Iron biofortification and fortification of lentil (*Lens culinaris* Medik.)

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ABSTRACT

Biofortification and fortification strategies for lentil (*Lens culinaris* Medik.) were investigated to increase bioavailable iron (Fe) in the human diet. Biofortification studies included, firstly, development of a precise protocol for Fe analysis of seeds of all (seven) *Lens* species using flame atomic absorption spectrometry (F-AAS). Secondly, genotype (G) × harvest (H) timing interaction of seed Fe accumulation was determined during seed maturation stages in seven lentil species. Thirdly, estimates were made of seed Fe concentration (SFeC), its inheritance, and the effect of genotype (G) × environment (E) interaction for two interspecific recombinant inbred line populations (RILs) of lentil. Finally, molecular markers associated with SFeC across 138 diverse cultivated lentil accessions were identified by phenotyping in four environments in Saskatchewan, Canada. For the fortification strategy, appropriate methods and dosage were determined for Fe fortification of lentil dal with $FeSO_4 \cdot 7H_2O$, NaFeEDTA and $FeSO_4 \cdot H_2O$. A colorimetric study determined changes in appearance of fortified lentil at various Fe concentrations over three storage periods. Sensory evaluation with panelists in Saskatoon and Bangladesh evaluated cooked and uncooked fortified lentil using a 9-point hedonic scale (1 = dislike extremely to 9 = like extremely). Finally, Fe and phytic acid (PA) concentration and relative Fe bioavailability (RFeB%) were estimated in 30 traditional Bangladeshi dal meals featuring either fortified (fortificant Fe concentration of 2800 µg g^{-1}) or unfortified lentil.

The first study determined the minimum lentil seed sample (0.3 g and 0.5 g of wild and cultivated species, respectively) required for an accurate and precise estimation of SFeC. The G × H timing interaction study revealed significant variation for SFeC among genotypes, but a similar seed Fe accumulation trend over the harvest period. Field evaluations revealed significant

variability for SFeC among lentil RILs and for G × E interactions with high broad sense heritability for SFeC. Association mapping studies revealed wide variation for SFeC among genotypes. Two SNP markers were tightly linked to SFeC (−log10 P ≥ 4.36) and also seven additional markers were also significant (−log10 P ≥ 3.06) for SFeC. Most (six) markers were found on chromosome 5. Putative candidate genes were identified underlying alleles encoding Fe related functions. The fortification study revealed that NaFeEDTA was the most suitable Fe fortificant for lentil dal, and at 1600 µg g^{-1} fortificant Fe concentration, it provided 13-14 mg of additional Fe per 100 g of dal. Total Fe and PA concentrations, and RFeB% differed significantly between cooked unfortified and fortified lentil. Significant differences in sensory quality were observed among all uncooked and cooked samples when tested in Canada and Bangladesh. NaFeEDTA had the least effect on consumer perception of colour, taste, texture, odour and overall acceptability of cooked lentil. The meal study revealed that NaFeEDTA fortified lentil increased Fe concentration in lentil from 60 to 439 µg g^{-1} and RFeB% by 79% as estimated by Caco-2 cell ferritin formation. Phytic acid levels also were reduced from 6.2 to 4.6 mg g^{-1} when fortified lentil was added, thereby reducing the PA:Fe molar ratio from 8.8 to 0.9. The overall outcomes of this research could help to significantly and cost-effectively increase the amount of bioavailable Fe in lentil, and the consumption of fortified lentil could help to provide a significant part of the consumer's daily Fe requirement.

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DEDICATION

This thesis is dedicated to my Wife, Daughter and parents for their support, love and unconditional sacrifice for my academic career.

In addition, I would like to dedicate my research to my Supervisor and all well-wishers.

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LIST OF ABBREVIATIONS

AM	Association Mapping
ANOVA	Analysis of variance
CA	Cronbach's alpha
CDC	Crop Development Centre
CSFL	Crop Science Field Laboratory
DTPA	Diethylene tri-amine penta-acetic acid
DCYTB	Duodenal enzyme cytochrome
DMT1	Divalent metal ion transporter1
EARs	Estimated Average Requirements
F-AAS	Flame atomic absorption spectrometry
Fe	Iron
LSD	Least significant difference
MCMC	Markov Chain Monte Carlo
NIST	National Institute of Standards and Technology
NS	Not significant
GLM	General Linear Model
FP	Ferroportin
K	Kinship matrix
MLM	Mixed Linear Model
NaFeEDTA	Ethylenediaminetetraacetic acid iron (III) sodium salt
PA	Phytic acid
PA:Fe	Phytic acid : Fe molar ratio
PCA	Principal component analysis
Q	Population structure

QTL	Quantitative trait loci
RDA	Recommended daily allowance
RFeB	Relative iron bioavailability
RCBD	Randomized complete block design
RIL	Recombinant inbred line
SAS	Statistical analysis software
SD	Standard deviation
SE	Standard error
SEM	Standard error of mean
[SFeC]	Seed Fe concentration
SNP	Single nucleotide polymorphism
SPG	Saskatchewan Pulse Growers
STH	Sutherland
TASSEL	Trait Analysis by Association, Evolution, and Linkage
Tf	Transferrin

CHAPTER 1

INTRODUCTION AND RESEARCH HYPOTHESES

Iron (Fe) deficiency is one of the most prevalent health concerns worldwide, especially in developing countries where diets are Fe deficient. About one fourth of the total world population is affected by anemia - an indirect indicator of Fe deficiency (McLean et al., 2009). The severity is much higher in developing countries due to inadequate supply of nutritionally balanced food in the context of geometric population growth rates, diverse food habits and socio-economic standing of populations. Fe is needed to regulate a number of metabolic processes and since the human body cannot produce it, adequate amounts of bioavailable Fe should be consumed in the diet to escape the risk of Fe deficiency.

Among the food legumes, lentil (*Lens culinaris* Medik.) is an important grain legume that provides both protein and micronutrients for human and animal diets. Lentil also is one of the cheapest sources of protein and micronutrients for vegetarian diets, in which animal product consumption is very low. This crop is consumed as a staple food in some developing countries where malnutrition due to Fe deficiency is more prominent. Improving Fe concentration and bioavailability potentially can be achieved by biofortification, a genetic approach, or by fortification, a food processing approach. Research has been initiated to increase Fe concentration and bioavailability through biofortification, although to this point limited investigation has occurred in the area of genetic strategies for increasing bioavailability of Fe in lentil. In this body of research, we report on a series of studies that can make contributions toward achieving the goal of improving both Fe content and bioavailability of Fe in lentil. The overall hypothesis of the body of work in the thesis was that both Fe biofortification and fortification can increase the concentration and bioavailability of Fe in lentil.

1.1. Biofortification studies in lentil

The concept of biofortification of staple seed crops is predicated on the idea that sufficient variability for Fe concentration exists in the available gene pool of the crop. The cultivated lentil gene pool has one species, *Lens culinaris,* plus six wild species, *L. orientalis, L. odemensis, L. tomentosus, L. nigricans, L. ervoides and L. lamottei* (Wong et al., 2015). The species of the wild gene pool have not been investigated extensively from the standpoint of their potential contribution to nutritional improvement of cultivated lentil.

1.1.1. *Optimizing seed sample size for Fe analysis of wild and cultivated lentil*

When using flame atomic absorption spectrophotometry (F-AAS) to measure Fe concentration in lentil seeds, sufficient amounts of seed are required to provide reliable estimates of Fe concentration. Consideration of how many seeds are used in analysis of Fe concentration is especially important for wild lentil. Seeds of wild lentil are very small, plants are indeterminate, and the seed pods are dehiscent. A wide range of variability is found in key biological traits such as seed dormancy, flowering, maturity, seed shattering, seed size and shape, seed yield per plant and disease resistance. Plants are difficult to grow and produce low seed yield, making them expensive to grow and produce large amounts of seed for nutritional analysis. The cultivated species has many different market classes that vary in seed size, seed shape, seed coat and cotyledon colour, and consumer preference. There is a need to quantify the necessary amount of seeds needed to assess the seed Fe concentration of the wild species and the different market classes of cultivated lentil to reduce cost, time and labour using F-AAS.

***Hypothesis*:** The quantity of seeds and seed weight of lentil species have an effect on consistent and accurate estimation of Fe concentration using flame atomic absorption spectrophotometry (F-AAS).

