (W1) was determined. 5gm samples was weighed in the dry crucible (W2) and

dried at 105^{o}C for 3 hours and after cooling to room temperature in desiccators it was again weighed (W3). The moisture content was determined by using Eq. (1).

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

3.2.2 Proximate composition determination

Proximate chemical analysis of the two samples i.e. laboratory threshed and field threshed teff samples was determined according to AOAC, 2000.

3.2.2.1 Determination of crude protein

Protein content was determined according to AOAC (2000) using the official method 979.09. **Digestion:** About 0.5 g of fresh samples (in triplicate) was added to a Tecator tube and 6ml of acid mixture (5parts of concentrated ortho-phosphoric acid and 100 parts of concentrated sulfuric acid) was added and mixed, and 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 gram of catalyst mixture (ground 0.5 g of selenium metal with 100 g of potassium sulfate) was added into each tube, and allowed to stand for about 10 minutes before digestion. When the temperature of the digester attained 370^{0}C, the tubes were lowered into the digester. The digestion was continued until a clear solution was obtained (about 4 hour). 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 (Manual, 1979).

The crude protein was determined as follows:

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

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

Where:

V is the volume of HCl in L consumed to the end point of titration, N is the normality of HCl (used often is 0.1N), Wo is sample weight on dry matter basis and 14.00 is the molecular weight of nitrogen. The % of nitrogen is converted to % of protein by using appropriate conversion factor.

(% protein = 6.25*%N for both the teff samples (laboratory and field threshed).

3.2.2.2 Determination of crude fat

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

About 2g of flour was extracted with 50 ml diethyl ether for a minimum period of 4 hours in the soxhlet extractor (2010 05 025, China). The solvent was then evaporated and the extracted fat was dried in the oven and cooled in a desiccator. The crude fat was determined as follows:

$$\text{Crude fat } \% \text{ by wt} = \frac{\text{W2} - \text{W1}}{\text{W}} * 100 \qquad 4$$

Where:

W_1= weight of the extraction flask (g)

W_2 = weight of the extraction flask plus the dried crude fat (g)

W = weight of sample (g)

3.2.2.5 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 30min at 550^{o}C. The dishes were cooled in desiccators (with granular silica gel) for about 30 minutes at room temperature and were weighed to the nearest milligram (M1). About 2.5g 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 the samples became thoroughly charred. The dishes with the samples were placed inside a muffle furnace (Carbolite CSF 12/13, England) at 550^{o}C for 5 hours and 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 to the nearest milligram (M3).

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

Where:

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

3.2.2.6 Determination of mineral contents

Iron content was determined using atomic absorption spectrophotometer method of (Osborne & Voogt, 1978).

Ash was obtained from dry ashing of food samples. The ash was wetted completely with 5ml of 6N HCl, and dried on a low temperature hot plate. 7ml of 3N HCl was added to the dried ash and heated on the hot plate until the solution boiled. The ash solution was cooled to room temperature in a hood and filtered into a 50ml graduated flask using a filter paper (whatman 45,125mm). 5ml of 3N HCl was added into each crucible dishes and heated until the solution boiled, was then cooled and filtered into the flask. The crucible dishes were again washed three times with de-ionized water; the washing was filtered into a flask. Then, the solution was cooled and diluted to 50ml with de-ionized water. A blank which contains 12ml 3N HCl and deionized water in 50ml volumetric flask was also prepared for FAAS reading.

Standard solutions: Four series of working standard metal solution (Table 1) was prepared by appropriate dilution of the metal stock solution (nitrate of the metal) with deionized water containing 2.4ml 3N HCl in 10ml volumetric flask. Calibration curve (concentration versus absorbance) for each element using the prepared standard solution was prepared.

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

Table 3.1 Series of working standard solution for iron determination

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

$$\text{Metal content } (\frac{\text{mg}}{100\text{gm}}) = \frac{[(a - b) * V]}{10W} \qquad 6$$

Where:

W= weight (g) of samples

V= 50ml = volume (V) of extract

a= concentration (µg/ml) of sample solution

b= Concentration (µg/ml) of blank solution

3.3 Changes in pH

During fermentation, the pH of both laboratory and field threshed teff fermented dough was recorded using pH meter (MP511,China) which was calibrated at PH 4, 7 and 9 buffer solutions(Galster, 1991). The rate of change of pH with time was determined.

3.4 Mineral absorption inhibitors

3.4.1 Determination of phytates

The spectrophotometric method described by (Latta & Eskin, 1980) was used to determine phytic acid content of the samples. Approximately 0.02 g of sample was measured and extracted with 10 ml of 0.2 N HCl solution for 1 hour at an ambient temperature and centrifuged at 3000 rpm and

the clear supernatant was used for the phytate determination. 2 ml of wade reagent was added to 3 ml of the supernatant sample solution then homogenized and centrifuged for 10 minute at 3000 rpm. Meanwhile, the standard phytic acid solution was prepared containing 4-40 microgram per gram in 0.2 N HCl. 3 ml of each standard was pipetted into 15 ml centrifuge tubes and 3 ml of water was used as a zero level (blank). 2 ml of wade reagent was added to each test tube and the solution was mixed on a vortex mixer for 5 seconds. The mixture was centrifuged for 10 minute at 3000 rpm. Both the extractant solution and the standard solution concentration was measured using UV-Vis spectrophotometer at 500nm. The amount of phytic acid was calculated using phytic acid standard curve and the result was expressed as phytic acid in microgram per gram fresh weight.

$$\text{Phytic acid } (\mu\frac{\mathbf{g}}{\mathbf{g}}) \quad \frac{[(\text{Ab} - \text{As}) - \text{Intercept}] * 10}{\text{slope} * \text{W} * 3} \qquad 7$$

Where:

As, Absorbance of the test sample; Ab, Absorbance of the blank; W, Weight of sample;

3.4.2 Determination of tannin

Tannin content of the test sample was determined by the Follins-Dennis spectrophotometer method by 6(Pearson). Approximately 1 gram of sample was measured in a test tube and 10 ml of 1% HCl methanol solution was added to the sample. The test tube was placed in a shaker for 24 hour at room temperature, and then the solution was centrifuged for 5 minute at 1000 rpm. 1 ml of clear supernatant solution was taken and mixed with 5ml of vanillin-HCl reagent in another test tube. The mixture was left for 20 minute before reading. On the other hand, 40 mg of D-Catechin in 100 ml of 1% HCl in methanol was prepared and a series of dilution was done having

a concentration of 0.2, 0.4, 0.6, 0.8 and 1ml of solution in a test tube. The volume of the standard solution was adjusted to 1ml using 1% HCl in methanol and 5 ml of vanillin-HCl reagent was added in each test tube. Finally, the solution was left for 20 minutes for a complete reaction. The extractant and the standard solution concentration were determined using spectrophotometer set at 500nm.