1.1.2. *Variation of Fe uptake at different plant maturity stages of lentil*

Lentil is an indeterminate plant and this growth habit is influenced by environmental condition (Shrestha et al., 2006). The time to maturity of different genotypes from different species, and even within a species, may vary due to their genetic constitution as well as the influence of the macro and micro environments which fluctuate widely based on temperature, moisture and day length. Fe accumulation in seeds during the maturation period also is important to understand, especially because the plants are dehiscent and continue to flower and produce seeds until they experience environmental conditions (frost, heat or drought) that end the life cycle. Variation may occur not only in total nutrient accumulation in seeds, but also in the rate of accumulation of nutrients in lentil seed during different seed maturation stages. A study was initiated with 12 wild and two cultivated species and seeds from a single plant were harvested three times at intervals of 10 days. The results from this study can provide an idea about Fe accumulation in lentil seed during maturation.

***Hypothesis*:** The time of seed development during the growing season influences seed Fe concentration and Fe accumulation in lentil seeds among the *Lens* species and is influenced by genotype × harvest interaction.

1.1.3. *G × E interaction effects on Fe accumulation in lentil interspecific hybrids*

Environmental factors and agronomic practices can interact with plant gene expression, which in turn can play a substantial role in differential micronutrient accumulation from soil (Bouis

& Welch, 2010). Apart from genotypic variation, the lentil production environment, such as geographical location, soil factors, temperature and other environmental conditions, have significant effects on micronutrient concentrations in lentil (Thavarajah et al., 2010). The influence of temperature and soil conditions on concentration of phytic acid, Fe and Zn in Saskatchewan grown lentils was reported by Thavarajah et al., (2011). Soil pH is an important factor that influences the availability of Fe for uptake by plants. Under natural alkaline pH conditions, soil Fe precipitates and limits availability and abundance of Fe in soil (Pandian et al., 2011). Kumar et al., (2013) also reported highly a significant influence of genotype, environment and location on Fe and Zn concentration in lentil. The Fe concentration in lentil will be influenced by environment and it may vary among genotypes. Genotype × environment interaction can reduce the genotypic stability of crop genotypes irrespective of environment. It would be useful to reliably identify stable genotypes with reliably higher concentrations of bioavailable Fe. Moreover, since interspecific hybridization is now used to improve disease resistance in lentil (Tullu et al., 2013) it is important to ascertain how seed Fe concentration may vary in interspecific lentil hybrids and their progenies which contribute genetic diversity to cultivated lentil breeding. The fundamental question is whether or not interspecific hybridization can result in development of lentil germplasm with more variation in seed Fe concentration, which would be essential to make progress in biofortification. This type of information has never been reported.

***Hypothesis*:** The concentration of Fe in seeds of *Lens culinaris x Lens ervoides* interspecific hybrids and their parents is the same across environments.

1.1.4. Marker-trait association analysis of Fe concentration in lentil seeds

Marker-trait association can help to determine the genetic basis for uptake of micronutrients, such as Fe, Zn, Se and other nutritional components of food legumes. A set of 138

diverse cultivated lentil accessions from 34 countries was previously evaluated for morphological and phenological traits in four environments (2 sites × 2 years) in Saskatchewan, Canada. The collection was genotyped using 1150 SNP (single nucleotide polymorphism) markers that are distributed across the lentil genome. Results from this study can reveal if there is any accessible variation for seed Fe concentration. The marker-trait association analysis can also detect SNP markers tightly linked to seed Fe concentration.

***Hypothesis*:** Genomic regions controlling seed Fe concentration of lentil can be identified through association mapping.

1.2. Iron fortification of dehulled lentil

Food fortification is a potentially cost-effective way to add micronutrients to processed foods that could rapidly mitigate micronutrient malnutrition (WHO & FAO, 2006). Fortifying lentil with suitable Fe fortificants during processing is a research area with potential to reduce Fe deficiency. In this approach, dehulled lentil can be enriched with extra Fe to prevent Fe deficiency in humans. This research is unique in the context of food fortification and requires addition of Fe, measurement of Fe concentration, sensory evaluation and assessment of bioavailability in fortified lentil.

1.2.1. *Optimization of Fe fortification method*

Initial research was focused on identifying the most appropriate Fe fortificant for fortifying dehulled lentil products. Known Fe fortificants such as ferrous sulphate heptahydrate, ferrous sulphate monohydrate, sodium-iron-EDTA, ferrous fumarate and ferric orthophosphate are acceptable fortificants (WHO & FAO, 2006) that were used to fortify dehulled lentil. Before fortification, some preliminary studies, such as, selection of lentil genotype for fortification, choice

of appropriate lentil product type, selection of appropriate method of fortification, assessment of appropriate temperature for drying of lentil after soaking with fortificants, assessment of the appropriate dose of Fe solution, effect of storage on changes in appearance, effect of fortification on boiling time, and determination of the fortification protocol that can be merged with current lentil processing techniques, would provide information that might help in standardizing the protocol for fortification of lentil.

***Hypothesis*:** It is possible to fortify Fe in de-hulled lentil in a biologically and culturally meaningful way.

1.2.2. *Sensory evaluation of Fe fortified lentil*

Sensory evaluation is a necessary component of the fortification technique when considering the production of processed or value-added foods for the marketplace. A series of techniques was used in this process to measure the human response to foods and reduce the bias effects of brand identity and other information that may create impact on stakeholder perception (Lawless & Heymann, 2010). Fortified lentil has some distinguishing characteristics in comparison to unfortified lentil. The changes in organoleptic properties of fortified lentil can be evaluated by consumers and their remarks would provide valuable information that would aid in making recommendations to food scientists or product developers for commercial food production.

***Hypothesis*:** Unfortified and Fe-fortified lentil are accepted similarly by consumers with respect to sensory attributes.

1.2.3. *Assessment of bioavailability of fortified lentils under relevant Bangladeshi meal preparation methods*

Ensuring sufficient amounts of mineral micronutrient intake to prevent deficiency disorders is a well-established concept, but whether or not adequate amounts of the supplemented mineral is absorbed is an important question for improvement of mineral status of humans. Different methods such as haemoglobin repletion, plasma appearance, fecal monitoring (chemical balance), and the invitro Caco-2 cell bioassay are used to assess Fe bioavailability (Fairweather-Tait, 2008). In this study, fortified lentil would be assessed through an in vitro system, the Caco-2 cell bioassay that is widely used to estimate bioavailability of Fe. Based on the results of bioavailability of fortified lentil, bioavailability of Fe in fortified lentil under relevant meal preparation methods also would be determined.

***Hypotheses*:** Using Fe-fortified lentil in relevant meal preparations will have a significant effect on increasing Fe concentration and bioavailability.

1.3. Summary of the significance of the research

The research in the thesis was designed to contribute to knowledge of the genetic potential of lentil for biofortification, and also to initiate new approaches to increase Fe bioavailability through consumption of fortified lentil. The possible outcomes of these studies include measurement of the stability of Fe concentration across different environments, estimation of Fe in wild species of the genus *Lens*, QTLs conferring seed Fe concentration, development of efficient Fe concentration measurement protocols and the identification of appropriate Fe fortificants and the bioavailability of Fe in fortified lentil.

1.4. Experimental objectives

Biofortification

1. Determination of the minimum quantity of lentil seeds required to consistently quantify Fe concentration using whole seed digestion with nitric acid and atomic absorption spectrophotometry (F-AAS).
2. Estimation of variation in Fe accumulation in seed during different growth stages and to determine the genotype × harvest timing interaction that influences Fe accumulation in seed of seven lentil species.
3. Determination of seed Fe concentration of interspecific RILs grown across a wide range of environments and assessment of inheritance and effect of genotype × environment interaction on seed Fe concentration.
4. Identification of significant marker-trait associations for Fe concentration in lentil seed via association mapping.

Fortification

1. Determine the most suitable Fe fortificant for de-hulled lentil based on ease of fortification, and to determine the optimal processing technology to fortify Fe in de-hulled lentil based on current processing practices.
2. Determine the sensory acceptability of fortified lentils – appearance, odour, texture, taste and overall acceptability.
3. Determine the concentration and bioavailability of Fe in fortified lentil when used in relevant meal preparations.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

The research topic selected here "Iron biofortification and fortification in lentil (*Lens culinaris* Medik.)" revealed a combination of three major areas - plant science, food science and human nutrition. A number of currently published articles, review papers, dissertations, websites, reports and short communications related to the selected research topic were organized and synthesized in this review to provide an update on current knowledge and gaps in this research area. Five main components of the review are described below. The first sub-chapter (2.2) is a brief introduction to the lentil crop. The second and third sub-chapters (2.3 & 2.4) are focused on a review of the relationship of Fe with plants and humans, respectively. The fourth and fifth sub-chapters (2.5 & 2.6) reviews Fe biofortification and Fe fortification, respectively.

2.2. The lentil crop

Cultivated lentil is a self-pollinated, diploid crop which in the small but genetically diverse genus *Lens* (Ladizinsky & Abbo, 1996). The term *Lens* was first coined by Tournefort to designate as a specific genus reviewed in Cubero et al., (2009). Perceptions of speciation within genus *Lens* evolves over time. The classical species relationships described for the genus *Lens* is that it is comprised of six different species: the cultivated lentil *L. culinaris* (Medik.) subsp. *culinaris* (i) *and* subsp. *orientalis* (Boiss.) Ponert (ii), *L. odemensis* (Ladiz.) (iii), *L. tomentosus* (Ladiz.) (iv) *L. nigricans* (M. Bieb.) Godr. (v), *L. ervoides* (Brign.) Grande (vi) and *L. lamottei* Czefr (vii) (Van Oss et al., 1997). All *Lens* species have the same chromosome number (2n = 14) (Sonnante et al., 2009). These six species are classified into three gene pools when considering hybridization

barriers among them (Cubero et al., 2009). Among the six species (i to vi) mentioned above, the primary gene pool includes (i) and (ii), the secondary gene pool includes (iii) and (iv) and the remaining three belong to the tertiary gene pool. The most recent classification of the lentil species is that of Wong et al., (2015) who used genotyping by sequencing and placed all the lentil species in four different gene pools. The primary gene pool consists of three species, *Lens culinaris*, *L. orientalis* and *L. tomentosus* and the secondary gene pool consists of two species, *L. lamottei* and *L. odemensis*. The tertiary and quaternary gene pool consists *L. ervoides* and *L. nigricans*, respectively.

Lentil is one of the most economically important legume crops. It has been used as a protein source in human and animal diets (AL-Asbahi, 2011) since prehistoric times. This crop is considered to have originated as part of the Near Eastern complex with many of the oldest domesticated crops such as einkorn, emmer, barley, linseed and pea (Harlan, 1992). The first report on lentil domestication in the Hindu-Kush region of central Asia was suggested by (Barulina, 1930). Pearman, (2005) reported that the Fertile Crescent is the source of wild ancestor of cultivated lentil (subsp. *orientalis*). Cubero et al., (2009) reported that, on the basis of archeological data, the wild and cultivated *Lens* species originated from the Near East. From the center of origin, lentil cultivation spread and is now grown in ~50 countries including the entire Mediterranean region, central West and South Asia, Ethiopia, Australia, temperate regions of North and South America, and even in some tropical regions (FAOSTAT, 2017).

Lentil ranks as the fifth most important grain legume crop of the world in terms of total production and area under harvest and fourth in terms of yield (FAOSTAT, 2017). The world production of lentil in 2014 was 4.82 Mt at 1.06 t/ha (FAOSTAT, 2017). In Western Canada, lentil was introduced in the early 1970s (McVicar et al., 2017) from the Palouse region. From 600 ha at

that time, the area increased in Saskatchewan (SK) to as much as 2.1million ha in 2016 (McVicar et al., 2017). Lentil has become a very important crop in SK due to its value in crop diversification, extension of crop rotations, reduction of the requirements for nitrogen fertilizer and its ability to improve economic returns to the growers. Canada has become the top lentil producer and exporter in the world, accounting for up to 40% of global production, and 90% of Canadian production. The value of lentil exports from Canada reached $2.4 billion in 2016 (Saskatchewan Pulse Growers, 2017). The Middle East and North Africa (MENA) region ranked as the region for highest per capita availability of lentil for consumption in 2006-08, followed by the South Asian region where consumption rate of lentil was 1.03 kg/person/year out of 9.70 kg/person/year for total pulses (Akibode & Maredia, 2011). Among the South Asian countries, India is the largest importer and consumer of lentil, especially red lentil exported from Canada (Manawaria, 2014).

Among the pulse crops, lentil contains a substantial amount of protein, complex carbohydrates, and micronutrients including Fe (DellaValle et al., 2013). Additional nutrients include amino acids, vitamins, phenolic compounds, dietary fiber and resistant and slowly digestible starch, making lentil one of the healthiest foods (Tosh et al., 2013). The main source of protein in South Asian region is believed to be the pulses. Lentil is increasingly deemed a whole food, and Canadian lentils are becoming more popular to consumers worldwide due to presence of considerable amounts of Fe (73-90 mg kg-1), Zn (44-54 mg kg-1), Se (425-673 mg kg^{-1}) and relatively low amounts of the micronutrient absorbance inhibitor phytic acid (2.5-4.4 mg g^{-1}) (Thavarajah et al., 2011).

2.3. Iron and plants

2.3.1. *Fe for plants*

Iron plays a significant role in increasing the quality and quantity of crop yield, which leads to effects on the health of humans and animals through dies. Iron is required in minimal amounts for plant growth, but it is essential for plant biological activities. It plays a vital role in all fundamental mechanisms in plants such as photosynthesis, respiration, and metabolic processes through its role in enzyme systems (Vigani, 2012) or as an electron donor in the electron transport chains of photosynthesis and respiration (Connolly & Guerinot, 2002). Iron deficiency reduces chlorophyll synthesis and thus causes chlorosis in plants (Hochmuth, 2011).

2.3.2. *Iron uptake in plants*

The mechanism of micronutrient acquisition in plants is becoming an important issue in modern agriculture due to the relationship between the micronutrient content of food and human health and nutrition (Kochian, 2000). The author also reported several reasons responsible for complicating the acquisition of micronutrients. The relative availability of micronutrients and their magnitude in soil is one of the obstacles for iron uptake into the plant. Another reason is the formation of "metallorganic complexes" by the micronutrient cations in the soil, their presence in the rhizosphere, and the breakdown of metal chelates for transport into the plant cell (Kochian, 2000).

Iron is abundant in soil (Peiffer et al., 2012; Schmidt, 1999), and plants require a minimal amount of Fe. Most annual plants require 1 to 1.5 lb Fe $acre^{-1}$, compared with nitrogen (N) at 80 to 200 lb $acre^{-1}$ (Hochmuth, 2011). The Fe availability in soil is highly influenced by soil pH and aeration (Schmidt, 1999). Alkaline conditions (pH > 8) make Fe^{3+} (ferric Fe) unavailable. The Fe^{2+} (ferrous Fe) form is available from soil at pH 6.5-7, and plants can easily uptake and use it (Havlin et al., 1999). The reduced form of Fe^{2+} is reported to be more available than Fe^{3+} (Kochian, 2000).

The soluble inorganic form of Fe found in soil in chelated condition is the dominant form in which plants take up the major part of Fe required for their growth.

Most of the Fe present in soil is insoluble and thus plants may suffer from Fe deficiency stress. Under stress conditions, plants can induce physiological and biochemical responses to make required Fe soluble and available for their growth. Two different strategies are used by plants to solubilize and take up Fe. Plants of the Poaceae (grass) family excrete highly soluble Fe^{3+} binding agents termed "phytosiderophores" that help to solubilize the Fe^{3+} ion for absorption. In most monocots and in dicots including legumes, Fe^{3+} is reduced to Fe^{2+} at the cell surfaces at 1-4 cm behind the root tip where the maximum amount of protons and reductants are released (Hochmuth, 2011). In dicot and non-poaceous plants, several responses to Fe deficiency were briefly described by (Kochian, 2000; Li & Lan, 2017). A model was used to describe the absorption of Fe in dicots from the rhizosphere by a two-step process. The first step is the reduction of extracellular Fe (III) chelates by ferric reductase and release of the bivalent Fe^{2+} ion. The second step is the transport of Fe^{2+} into the cytoplasm with the help of a "specific Fe^{2+} transporter". Kobayashi & Nishizawa, (2012) reviewed current understanding of Fe uptake, translocation, subcellular translocation, and regulation in response to Fe shortage or excess in higher plants at the molecular level. The authors summarized the studies that represented the central genes responsible for Fe homeostasis in plants.