$$\text{Tannin } \% = \frac{[(\text{As} - \text{Ab}) - \text{Intercept}]}{\text{slope} * \text{d} * \text{W}} \qquad 8$$

Where

As= Absorbance of sample

Ab= Absorbance of the blank

D= Density of the solution

W= weight of sample in gram

3.4.3 Iron-binding polyphenols

Iron-binding polyphenols (galloyl and catechol groups) were analyzed using the method of (Brune et al., 1991). The amount of galloyl and catechol groups was determined using ferric ammonium sulfate (FAS) reagent after 16 h extraction of samples with 50% dimethylformamide in acetate buffer (pH 4.4). This method is based on FAS's ability to form colored complex with iron-binding galloyl and catechol groups. The absorbance of the colored complex was measured at 680 nm and 578 nm, corresponding to the absorption maxima of Fe-galloyl and Fe-catechol complexes, respectively. After subtracting food blank absorbance, the content of galloyl groups

(expressed as tannic acid equivalents) and catechol groups (expressed as catechin equivalents) were calculated from standard curves for catechin and tannic acid at both wavelength.

3.5 Sequential extraction method for iron fraction determination

At the beginning of the analysis, fresh reagent solution was prepared for the purpose of extraction. The type of reagents prepared were 1M magnesium chloride, 1M sodium acetate (at pH=5 adjusted using acetic acid), 0.04M hydroxylamine hydrogen chloride in 25% acetic acid, 0.02M nitric acid,30% hydrogen peroxide (at pH=2 adjusted with nitric acid), 3.2M ammonium acetate in 20% nitric acid. For residual fraction 6N HCl, 3N HCl and 10% HNO3 was employed.

Five iron fractions (exchangeable, carbonate bound, oxide bound, organic bound and residual) were obtained from teff flour, fermented dough (lyophilized), baked injera (lyophilized) and that of soil sample.

Generally the extraction was carried out from the most mobile iron fraction to the least mobile iron fraction.

Quality control

To minimize the risk of contamination, all glassware and plastic bottles used were immersed in 10% solution of nitric acid for 24 h, washed with distilled water and rinsed with deionized water before use. All chemicals used were of analytical grade.

3.5.1 Exchangeable fraction

One gram of sample (teff flour and soil) was weighed in four digit analytical balance and the sample was transferred to a clean and de-ionized water rinsed volumetric flask in triplicate. 8 ml of 1M magnesium chloride solution was added into the volumetric flask and continuously

agitated in a shaker for one hour at room temperature. The sample solution was then transferred to a plastic test tube and then centrifuged for 30minute at 3000 rpm. Finally, the clear supernatant solution was taken from the test tube for FAAS for reading. Leftover from the exchangeable fraction was used for the next carbonate bound fraction (Simpson, Sidhar, & Peters, 1992).

3.5.2 Acid soluble fraction

Precipitate from the exchangeable fraction was treated with 8ml 1M sodium acetate at pH=5 adjusted with acetic acid. The residue was extracted for 4 hour at room temperature. After agitation, the sample solution was transferred to plastic test tube and then centrifuged for 30minute at 3000 rpm. Finally, the clear supernatant solution was taken from the test tube for flame atomic absorption spectroscopy for element iron reading. Leftover from the carbonate bound fraction was used for the next oxide bound fraction (Simpson, Sidhar, & Peters, 1992).

3.5.3 Reducible fraction

Precipitate from the carbonate bound fraction was treated with 20 ml of 0.04M hydroxylamine hydrogen chloride (in 25% acetic acid) to get the oxide bound iron fraction. The mixture was agitated for 24 hour at room temperature. After agitation the sample solution was transferred to plastic test tube and then centrifuged for 30minute at 3000 rpm. Finally, the clear supernatant solution was taken from the test tube for element iron determination using flame atomic absorption spectroscopy. Precipitate from the reducible fraction was used for the next organic bound fraction (Simpson, Sidhar, & Peters, 1992).

3.5.4 Oxidizable fraction

Precipitate from the oxide bound fraction was first treated with 3ml 0.02M nitric acid and 5ml 30% hydrogen peroxide adjusted to pH=2 using nitric acid and the mixture was heated at 85^{o}C in

a water bath for 2 hour with occasional agitation. Secondly, the mixture was cooled and treated with 5ml 3.2M ammonium acetate (in 20% nitric acid) solution. The solution was diluted into 20ml using de-ionized water and agitated for 30 minute at room temperature. After agitation the solution was centrifuged for 30 minute at 3000rpm. Finally, the clear supernatant solution was taken from the test tube for flame atomic absorption spectroscopy for element iron reading. Leftover from the oxidizable fraction was used for the next residual fraction (Simpson, Sidhar, & Peters, 1992).

3.5.5 Residual fraction

Residual fraction was determined by ashing and acid dissolution. First, all crucibles and glass wares were washed and rinsed with distilled de-ionized water. Additionally, crucibles and glass wares were washed with 6N HCl and 10% HNO_3, respectively. Secondly, the crucible was placed in an oven for 30 minute at 100^{o}C and the crucible was placed in a desiccator for cooling. The residual fraction was measured and placed on the hot plate at the start from low temperature under a hood until it was charred. The sample was placed in a muffle furnace at 550^{o}C for 1 hour. The sample was taken out from the furnace and allowed to cool and then moistened with five drops of distilled de-ionized water. The sample was ashed for the second time in a furnace for 30 minute at 550^{o}C and allowed to cool for 30 minutes. Some drops of distilled de-ionized water and 5 drops of concentrated nitric acid was added and evaporated in a hot plate under the hood.

Finally the sample was ashed in a furnace at 550^{o}C for 30 minute and the crucible was cooled for 1 hour.

Acid dissolution was done by adding 6ml of 6N HCl (to wet it completely) and carefully placed on a low temperature hot plate under the hood to take the sample to dryness. Additionally, 7 ml

of 3N HCl was added and the crucible was heated until the solution was boiled. The solution was cooled and filtered through filter paper to 50 ml graduated flask; moreover, 5ml of 3N HCl was added to the crucible and allowed to boil in a hot plate. The solution was again filtered in to graduated flask through filter paper and the crucible was washed with distilled de-ionized water three times and collected in graduated flask. Finally, the iron content in the solution was determined by flame atomic absorption spectroscopy (Gleyzes, Tellier, & Astruc, 2002).

The total iron in all fractions was summed and the content of each fraction expressed as the percent of the total.

$$\text{Total iron} = \text{Fe1} + \text{Fe2} + \text{Fe3} + \text{Fe4} + \text{Fe5} \qquad 9$$

$$\text{Content of iron fraction (X)} = \frac{[\text{Amountof iron fraction X}]}{\text{Total iron}} * 100 \qquad 10$$

Where, X represents fraction 1-5.

3.6 Statistical analysis

All analytical determinations were conducted in triplicate and the values were averaged. The data was analyzed by one way analysis of variance (ANOVA) and independent t-test to investigate if there was any significant difference between laboratory and field threshed teff injera samples and to see any fraction difference due to processing. Statistical analyses were carried out using SPSS 20 (statistical product and service solution) software version and homogeneity of variance was checked.

Chapter four

Results and Discussion

4. Results and Discussion

Two samples of teff were collected. One harvested and threshed in the laboratory and the other harvested and threshed traditionally. The samples were from the same variety and were grown under the same conditions. Flours, fermented dough and *injeras* prepared from the two samples were characterized for their proximate composition, mineral absorption inhibitors, and total iron. Moreover, the distribution of iron in the different fractions of a sequential extraction scheme and the way they evolve during *injera* processing was evaluated.

4.1 Change in pH during injera fermentation

Decrease in pH was observed in both field and laboratory threshed injera fermentations. The pH changed from an initial value of 5.2 in LT and 5.3 in FT injera to reach a final pH of 4.1 and 3.9, respectively **(Fig. 4.1)**.

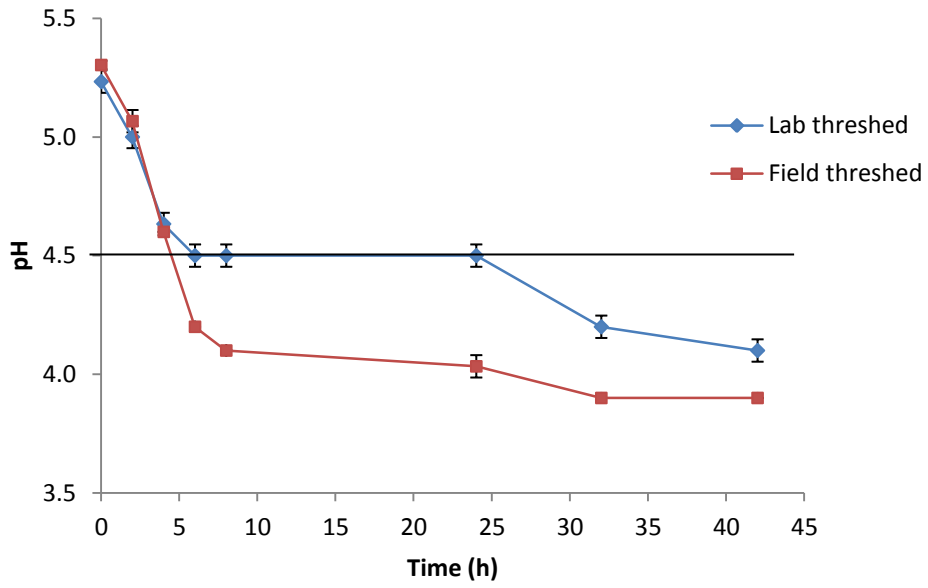

Fig 4.1: Change in pH during lab- and field- threshed teff *injera* fermentation

The rate of pH change of FT injera fermentation was higher than that of the LT. Similarly, the maximal rate in pH change ($-dpH/dt_{max}$) in FT was higher (0.23) than that of the LT (0.18). This difference in the rate was noticed starting from 4h of fermentation. However, towards the end of the fermentation, the difference in the rate of pH change was narrowed.

The pH decrease during fermentation is likely to be explained by the production of organic acids, mainly lactic acid. Depending on fermentation conditions such as grain variety, flour type, temperature, etc., changes in the rate of pH change is expected; however in the present study, the rate of pH change between LT and FT injera fermentations was found different despite the same grain variety and fermentation conditions like temperature, back-slop starter, amount of water, etc., was applied. This suggests that the difference in rate is likely to be due to changes incurred during threshing.

Indeed, threshing of teff has been previously associated with extreme soil contamination. It is likely that teff is also contaminated by cow dung used as a plastering for the site where threshing takes place. The mechanism by which soil contamination is to influence rate of pH change is unknown, however it can be hypothesized that flour with soil contamination may have higher microbial diversity than non-contaminated cereal flours. This is likely to speed-up the fermentation.

Elsewhere, the contamination by cow dung can provide additional substrate, perhaps more available, for microbes to thrive. The rate of pH change in the FT injera fermentation is similar to that reported for injera fermentation based on field threshed teff and sorghum (Baye, Mouquet-Rivier, Icard-Vernière, Rochette, & Guyot, 2012).

Contamination by soil and cow dung can have a negative impact on the sanitary quality of injera; however, as a result of fermentation pH dropped below 4.5, and hence better hygienic conditions is expected (Kingamkono, Sjögren, Svanberg, & Kaijser, 1994).

According to (Helland, Wicklund, & Narvhus, 2004) ethanol produced by yeasts, organic acids produced by bacteria and anaerobic conditions induced by fermentation inhibit the development of pathogenic microorganisms. However, the microbial safety of consuming kitta, unleavened bread, usually made from teff flour may be in question and needs further study.