2.3.3. *Fe storage in seeds*

Plant ferritin also known as "phytoferritin" is a broad super-family of storage proteins (Lv et al., 2015). One of the main goals of biofortification is to enrich the phytoferritin content of edible parts of plants. The ferritin also plays an important role in Fe metabolism and plants can store up to ~ 4500 Fe^{3+} in inner cavities of ferritin molecule in the form of an "iron oxyhydroxide-phosphate mineral" (Harrison & Arosio, 1996; Lv et al., 2015). Ferritin content in edible plant parts, such as

seed, stem, and leaf tissue should be an excellent source of Fe (Zielińska-Dawidziak, 2015). This protein can provide Fe especially for vegetarians and populations where Fe from meat is limited.

Most of the Fe uptake from the soil is accumulated in leaves. In legumes, nodules involved in nitrogen fixation are also rich in ferritin. Ferritin from leaves, roots, and nodules remobilizes in seeds (Zielińska-Dawidziak, 2015). Compared to cereals, legume seeds, such as, soybean (*Glycine max*), pea (*Pisum sativum*), lentil, and chickpea (*Cicer arietinum*) are rich in ferritin due to presence of nodules because Fe from root nodules translocate to the seeds (Burton et al., 1998). Ferritin concentration in soybeans seeds was reported in the range of 50-70 mg kg^{-1} and 100 g of fresh raw beans or seeds can provide only 12.5% and 6.66% of the RDA (recommended daily allowance) for non-vegetarian adult men and women, respectively (Sczekan & Joshi, 1987; Zielińska-Dawidziak, 2015). Lentil is also rich in micronutrients, such as Fe, Zn, Se etc. Ferritin Fe concentration in seeds may be influenced by the growing conditions (Zielińska-Dawidziak, 2015). Using biofortification strategies could be an attractive way to develop or explore new germplasm that can take up more Fe from the soil for deposition in seeds.

2.3.4. *Influence of environment on Fe accumulation by lentil plants*

Assessment of genotype by environment interaction for micronutrient dense germplasm is essential for determining the influence of growing environments on micronutrient content expression (Bouis & Saltzman, 2017). The interaction can also reduce the genotypic stability of micronutrient dense genotypes. Plant gene expression can be influenced by environmental factors and agronomic practices that can differentiate the amount of micronutrient accumulation from soil (Bouis & Welch, 2010). Lentil is cultivated in many different agro-ecological regions around the world, therefore geographical location, soil factors, temperature and other conditions can have significant influence on lentil seed Fe concentration (Thavarajah et al., 2010). Thavarajah et al.,

(2011) reported on the influence of temperature and soil conditions on the concentration of phytic acid, Fe and Zn in Saskatchewan grown lentils. For instance, Fe availability is highly influenced by soil pH condition. In natural alkaline pH conditions, soil Fe precipitates and thus decreases availability (Pandian et al., 2011). Kumar et al., (2013) also reported the significant influence of genotype, environment, and location on Fe and Zn concentration in lentil seeds.

2.4. Iron and humans

2.4.1. *Nutritional aspects of Fe and its homeostasis in human*

Fe is also an essential micronutrient for humans. A human requires more than 22 mineral elements (White & Broadley, 2005) and Fe must be supplied by the diet. Iron deficiency anemia is the most common and prevalent form of micronutrient malnutrition, affecting one-third of the world population (WHO & FAO, 2006). Anemia, resulting from Fe deficiency, is considered one of the most predominant health risks in developing countries and in a few developed countries (Maheshwari & Chandra, 2012). Two out of every three persons from the developing world suffer from Fe deficiency and its resulting anemia (Baltussen et al., 2004). Anemia significantly affects psychomotor and mental development of infants, cognitive development of pre-school children, cognitive function and educational achievement of school-age children, pregnancy outcomes, and adult work productivity (Baltussen et al., 2004). The WHO reported that prevalence of anemia was 50% for pregnant women, infants, and children aged 1-2 years, followed by 40% for school children (WHO & FAO, 2006). The anemic condition of preschool-aged children, adolescents, and non-pregnant women are also estimated to be about 25%, 30-55% and 35%, respectively (WHO & FAO, 2006). In developing countries, the major concern is the increasing rate of morbidity and mortality rate of preschool-aged children and pregnant women, mostly due to Fe

deficiency (McLean et al., 2009), caused by poor diet. The RDAs for Fe (in mg/day) for infants, children and adults are summarized in Table 2.1 (Zhao et al., 2014)).

Table 2.1. Recommended dietary allowances for iron for infants, children and adults*

Age	Males (mg/day)	Females (mg/day)	Pregnancy (mg/day)	Lactation (mg/day)
7 to 12 months	11	11	N/A	N/A
1 to 3 years	7	7	N/A	N/A
4 to 8 years	10	10	N/A	N/A
9 to 13 years	8	8	N/A	N/A
14 to 18 years	11	15	27	10
19 to 50 years	8	18	27	9
51+ years	8	8	N/A	N/A

*(Zhao et al., 2014)

Mammalian Fe metabolism or Fe homeostasis is reviewed or reported in much literature, for example in (Anderson et al., 2012; Hentze et al., 2010; Hoppler et al., 2008). Many molecular structures and metabolic pathways are involved in Fe homeostasis in the human body. The regulation of adequate plasma Fe levels is the key to systemic Fe supply and homeostasis (Hentze et al., 2010). This plasma Fe is bound to the glycoprotein transferrin that indicates the Fe overload and Fe deficiency in human. Fe deficiency occurs when the plasma transferrin saturation < 16% and Fe overload occurs when plasma transferrin saturation is > 45% (Hentze et al., 2010). Fe is absorbed first by the epithelial mucosa cells, mainly in the duodenum and upper jejunum (Hoppler et al., 2008). The cellular uptake of Fe also depends on whether or not it is in non-heme or heme Fe form (Hoppler et al., 2008), and cellular Fe homeostasis is influenced by the amount of Fe

uptake, storage, utilization, and export. These functions are regulated by Fe regulatory proteins 1 and 2 (IRP 1 and IRP 2) (Anderson et al., 2012).

Both the heme and non-heme Fe partly share a similar pathway across the mucosal border (Hoppler et al., 2008). Non-heme Fe is more efficiently absorbed than heme Fe. A saturable heme carrier protein (HCP1) has been identified, and it is regulated by the present Fe status. Heme Fe is primarily absorbed as the form. The Fe^{3+} form of dietary Fe is first reduced to Fe^{2+} ferrous by a duodenal enzyme cytochrome (DCYTB). Then the Fe^{2+} enters the enterocyte with the help of divalent metal ion transporter1 (DMT1). Inside the enterocyte, both heme and non-heme Fe combine with plasma carrier transferrin (Tf) with the help of a ferroportin (FP) protein. This Tf transports Fe throughout the body cells and the absorbed ion is mainly used for hemoglobin formation (Hoppler et al., 2008).

2.4.2. *Iron absorption inhibitors present in legumes including lentils*

Pulses do have some protein or non-protein antinutritional compounds that reduce consumer acceptability. In some regions of the world, especially in the developing countries, people traditionally consume pulse crops as a partially staple food and to feed animals. This practice might have made them tolerant to these antinutritional compounds. But in some regions, consumers have expressed concerns about pulse consumption due to feeling stomach discomfort, hemagglutination, bloating, vomiting and pancreatic enlargement (Roy et al., 2010). Some of the antinutritional compounds in pulse crop seeds are alkaloids, antigenic factors, trypsin inhibitors, vicine-convicine, lectins, oligosaccharides, tannins and phytates (McPhee & Muehlbauer, 2002). Almost all pulses contain phytic acid or inositol hexaphosphate (IP6) in variable amounts. It is considered to be antinutritional due to its effects on reducing the absorption of micronutrients in human and animal diets. Phytic acid has a significant role in inhibition of Fe absorption which can

be increased four to five-fold by reducing the phytate level in grains (Hurrell et al., 1992). Another study showed that phytic acid is a proactive component that chelates metal ions, thus helping to reduce Fe-mediated colon cancer and blood pressure (Zhou & Erdman, 1995). However, though many components can influence the quality of human and animal diets, to attract people to consume more pulses, it is essential to reduce antinutritional components.

2.5. Iron biofortification

WHO (2018a) defined biofortification as "the process by which the nutritional quality of food crops is improved through agronomic practices, conventional plant breeding, or modern biotechnology". One advantage of biofortification over conventional fortification or supplementation is that the former can reach populations where the latter two activities are difficult to implement and/or have limits. To enrich the nutritional quality of staple crops such as rice, wheat, maize, and common bean it is imperative to supplement the essential micronutrients. Biofortification can help to increase the micronutrient level in the edible part of the staple foods, which can improve the nutritional health of micronutrient deficient populations (Bouis & Welch, 2010).

Biofortification research over the last two decades was focused on use of conventional plant breeding and other modern genetic technologies. Genes conferring regulation of Fe uptake in food crops are now identified using molecular, genetic and biochemical techniques. Kobayashi & Nishizawa, (2012) reviewed representative genes that are responsible for Fe deficiency in both monocot and dicot plants. These genes affect Fe uptake, translocation, subcellular translocation, and regulation in response to Fe shortage or excess at the molecular level. Nestel et al., (2006) summarized the multiple advantages of biofortification of staple food crops. The authors mention that (i) biofortification can capitalize on the micronutrients in daily diets or staple foods of low

income people, and a onetime investment to develop genetically improved micronutrient rich seed can allow people to produce seed by themselves so it will be cost effective; (ii) biofortified crops will be sustainable; (iii) biofortified crops are more readily available than the commercially fortified foods, so it can target people living in both suburban and the remote areas; (iv) biofortification is an environmentally feasible method and breeding to increase higher micronutrient component will not incur a yield penalty.

2.5.1. *Using gene bank germplasm for biofortification*

Availability of suitable genetic resources of any crop are important for initiation of any breeding program that involves creating variation followed by selection of desirable phenotypes. The main goal to use the genetic resources to achieve optimum yield and resistance to abiotic and biotic stresses. Considerable diversity is observed in lentil germplasm collections conserved *in situ* at different national and international germplasm banks around the world. Crop Trust, (2017) has recorded 43214 accessions in different gene or institutions of 41 countries including International Centre for Agricultural Research in the Dry Areas (ICARDA), the Australian Temperate Field Crops Collection in Australia and the Seed and Plant Improvement Institute, Iran. The collections include wild relatives, landraces and breeding materials developed by using germplasm from the genus *Lens*. The University of Saskatchewan has received landraces and wild lentil accessions from the gene banks of ICARDA and the USDA. These have been incorporated into the lentil breeding program to develop recombinant inbred lines (RILs), advanced backcross populations and new varieties. Recent, some accessions of *Lens lamotte*i were found to have potential to take up higher amounts of micronutrients such as Fe and Zn from soil (Da. It could be worthwhile to use the broad genotypic variation that is present in the landraces and wild accessions in future

breeding program. Breeding has been initiated to develop RILs to further investigate introgression of genes into cultivated lentil for developing lentil germplasm with improved ability to take up Fe.

2.5.2. *Molecular marker and QTL associations for Fe uptake in lentil*

QTL linkage mapping and association mapping techniques are used to identify molecular markers associated with desired traits. Selection using molecular markers tagged with specific traits could help to develop effective breeding programs for new varieties of interest to end-users. Genetic variation for micronutrients such as Fe, Zn and Se are available in both cultivated and wild species (Khazaei et al., 2017; Kumar et al., 2013; Thavarajah et al., 2011). Development of molecular markers or QTL for Fe concentration could accelerate lentil breeding for this objective.

QTLs and candidate genes for Fe concentration have been identified in different crops, mostly in rice which has a fully sequenced genome. Anuradha et al., (2012) identified 14 QTLs and 10 candidate genes for both Fe and Zn concentration in rice. (Peiffer et al., 2012) identified QTLs explaining 70% of the genetic variation for Fe efficiency in soybean (*Glycine max*). In lentil, four QTL regions were found to be distributed across two linkage group (LG2 and LG5) for seed Se concentration (Ates et al., 2016). Blair et al., (2010) reported a set of across-site overlapping Fe and Zn QTL on linkage group b06 of a Mesoamerican common bean (*Phaseolus vulgaris* L.) population. Limited research has been done to measure the QTL for Fe uptake in lentil. The first high density linkage map was constructed using genotyping by sequencing and mapped QTL for seed Fe uptake in lentil by Aldemir et al., (2017). A recently initiated lentil genomics project is characterizing global lentil germplasm from all over the world and screening it under a wide range of environmental conditions. This may lead to development of some functional markers associated with desirable nutritional traits.

2.5.3. *Marker-trait association studies of seed Fe concentration in lentil*

Two different strategies, linkage analysis or QTL mapping, and association mapping (AM) have been used widely by plant geneticists and breeders to associate desired traits of interest with molecular markers. The AM approach is the more promising tool, using modern genomic technologies to exploit natural diversity through assessment of historical and evolutionary recombination events that occur at the population level (Nordborg & Tavaré, 2002; Zhu et al., 2008). Association mapping also helps with selection of molecular markers that can inherit with or associate with the trait. This helps the breeder to select genotypes or predict the phenotype of a particular genotype before going to the field (Fedoruk, 2013). Yu & Buckler (2006) reported three advantages of AM over linkage analysis. It provides much higher mapping resolution, it uses greater allele numbers and broader reference populations, and ultimately, it reduces research time.

Candidate gene association mapping and genome-wide association mapping (GWAS) are two broad categories of association mapping reported in the literature. The former helps to detect polymorphisms of selected candidate genes responsible for controlling phenotypic variation of a specific trait, whereas GWAS is a more comprehensive approach that systematically searches the whole genome to find the signals for various complex traits (Zhu et al., 2008).

2.5.4. *Association mapping for Fe concentration in other crops*

Some recent studies that have been conducted to identify the marker-trait association for micronutrients including Fe in different crops is summarized in Table 2.2.

Table 2.2. Marker-trait associations for Fe concentration in different crops

Crop	Marker type/ total markers used	Trait/s	Marker trait association	Reference
Maize	457,650 SNPs	Fe deficient and Fe sufficient regions	18 and 17 significant SNPs found associated in Fe deficient and Fe sufficient regions	(Benke, Urbany, & Stich, 2015)
Rice	143 markers including 100 simple sequence repeats (SSR) markers	Content of 5 minerals in whole grain (including Fe)	Three QTLs were identified for Fe concentration	(Y. Huang et al., 2015)
298 Barley landraces	7842 SNP markers	Grain Fe concentration	No QTL was reported	(Mamo, Barber, & Steffenson, 2014)
219 Brown rice accessions	155 SSR markers	8 macro and micronutrient concentrations including Fe	155 SSR markers. The highest number of markers (16) were detected for Fe concentration.	(Nawaz et al., 2015)

2.5.5. *Association mapping studies related to Fe accumulation in legumes including lentil*

Several studies report association analysis in various legume crops with the goal of identifying the association between the marker and specific traits related to mineral micronutrients. Most of the research is AM to identify Fe deficiency chlorosis loci in soybean. Wang et al., (2008) reported two significant associations (Satt 114 and Satt 239) with Fe deficiency chlorosis in soybean. Diapari et al., (2014) identified 8 SNP loci associated with Fe and Zn concentration in a

set of 94 diverse chickpea germplasm with 1186 SNPs. A large-scale set of 16591 SNPs was used in a genome-wide association study of 92 desi and kabuli chickpea accessions (Upadhyaya et al., 2016). The results showed 16 loci associated with seed Fe and Zn concentration. Studies of marker-trait associations for Fe concentration in lentil are limited. Fedoruk, (2013) observed associations using a GLM model for four different traits and found 30 different associations for three of them, including 15 associations for seed diameter, 9 for seed plumpness and six for seed thickness. No associations were observed for flowering date.

2.6. Iron fortification

Several approaches have proven potential to address micronutrient malnutrition. All have limitations depending on sociocultural and economic factors, including the age and gender of the target population (Northrop-Clewes, 2013). Some approaches are long term, such as increasing micronutrient status in staple food crops using modified agronomic approaches, and food-based techniques including food fortification, micronutrient supplementation, and dietary diversification. Other approaches, such as nutrition education, public health interventions and food safety measures also play a role in reduction of micronutrient malnutrition. All of these approaches can be used individually or in combination be applied to address micronutrient deficiency in a target population (Northrop-Clewes, 2013).