4.2 Proximate composition of teff flour, fermented dough and baked injera

Table 4.1: Proximate composition of Laboratory threshed and Field threshed Teff-injera

	Dry matter (g/ 100g)		**Fat** (% DM)		**Protein** (% DM)		**Ash** (g/100g DM)	
Threshing	**Lab**	**Field**	**Lab**	**Field**	**Lab**	**Field**	**Lab**	**Field**
Flour	82.3 ± 0.22	89.2 ± 0.12	2.9 ± 0.06^{a}	2.9 ± 0.03^{a}	10.7 ±0.06^{a}	10.6± 0.05^{a}	2.6 ± 0.02^{a}	2.7±0.05^{b}
Fermented	90.3 ± 0.09	90.4 ± 0.11	2.8 ± 0.07^{k}	2.8 ± 0.03^{k}	9.3 ± 0.02^{k}	9.3 ± 0.06^{k}	2.6 ± 0.04^{k}	2.7±0.01^{l}
Injera	92.5 ± 0.14	92.4± 0.13	2.1 ± 0.05^{m}	2.1 ± 0.04 ^{m}	9.3 ± 0.04 ^{m}	9.2 ± 0.03 ^{m}	2.6 ± 0.02 ^{m}	2.8±0.04^{n}

Different superscripts within a raw represent statistically significant difference (P<0.05)

Protein content of both laboratory threshed and field threshed teff injera was similar and was about 10.6 % DM **(Table 4.1)**.

Fermentation of teff flours reduced the protein content by about 12.3 % but baking had no significant effect. Similar results were reported by (KHETARPAUL & Chauhan, 1989), and was hypothesized that this was due to protein catabolism associated liberation of ammonia during fermentation. However, this hypothesis has not been confirmed.

Similarly, no significant difference was noted in fat content between laboratory and field threshed teff injera (**Table 4.1).** Baking reduced the fat content from 2.8% to 2.1 % (P<0.05) in both

laboratory and field threshed teff injera. Although this decrease was statistically significant it may not be nutritionally relevant. Similar trends were reported by (Mohammed, Ahmed, & Babiker, 2011).

Ash content was different between lab and field threshed teff flour, fermented dough and injera ($P<0.05$). Field threshed teff flour had 4.5% greater ash content than that the lab threshed one. This is expected as soil contamination adds up to the mineral content of the field threshed teff.

4.3 Effect of injera processing on mineral absorption inhibitors

4.3.1 Effect of injera processing on phytate contents

Phytate content of both laboratory threshed and field threshed teff injera was similar and was about 255 mg/100g DM **(Table 4.2)**. Fermentation of teff flours reduced the phytic acid content by about 21 %. Further degradation (about 6%) was observed during baking and altogether injera processing allowed about 27% phyate degradation. These levels of phytate degradation during fermentation and baking are in the range reported in other studies (Erdman & Pneros-Schneier, 1994).

Several reasons may explain the degradation of phytates during fermentation. The lowering of pH to optimal conditions for endogenous phytase activities may degrade phytate (Poutanen, Flander, & Katina, 2009; Reale, Konietzny, Coppola, Sorrentino, & Greiner, 2007). Furthermore, microbial phytases may be produced. Indeed, lactic acid bacteria and some strains of yeasts have been reported to produce phytase (Palacios, Haros, Rosell, & Sanz, 2008).

Such reduction in phytate contents may increase the amount of soluble iron, zinc and calcium (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003). Additionally, the organic acids produced during fermentation may bind iron and thus make it unavailable for phytates. Furthermore, the produced organic acids might reduce ferric iron to ferrous iron and thus making

the iron more soluble and thus potentially more bioavailable (Gibson, Perlas, & Hotz, 2006). However, not only phytate but also polyphenols chelate minerals.

4.3.2 Effect of injera processing on polyphenol contents

Tannin content of both laboratory threshed and field threshed teff injera was similar and was about 71 mg/100g DM **(Table 4.2)**. Fermentation of teff flours reduced the tannin content by about 48.5 %. No further degradation was observed during baking. These levels of tannin degradation for fermentation and baking are in the range reported in other studies (Gamboa & Ekris, 2011) .

Several reasons may explain the degradation of tannin during fermentation. The incubation of the flours in optimal pH condition (pH about 5) in the initial phase of the fermentation might have activated endogenous tannase (Greiner & Konietzny, 2006). Whether these degradations are likely to improve iron bioavailability needs to be investigated. However, the iron binding property of polyphenols was ascribed for the galloyl and catechol groups (M. Brune, Rossander-Hultén, Hallberg, Gleerup, & Sandberg, 1992; Leif Hallberg, Rossander-Hultén, Brune, & Gleerup, 1992).

Table 4.2 Effect of processing on mineral absorption inhibitors

Teff Sample type	Phytate (mg/100gm DM)	Tannin (mg/100gm DM)	Catechol (mg CE/100 g DM)	Gallyol (mg TAE/100g DM)
		Lab threshed		
Flour	256.66 ± 5.18a	70.2 ± 1.51a	0.54 ± 0.04a	0.40 ± 0.02a
Fermented dough	201.57 ± 3.87b	36.78 ± 1.36b	0.38 ± 0.04b	0.30 ± 0.01b
Injera	182.11 ± 2.00c	36.57 ± 1.36b	0.47 ± 0.01a	0.35 ± 0.01b
		Field threshed		
Flour	254.64 ± 9.38a	70.81 ± 1.68a	0.46 ± 0.02a	0.34 ± 0.01a
Fermented dough	202.52 ± 3.67b	35.87 ± 1.39b	0.24 ± 0.01b	0.24 ± 0.03b
Injera	184.5 ± 2.01c	36.51 ± 0.69b	0.52 ± 0.06a	0.35 ± 0.04a

Different superscripts within a column represent statistically significant difference ($P<0.05$)

CE, catechin equivalent; TAE, tannic acid equivalent

Catechin content of both laboratory and field threshed teff was similar and was about 0.5 mg CE/100 DM whereas the galloyl content was in the range of 0.3-0.4 mg TAE/100g DM **(Table 4.2)**. Fermentation of teff flours reduced the catechol and galloyl contents ($p<0.05$). This might be related to the activation of endogenous polyphenoloxidase or the production of microbial polyphenoloxidase (El Hag, El Tinay, & Yousif, 2002). However, baking led to increases in the catechol and galloyl contents except in the case of LT teff where it did not increase. The increases observed may be attributed to possible changes in redox potential.

4.4 Total iron content in teff flour, fermented dough, injera and soil

Very high iron contents were found in both LT and FT teff grains. The total iron content of FT teff flour was about 37% greater than that of LT teff flour (**Table 4.3**). During fermentation, the

iron contents of LT and FT teff injera increased by about 19 % and 18%, respectively. This might be due to the additional iron provided by the back-slop used to trigger the fermentation. No increase in total iron contents was observed during baking.

Table 4.3 Total iron content of laboratory threshed and Field threshed teff-injera

Sample type	**Type of threshing**	**Fe (mg/100 g DM)**
Flour	Lab	16.15 ± 0.54a
	Field	25.53 ± 0.11c
Fermented dough	Lab	20.04 ± 0.08b
	Field	31.09 ± 0.45d
Injera	Lab	20.01 ± 0.11b
	Field	31.05 ± 0.63d

Different superscripts within a column represent statistically significant difference ($P<0.05$)

The difference in the total iron content of LT and FT teff is due to the significant contribution of contaminant iron from both traditional threshing and injera processing. Extreme contamination of teff with soil iron is associated with the traditional threshing of grains under the hooves of cattle. Previous studies have shown that iron intake of Ethiopians can be very high (300-500 mg) the bulk of which can be attributed to contamination of grains by soil (Bothell al., 1979). Similarly, comparing food composition data of 20 developing nations, (Beaton, 1974) showed that iron contents of the foods were overestimated by as much as twice. This was attributed to contamination by extrinsic sources (i.e. food containers, dirt and milling devices).

4.5 Iron fractionation in lab and field-threshed teff flour

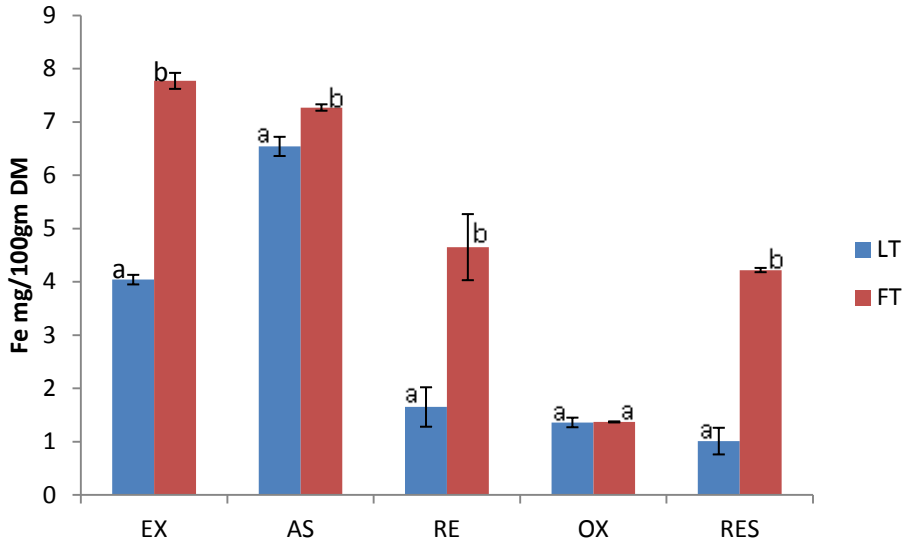

Figure 4.2: Changes in Iron Fraction Percent Lab and Field Threshed Teff-Flours. **Ex**-Exchangeable, **AS**-Acid soluble, **RE**-Reducible, **OX**-Oxidizable, **RES**-Residual**.** Different superscripts within a raw represent statistically significant difference (P<0.05)

The acid soluble fraction in the laboratory threshed teff was the highest and accounted for 44% of the total iron. The exchangeable fraction was the second highest and accounted for 27.6% of the total iron. However, in field threshed teff, the exchangeable fraction is the highest of all fractions and is followed by the acid-soluble and then the reducible fraction. Marked differences in the exchangeable, reducible and residual fractions were observed between the field threshed and the lab threshed teff flours. Except for the oxidizable fraction, significantly higher iron content was obtained in the field-threshed flour than that threshed in the lab. This was particularly the case for the exchangeable, reducible and residual fractions. This may suggest that soil iron is mostly constituted of these three fractions. However, iron fractionation of the soil on which the teff was grown revealed that the major fractions were the reducible, oxidizable and residual fractions. This discrepancy may be due low recovery of the exchangeable fraction in analyzing our soil sample. However, the amount of exchangeable iron was in the levels found in previous studies

(Stone & Droppo, 1996). In soil, the exchangeable fraction usually represent not more than 2% of the total iron (Krishnamurti, Huang, Van Rees, Kozak, & Rostad, 1995; Rauret, 1998). Therefore, the discrepancy observed may be related to sampling of soil in a way that represents both the inner and outer surface soil, while contamination of teff might have been mainly due to contact with surface soil.

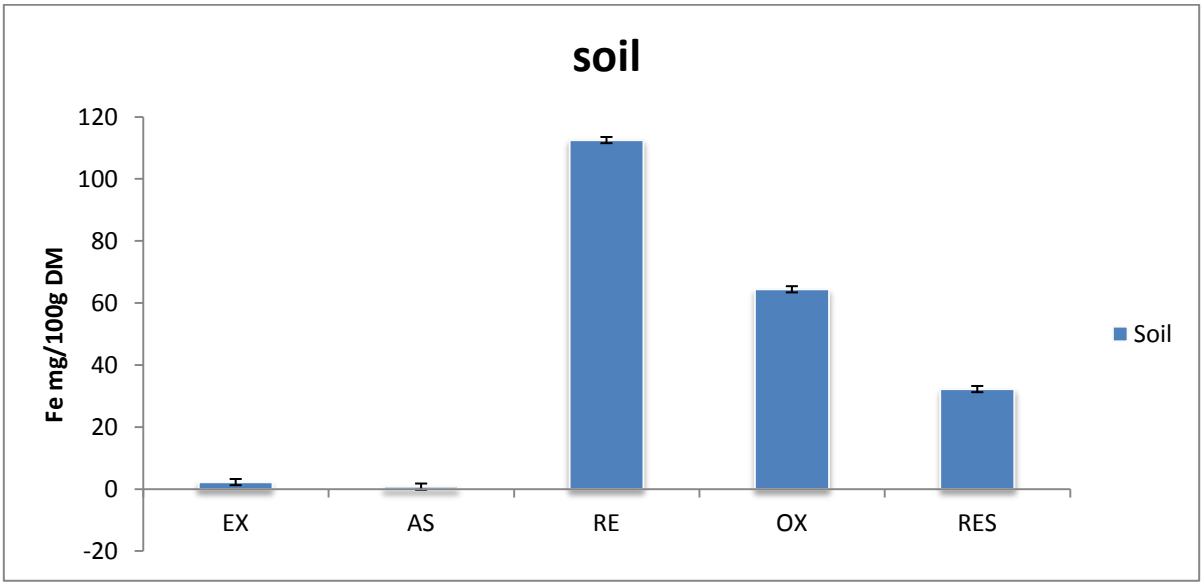

Figure 4.3: Changes in iron fraction in Soil. **Ex**-Exchangeable, **AS**-Acid soluble, **RE**-Reducible, **OX**-Oxidizable, **RES**-Residual

4.6 Effect of fermentation on the distribution of iron fractions

In both lab and field threshed teff, fermentation increased the oxidizable and the exchangeable fractions, while it decreased acid soluble and residual iron fractions. Fermentation decreased the reducible fraction in Field threshed teff but had no effect on the lab-threshed teff (Fig 4.4 A &4.4 B). Looking at the distribution in % relative to the total iron content similar trends has been observed (Fig 4.5).