2.6.1. *Fortification*

Food fortification with micronutrients is a rapid and cost-effective way to increase micronutrient intake or to mitigate the micronutrient deficiency. Fortifying complementary foods is a cost-effective and sustainable approach to provide micronutrients to a target population without changing their food habits (Northrop-Clewes, 2013). Various foods or food products have

been fortified to enrich or improve micronutrients intake levels and are used in different regions of the world to target specific health problems. Examples include Fe-fortified cereals to correct anemia, and vitamin-D fortified milk to prevent rickets disease (Bishai & Nalubola, 2002). The US Agriculture and Consumer Protection department and FAO of the United Nations state 10 general principles for addition of nutrient to foods in a published technical Consultation on Food fortification, Technology and Quality Control (1995). A recent report indicated that food fortification with various micronutrients and vitamins was mandatory by legislation in 84 countries (Food Fortification Initiative, 2015). Several studies prove that fortification with Fe can improve the Fe status in humans. An example is a systematic review revealing that fortification with micronutrients including Fe significantly increased serum Fe concentrations with no significant adverse effect on hemoglobin levels (Das et al., 2013).

2.6.2. *Fortificants used for fortification*

Several Fe fortificants are approved for use to improve Fe status, including ferrous sulfate, ferrous fumarate, sodium iron EDTA, ferrous orthophosphate, etc. A successful Fe fortification program depends on the choice of a complementary food vehicle, choice of Fe fortificant, and absorbability of the added Fe. Obstacles such as safety, technological and economical consideration also require consideration (Haas & Miller, 2006). Moreover, Fe interacts with food constituents and develops undesirable organoleptic changes that influence consumer acceptability of Fe-fortified food. The wide variety of Fe fortificants used as food fortificants are divided into three broad categories on the basis of solubility (Hurrell, 2002a; WHO & FAO, 2006) as follows: (i) water soluble, (ii) poorly water soluble but soluble in dilute acid, and (iii) water insoluble and poorly soluble in dilute acid. The water-soluble compounds/fortificants are widely accepted due to their high relative bioavailability, but the negative relationship between relative Fe

bioavailability with other undesirable changes (Hoppe et al., 2008) is a constraint to their use as fortificants. The most widely used water-soluble Fe fortificant is $FeSO_4$ due to its ease of application in dry foods and its lower cost. But $FeSO_4$ can also cause rancidity and off-color development. The lowest adverse effects on sensory attributes in food are developed from Fe compounds that are insoluble in water and poorly soluble in dilute acid (category 3), such as ferric phosphate compounds and elemental Fe. The most widely used Fe fortificant for legumes and cereals is NaFeEDTA because of some specific properties compared to other Fe fortificants (discussed in section 7.4).

Nineteen American countries now have national fortification programs in which at least one widely consumed food is fortified with Fe and other micronutrients (Dary et al., 2002). Different Fe compounds are suggested as the most suitable fortificants for specific food vehicles, such as wheat flour, corn flour and masa, different cereal-based complementary foods, dairy products, rice, cocoa products, soy sauce, salt (WHO & FAO, 2006). For instance, anhydrous ferrous sulfate is considered suitable to fortify low extraction (white) wheat flour and degermed corn flour, while NaFeEDTA is used for high extraction wheat flour, corn flour, and corn masa flour. No fortificants are reported for pulse crops like lentil.

2.6.3. *Use of NaFeEDTA as a food fortificant*

Sodium iron EDTA (NaFeEDTA) is a widely used, water-soluble Fe fortificant that has stability during processing and storage (WHO & FAO, 2006). NaFeEDTA is also preferred for use in fortifying foods that contain phytic acid because at lower pH, EDTA works as a chelating agent and prevents Fe from binding to phytic acid and some phenolic compounds. This can increase Fe absorption from food and from the food fortificant (Hurrell et al., 2000; Davidsson et al., 2002;

International Nutritional Anemia Consultative Group, 1998). Among the different fortificants used to increase Fe concentration in foods, NaFeEDTA was reported to be 2-4 times more effective for achieving higher absorption of Fe compared to $FeSO_4$ and ferrous fumarate (Hurrell et al., 2000). The authors suggested that combining Na_2EDTA with $FeSO_4$ in a 1:1 molar ratio can increase the absorption of Fe from $FeSO_4$. Thuy et al., (2003) reported NaFeEDTA to be a promising cost effective, water soluble and highly bioavailable Fe fortificant that improved Fe status of Vietnamese woman who had consumed NaFeEDTA-fortified fish sauce for 6 days $week^{-1}$ (10 mg Fe day^{-1}) for 6 months. The authors also reported that prevalence of Fe deficiency and Fe deficiency anemia were reduced from 62.5% to 32.8%, and from 58.3% to 20.3%, respectively, in the Fe- fortified group compared to the control group.

A significant improvement of Fe storing and Hb level increases were observed after intervention of NaFeEDTA in a semi-rural Guatemalan population (Viteri et al., 1995). A study by Viteri et al., (1978) in 7 children and 98 adults with three Fe fortificants ($(Fe_2(SO_4)_3$, NaFeEDTA and ferrrous ascorbate) revealed that NaFeEDTA was 2-3 times more effective than $Fe_2(SO_4)_3$ when added solely in the meal, due to its adequate bioavailability and higher tolerance to inhibitors present in the food. Another report showed that consumption of NaFeEDTA-fortified fish sauce significantly increased the amount of Hb and serum ferritin after providing it to Fe-deficient anemic school children in Cambodia (Longfils et al., 2008). Lena Davidsson, Kastenmayer, & Hurrell (1994) revealed no significant negative effect of NaFeEDTA-fortified bread (5 mg Fe/day) consumption on Zn and Ca, and that NaFeEDTA may also increase Zn absorption and Fe bioavailability. Another study by Davidsson et al., (1998) showed no influence of absorption or urinary excretion of Mn after consuming NaFeEDTA fortified foods. Li et al. (2015) reported that NaFeEDTA fortified soy sauce did not affect Zn bioavailability in children.

Vitamin C helps with absorption of Fe from fortificants. Trinidad et al., (2014) showed improvement of the Fe status of children after receiving NaFeEDTA fortified hot beverages, and absorption was increased by 1.5% by receiving additional vitamin C with the beverages. Chang et al., (2012) reported that Fe absorption was increased by using a mixture of $FeSO_4$ and NaFeEDTA instead of using NaFeEDTA or $FeSO_4$ alone.

2.6.4. *Sensory evaluation of Fe fortified foods*

Sensory analysis started in the mid-19th century, and it is considered a multidisciplinary science composed of different knowledge areas such as food science, psychology, statistics, human physiology, sociology and food preparation knowledge (Cruz et al., 2010; Moskowitz & Hartmann, 2008). Three major types of sensory evaluation techniques are generally used by the food industry to evaluate fortified foods or new processed foods. These are (i) descriptive testing, (ii) discriminative testing and (iii) consumer effectiveness testing. These tests are selected based on their primary purpose and most valid use. Selection of testing methods for food product evaluation should be appropriate for answering the questions under investigation (Lawless & Heymann, 2010). Sensory measurements of characteristics of any food product should be done very carefully by following an impartial presentation of the samples to the subjects, eliminating response biases and using an appropriate method that can help to demonstrate the consumer or panelist ability for evaluation (Jeannine, 2009).

The success of fortification programs depends on consumer acceptability of the fortified food. Some natural food components such as anthocyanins, tannins, and flavonoids can react with Fe to cause rancidity and other flavor changes (Bovell-Benjamin & Guinard, 2003). For instance, ferrous salts are more soluble and reactive with food components compared to ferric salts (Richardson, 1990). There is an obvious challenge for food fortification if the use of highly

bioavailable Fe results in off-color and off-flavor development attributable to catalytic degradation of vitamins and lipid oxidation (Mellican et al., 2003). Polyphenols containing ortho-hydroxyl groups react with ferric iron and develop off-color (Mellican et al., 2003). Sensory evaluation can help to determine the factors that affect the flavor of foods or drinks, and ultimately, the acceptability to and preferences of consumers.

2.6.5. *Bioavailability of Fe in humans*

Bioavailability of Fe is the key determinant that affects the success or failure of Fe status improvement programs that use dietary intervention (Fairweather-Tait & Teucher, 2002). In humans, bioavailability represents the efficiency of the nutrient that is used to improve nutrient status (Wienk et al., 1999). Several individual factors, such as present Fe status, pregnancy, nutritional deficiencies, genetic disorders and disease status can influence the bioavailability of non-heme or plant-based Fe (Hallberg 1981; Hurrell and Egli 2010). Usually, plant-based foods have poor Fe bioavailability compared to animal-based foods due to the presence of Fe absorption inhibitors such as phytate (Gibson et al., 2010). The primary source of non-heme Fe is the complementary foods which are a mixture of cereal grains and legume seeds. These two food groups have high levels of phytic acid which is considered a potential inhibitor of Fe (Hurrell, 2003).