The resulting increase in exchangeable fraction may however be ascribed to either the decrease in the residual fraction associated to the decrease in iron-chelating agents (i.e. phytate, iron-binding

polyphenols, etc) or to the acidification process that solubilized the acid soluble fraction. Acidification has previously been reported to increase the exchangeable fraction (Simpson et al., 1992). The present findings confirm the positive role of fermentation in mobilizing less mobile iron fractions into labile and potentially bioavailable fraction (exchangeable). Using in-vitro digestion, Grefeuille et al., (2011) reported that fermentation increased the bioaccessible iron fraction in iron contaminated food samples. However, the observed increase in the bioaccessible fraction was offset during baking. Similarly, decreases in the exchangeable and oxidizable fractions were observed in the field threshed and lab threshed teff respectively. This may be related to possible re-complexation of iron with organic matter during baking.

This suggests that indeed, the high iron content in teff whether intrinsic or extrinsic might have a role in preventing anemia. Earlier studies conducted in Ethiopia reported low prevalence of anemia and iron deficiency and partly attributed this to the frequent consumption of teff (Gebre-Medhin, Killander, Vahlquist, & Wuhib, 1976).

However, recent studies report that iron deficiency anemia is not a rarity in Ethiopia (J. A. Haidar & Pobocik, 2009) and represents a moderate level public health concern. This difference between earlier and recent studies may partly be explained by the decrease of teff consumption, mainly due to economic reasons.

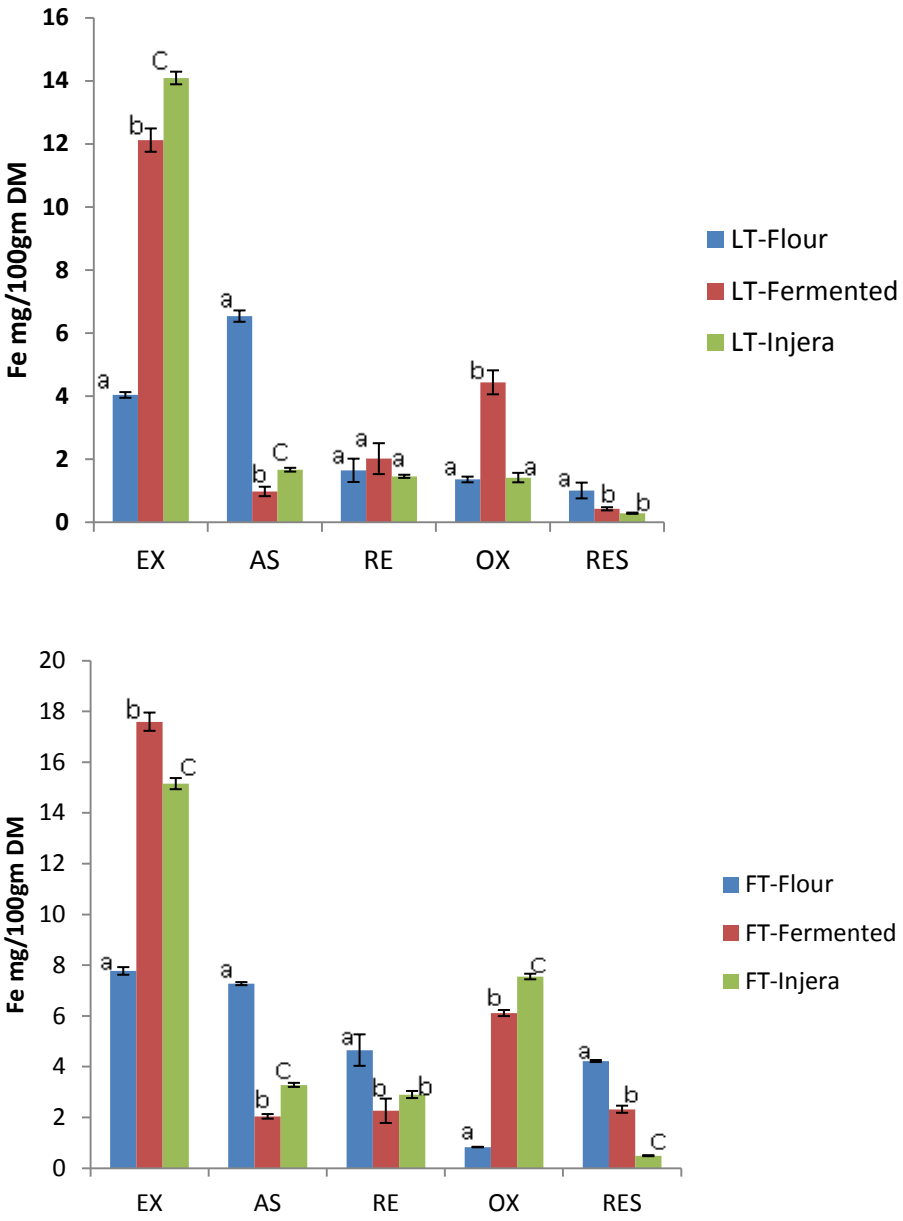

Figure 4.4: Effect of processing on iron fractions of lab-threshed **(A)** and field threshed **(B)**. **Ex**-Exchangeable, **AS**-Acid soluble, **RE**-Reducible, **OX**-Oxidizable, **RES**-Residual. Different superscripts within a raw represent statistically significant difference (P<0.05)