2.6.6. *In vitro models for assessing Fe bioavailability*

Bioavailability of Fe mainly depends on the form of Fe in the diet. Plant-based food primarily exists as non-heme Fe (Hoekenga et al., 2011) and its solubility is a significant factor influencing its bioavailability. An increase of Fe concentration does not necessarily increase Fe bioavailability. Therefore, it is important to assess the bioavailability of Fe before recommending

any food or food products in a diet. Four *in-vitro* methods are used to determine the bioaccessibility of Fe - solubility, dializability, the gastrointestinal model and the Caco-2 cell model (Etcheverry et al., 2012). The authors discussed the protocol, advantages and limitations for each one of the methods. In brief, before assessing the bioavailability of Fe, an *in-vitro* digestion is conducted to simulate the human digestive system via either a two-step or three-step digestion. Afterwards, the digested food samples are used to measure the Fe bioaccessibility using solubility, dializability or gastrointestinal models. Bioavailability can be assessed by determining the Fe uptake, transport, or both by Caco-2 cells.

2.6.7. *Estimation of Fe bioavailability using Caco-2 cell culture*

Caco-2 cells are the human epithelial cell line that was derived from a human colonic adenocarcinoma (Etcheverry et al., 2012). These Caco-2 cell lines have been used for a few decades as a model for studying intestinal human Fe uptake (Alvarez-Hernandez et al., 1991). These cells can express several biochemical and morphological characteristics of small intestinal enterocytes (Pinto et al., 1983; Sambuy et al., 2005). Glahn et al., (1996) developed a model to assess Fe bioavailability from food by combining simulated peptic and intestinal digestion followed by Fe uptake measurement using Caco-2 cell monolayers.

2.6.8. *In-vivo models for assessing Fe bioavailability*

Experiments using animals or other living organisms are referred as *in-vivo* techniques. The *in-vivo* methods require more ethical considerations compared to the *in-vitro* methods. The *in-vivo* methods are sometimes used as part of a validation procedure for the *in-vitro* methods (Tako & Glahn, 2010). The *in-vivo* model is the most appropriate model for estimation of Fe bioavailability is humans, although it is more expensive and time-consuming (Dias et al., 2017).

Various animals such as rabbits, chickens, and pigs are used in micronutrient bioavailability studies (Liu, 2014). For *in-vivo* techniques, subjects are fed with the experimental diets for a specified period, and a blood sample is collected to assess the hemoglobin repletion efficiency (HRE), which is an indicator of iron bioavailability (Dias et al., 2017; Patterson et al., 2008). Iron bioavailability of biofortified foods using *in-vivo* techniques was reviewed in a recent article by (Dias et al., 2017). The *in-vivo* study designs reported by the authors included the rat model using the haemoglobin depletion-repletion method, the poultry model via haemoglobin maintenance efficiency, a human model using stable isotope in women with low Fe status, intervention studies with preschool children using ferritin and haemoglobin analysis, and randomized controlled, double-blind, longitudinal, intervention trials with anemic and non-anemic women. According to (WHO & FAO, 2006) blood hemoglobin level and serum ferritin status are commonly and reliably used to assess Fe status in anemic and iron deficient populations. Other indicators such as transferrin receptor, and transferrin saturation have also been used for all population groups (WHO & FAO, 2006).

Among the animal models, the pig models are usually preferred due to the similarity of gastrointestinal anatomy and physiology between pigs and humans (Tako et al., 2009). The authors also reviewed and suggested use of the pig model in bioavailability studies because (i) pigs are omnivorous and the digestive and metabolic processes in pigs are similar to those of humans, (ii) pigs readily consume diets that are similar to common human diets in resource-poor regions of the world and (iii) young pigs show Fe deficiency symptoms just after their birth unless they are given Fe injections.

2.6.9. *Improvement of Fe bioavailability*

Bioavailability of Fe is highly influenced by phytate and by some Fe-binding polyphenols present in legume crops including lentil. In legumes, phytate content is higher and located in the protein bodies in the endosperm (Sandberg, 2002). Seeds of soybean, red kidney bean, pea, and lentil have phytate-phosphorus within a range of 0.28–0. 63, 0.34–0.58, 0.06–0.33 and 0.08–0.30 g/100g, respectively (Reddy, 2001). Diets with low phytate or no phytate can help increase absorption of Fe from food. Degradation of phytate can help to make Fe more bioavailable. Hurrell et al., (1992) found significantly higher Fe absorption when phytate was degraded by adding a microbial phytase preparation in soy infant formula. Some polyphenolic compounds that inhibit Fe absorption were degraded by enzymes during processing (Reddy, 2001).

2.7. Research perspective

The Crop Development Centre of the University of Saskatchewan has conducted research on various aspects of pulse crop lentil biofortification for a decade. The primary objective of this research was to improve nutrient status in lentil seeds. CDC has a number of lentil cultivars with comparably higher amounts Fe, Zn, and Se in comparison to cultivars from other lentil growing areas of the world. Moreover, Canada is producing and exporting the largest amount of lentils to the world because of increased demand from consumers. Wild species of the genus *Lens* have proved to be a good source resistance for various diseases compared to the cultivated species. But little is known about Fe and other micronutrients status in wild lentils. Some preliminary work showed genotypes from some wild species have significantly higher concentration of Fe and other micronutrients than the cultivated genotypes. Research work to transfer potential genes that may confer higher seed micronutrient uptake is also a research topic at the CDC. The lower bioavailability of nonheme Fe from plant-based sources, and the high costs of developing and

marketing new varieties with higher micronutrient content have made the biofortification program of limited use to consumers so far. Compared to biofortification, a fortification program can overcome the limitations mentioned above. To our knowledge, there are no reports of efforts to fortify lentil or other grain legumes to improve micronutrient status. Success has been achieved for fortification of wheat flour, soy sauce, water, milk or milk products, rice, and edible oils using micronutrients and vitamins. Fortification of lentil with Fe is the first step in the attempt to improve the Fe status of lentil, a food that is in high demand for consumption on a regular basis in most of the South Asian countries. Both biofortification and fortification programs, in combination, have potential to improve the Fe status of lentils to help mitigate Fe deficiency of vulnerable people.

Prologue to Chapter 3

From the literature review (Chapter 2) it was revealed that both biofortification and fortification can help to improve the micronutrient concentration of lentil. Biofortification can be done using both genetic and agronomic methods. Genetic biofortification can be achieved by both conventional and transgenic approaches. In the following chapters, studies using both biofortification and fortification strategies to improve Fe content and bioavailability will be reported. For both biofortification and fortification approaches, appropriate measurement of Fe concentration is an important step. Fe concentration is usually measured by first using digestion of seeds, followed by different analytical techniques including spectrometry. The digestion procedure is a destructive method that requires digesting the samples to extract the Fe. Compared to the cultivated species, the productivity of the wild *Lens* species is reduced. Determining the minimum amount or number of seed that can be used to produce precise estimates of Fe is the foremost objective of conducting large-scale experiments with many genotypes and populations. Validation of a quick and simple technique is required to estimate Fe concentration using F-AAS in whole lentil seed. In consideration of this, the first study was undertaken taken to optimize seed sample size for Fe analysis of both wild and cultivated lentil using F-AAS.

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CHAPTER 3

OPTIMIZING IRON ANALYSIS OF SEEDS OF CULTIVATED AND WILD LENTIL BY F-AAS

3.1. Introduction and objectives

Iron (Fe) is an essential element for all forms of life on the planet. Fe plays a significant role in normal growth, development and reproduction in plants and animals. Fe also plays an important role in all fundamental mechanisms in plants such as photosynthesis, respiration and metabolism due its role as a constituent of enzymes (Vigani, 2012) or as an electron donor in the electron-transport chains of photosynthesis and respiration (Connolly & Guerinot, 2002). Plants have a transport mechanism to take up Fe from soil to different plant parts, a process knows as Fe homeostasis. The most important edible part of most of the cereal and legume plants is the seed where accumulated Fe is stored.