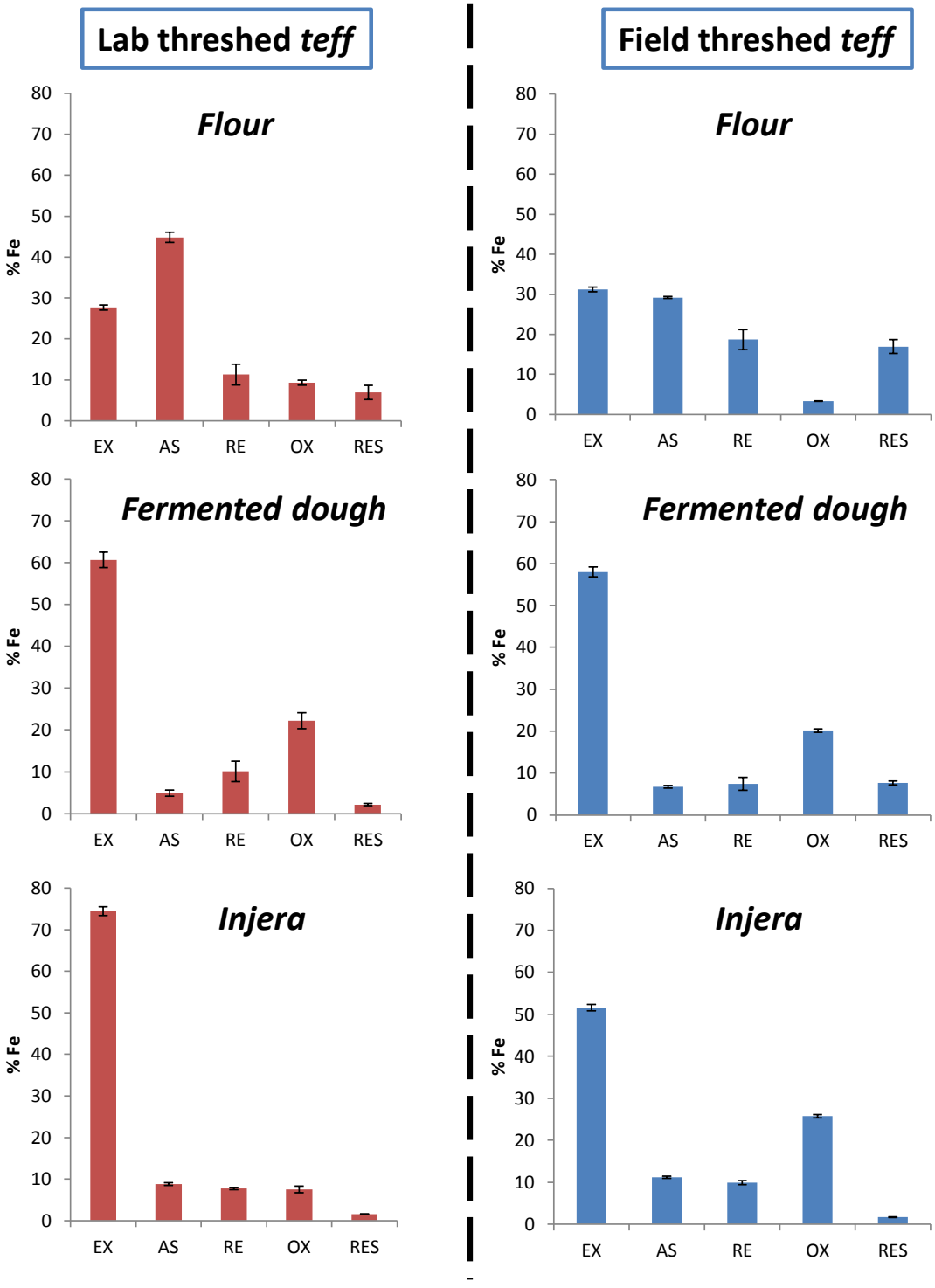

Figure 4.5: Distribution of iron fraction relative to total iron (%) in lab and field threshed teff flour, fermented paste and injera. **Ex**-Exchangeable, **AS**-Acid soluble, **RE**-Reducible, **OX**-Oxidizable, **RES**-Residual

4.7 Limitations of the study

The back-slop utilized to trigger the fermentation was an additional source of iron and may have had implications on the iron fraction distribution. However, given the low amount (62.3g/

460.27g) utilized this is unlikely. In this study both teff variety and soil type was controlled in order to see the effect of processing on iron fractions. Before generalizing the observed findings, several teff varieties grown under different soil types may need to be evaluated. Due to time and chemical limitations, experiments on iron speciation by valence was not conducted.

Chapter five

Conclusion & perspectives

5. Conclusions

Two samples from the same teff variety, grown under the same condition, but differing in the way they are threshed (laboratory vs traditional) were analyzed for their proximate, mineral absorption inhibitors, and total iron contents.

The effect of injera processing (fermentation and baking) on proximate composition, total iron, and mineral absorption inhibitors in both lab and field threshed teff was evaluated. Furthermore, the effect of soil contamination and injera processing on the different iron fractions was investigated using sequential extraction scheme.

Iron content of teff is high. However, the grain further increases in its iron content due to contamination with soil that occurs during traditional threshing under the hooves of cattle. Our study confirmed this by showing that field threshed teff has higher (about 37%) total iron content than lab threshed teff. Furthermore, except for the oxidizable iron fraction, significantly higher iron content was obtained in the rest of the iron fractions. This was even the case for the exchangeable fraction which is likely to be the most bioavailable fraction.

Food processing (i.e. fermentation) through acidification or decrease of mineral absorption inhibitors does have a significant effect on mobilizing different iron fractions. A shift from the acid soluble and residual fraction to the exchangeable fraction was noticed. This implies that teff is a good source of iron.

Furthermore, contaminant iron can be mobilized to a more bioavailable fraction during fermentation. Based on the present findings, we recommend fermenting teff as a good way of not only decreasing mineral absorption inhibitors but also a way of mobilizing both intrinsic and contaminant iron.

6. Recommendations

Iron bioavailability related findings in the present study may need to be confirmed using more common bioavailability assessment methods such as in-vitro digestion and human absorption studies. Speciation of iron by their valence could also complement the present findings.

The effect of grain variety and soil type on the distribution and mobility of iron fractions needs to be studied.

The level of heavy metals in teff growing soil shall be studied and the safety of teff threshed on heavily polluted soils needs to be evaluated using sequential extraction method.

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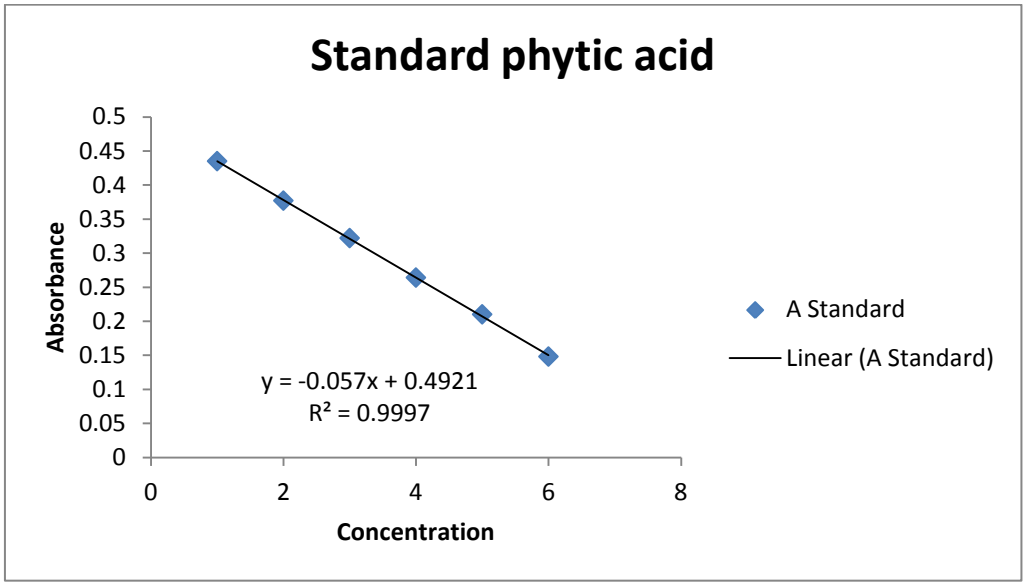
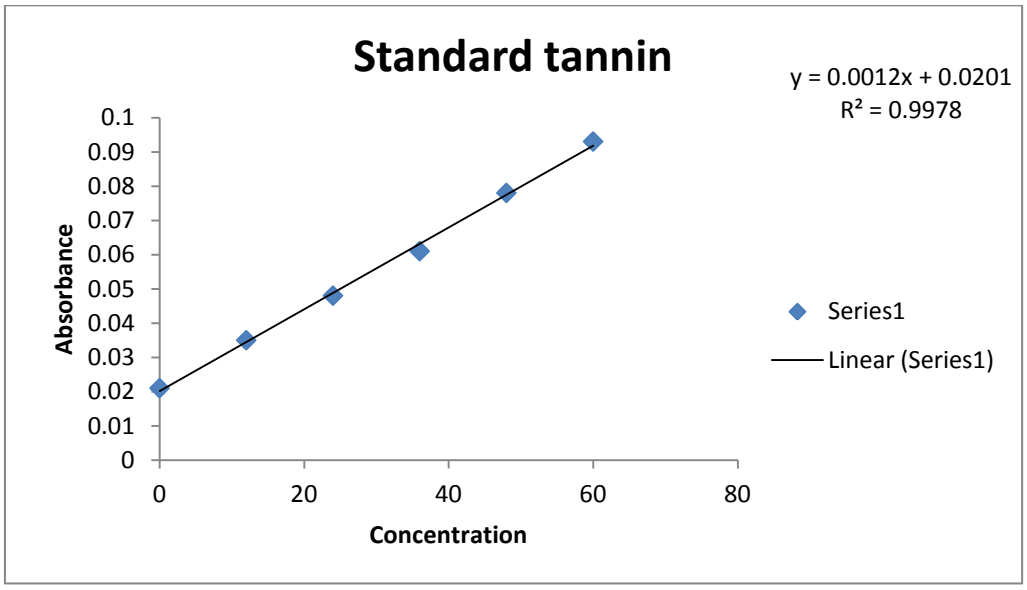
Annex

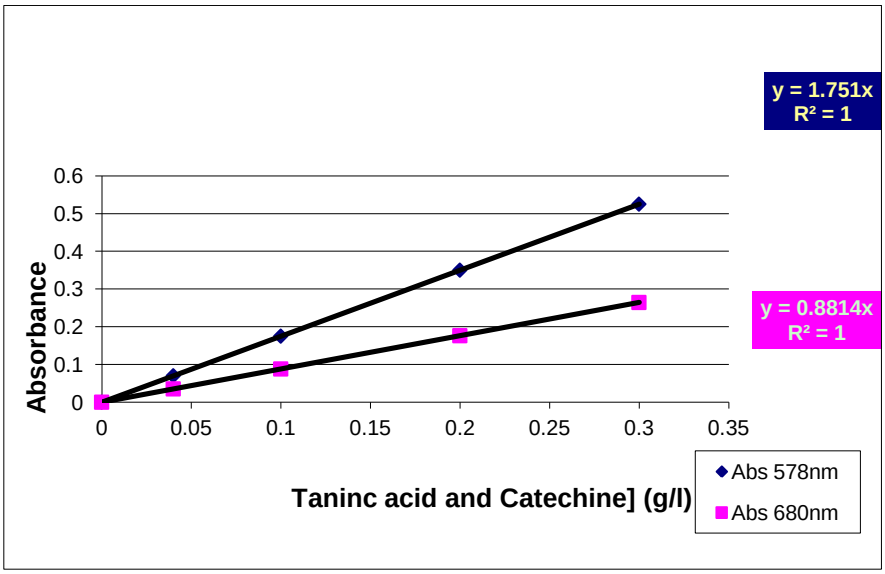
Annex A: Standard phytic acid calibration curve



Annex B: Standard tannic acid calibration curve



Annex C: Standard tannin acid and catechin calibration curve



Annex D: Teff type, growing condition and sampling point

Teff type: Red teff

Date of plaughing: August 30 2004 EC

Date of harvesting: October 25 2005 EC

Soil type and grade: Black soil and "B" grade

Soil name: Koticha

Type of weed and weed killer used: Weed types are (Megeresere and Bokusere) and 2, 4 D weed killer (200 liter water and 1liter weed killer per hectare is used)

Water source used: Rain water

Type and amount of fertilizer used per 2500 meter square: DAP and UREA. Firstly (DAP-Di-ammonium Phosphate=25kg), (UREA 15kg added after two weeks)

Sample collection point