Various analytical techniques are available for determining the concentration of trace metal elements in plant tissues. The three most available techniques are 1) flame atomic absorption spectrometry (F-AAS), 2) inductively coupled plasma mass spectrometry (ICP-MS) and 3) inductively coupled plasma atomic emission spectrometry (ICP-AES). F-AAS is the most commonly used method for micronutrient analysis in clean and complex matrix samples (Abarca et al., 2001) because of its lower cost, easier accessibility and analytical performance. Compared to other time-consuming and laborious digestion procedures, the HNO_3-H_2O_2 digestion procedure is the most frequently used digestion procedure for trace element analysis.

Wild relatives of crop species have been a valuable source of resistance to abiotic and biotic stresses (Tullu et al, 2010). Wild lentil species are increasingly being used to expand available genetic diversity in cultivated lentil. Wong et al., (2015) recently classified lentil species into four

gene pools using genotyping by sequencing. *Lens culinaris*, *L. tomentosus*, and *L. orientalis* were considered the primary gene pool, *L. lamottei* and *L. odemensis* as the secondary gene pool, *L. ervoides* into the tertiary and *L. nigricans* into the quaternary gene pool. Genetic resources of wild lentil species originating from different parts of the world revealed high variation in seed Fe concentration (Sarker et al. 2007; Kumar et al. 2014; Karaköy et al. 2012). The amount of micronutrients in lentil seeds depends on growing location, genotype, and genotype by location effects. Karaköy et al., (2012) found Fe concentration in Turkish lentil landraces ranged from 64-81 mg kg^{-1}. Thavarajah et al., (2011) reported Fe concentration in Canadian lentil cultivars ranged from 73-90 mg kg^{-1}. Sarker et al., (2007) reported a wide range of variation in total Fe concentration from 41-109 mg kg^{-1} in 1200 lentil genotypes including breeding lines, landraces and wild lentil species.

Lentil seed size varies across the species of *Lens* taxa, with significant size and weight difference among genotypes from the different centres of origin. Canadian lentil cultivars generally have greater seed weight compared to South Asian cultivars and wild lentil progenitors. On the basis of seed size, Barulina, (1930) classified cultivated lentils into two sub-species, microsperma (small seeded) and macrosperma (large seeded) which were considered two different lentil biotypes. Ferguson & Robertson, (1999) studied the morphological and phenological variation of 310 accessions of wild *Lens* taxa from the ICARDA germplasm collection. They reported that for cultivated lentil 100-seed weight (HSW) ranges from 1.6-10.1 g and wild lentil accessions had much lower 100 seed weight than the *Lens culinaris* laboratory standard. Wild lentil seeds and seeds of their interspecific hybrids are often difficult to produce, however, and are available only in small quantities from seed resources, making it difficult to assess micronutrient levels.

Canadian lentil production includes up to ten market classes including small red, extra small red, large red, small green, extra small green, medium green, large green, French green, green cotyledon and Spanish brown as sub-classifications of the three major market class groups (green, red and specialty market classes) (Saskatchewan Pulse Growers, 2017). Green lentils are usually large (>6g/100 seeds) with green seed coats and yellow cotyledons (Erskine, 1996). Red lentil typically has brown to gray seed coats with seed weight <3.5g/100 seeds and is consumed after dehulling to prepare a dish known as "dhal."

This study was initiated to assess the concentration of Fe in lentil genotypes available at the Crop Development Centre, University of Saskatchewan, Canada. Micronutrient concentration measurement is destructive and therefore, the specific goals of this experiment were (i) to determine the minimum amount of seeds required for precise estimation of Fe concentration in lentil seeds by F-AAS and (2) to validate a quick and simple analytical method for the estimation Fe concentration in whole lentil seeds. To our knowledge, this experiment is the first to identify the minimum amount of wild and cultivated lentil seeds necessary to analyze the accurate concentration of Fe in lentil seeds by using F-AAS, the most accessible and inexpensive analytic technique.

3.2. Materials and Methods

3.2.1. Apparatus

An electronic seed counter (ESC-1, Agriculex Inc. Guelph, Canada) was used to count lentil seed samples. The seed weight of lentil genotypes was determined by counting 100 seeds (at 12% moisture content) with an electronic balance. Estimations of all metal ion concentrations were performed using an Analytikjena (Jena, Germany), novAA®300 flame atomic absorption spectrometer (AAS) equipped with a computer processor. Deuterium background correction was

used with Fe hollow-cathode lamps as radiation sources. Operating conditions recommended by the manufacturer were used throughout the experiment. To maximise the absorbance signal for each metal burner, height and acetylene-air flow rate were adjusted by aspirating the analyte solution. To maintain discrete volume sampling, a final volume of 100 µl of analyte solution was injected automatically into the flame of the spectrometer through the nebulizer by sample aspiration tubing. Absorbance signals were measured in peak area mode by the spectrophotometer reader. Other instrumental parameters of this spectrophotometer for the estimation of Fe concentration are summarised in Table 3.1.

Table 3.1. Instrumental settings for the determination of Fe concentration by F-AAS

Parameter	Fe
Wave length (nm)	248.3
Slit width (nm)	0.2
Light source	Iron hollow cathode lamp
Power supply (mA)	6
Flame, flow setting (l min^{-1})	Air (6.67), Acetylene (1.08)
Integration time (s)	3
Usable burner height (mm)	6-10

3.2.2. Reagents and solutions

All reagents were analytical grade and distilled and deionized water that was further purified by a Nanopure high purity water (electrical resistivity of 16.0 MΩ cm^{-1}) (Barnstead, Massachusetts, USA). Laboratory glass wares were kept in 10% (v/v) HNO_3 for overnight and subsequently rinsed four times in distilled water followed by oven drying to avoid contamination. Stock standard solutions of Fe (1000 mg l^{-1}) were obtained from VHG, Manchester, USA. Working standard solutions were prepared by appropriate dilution of the standard stock solutions. A

standard solution of Fe was used for calibration. Different concentrations of Fe (0.0, 0.5, 0.1 and 3.0 mg l^{-1}) working standard solutions were used to confirm F-AAS accuracy. The standard stock solutions concentration calibration curves were linear (for Fe, r^2= 0.9993). Concentrated nitric acid, hydrochloric acid and hydrogen peroxide used in the digestion procedure were supplied by Fisher Chemicals and Anochemia, respectively. Four standard reference materials (Tomato leaves (NIST.1573a), Durum wheat (NIST.8436a), Bovine liver (NIST 1577a) and Rice flour (NIST 1568a)) supplied by National Institute of Standard and Technology (NIST, USA) were used as standard to compare.

3.2.3. *Sampling of seeds*

Six wild lentil genotypes (one representing each of the six species of genus *Lens*) and six popular cultivated lentil genotypes (one representative accession from each of the six most important market classes produced in Canada) were used in this study (Table 3.2). Seeds of wild lentils were grown in field at Crop Science Field Laboratory, Saskatoon in 2013. The seed samples of six cultivars were collected from the Lentil Regional Varietal Trial, 2013, at Limerick, Saskatchewan.

Table 3.2. Wild and cultivated lentil genotypes used for optimizing the estimation of Fe concentration in seeds by F-AAS.

Wild *Lens* species and genotypes		Cultivated lentil market classes and genotypes	
Species	Genotype	Market class	Genotype
Lens orientalis	IG 72611	Extra small red	CDC Robin
Lens tomentosus	IG 72643	Small red	CDC Maxim
Lens lamottei	IG 110813	Large red	CDC KR-1
Lens odemensis	IG 72760	Small green	CDC Viceroy
Lens ervoides	IG 72815	Large green	CDC Greenland
Lens nigricans	IG 116024	Green cotyledon	CDC QG-2

3.2.4. Procedure

Fe concentrations (mg kg^{-1}) in whole lentil seeds were measured to assess the validity of proposed digestion and analytical methods. Total Fe concentration in each replicated lentil seed sample was measured using HNO_3-H_2O_2 digestion followed by F-AAS analysis. Whole lentil seed samples were digested using the modified procedure described by (Lintschinger et al., 2000). Whole seed samples were thoroughly washed with distilled-deionized water to remove surface contaminants and then air-dried before weighing separately into 0.1, 0.3, 0.5 and 0.7 g sub-samples which were placed into specific digestion glass tubes (30 ml) of the Vulcan 84 automated digestion chamber (Vulcan 84, Questron Technology, Ontario, CA, USA). Every analysis set consisted of four blanks and four laboratory standards within a set of 84 digestion tubes. Each digestion tube had 6 ml of concentrated nitric acid (HNO_3) injected into it. The digestion plate temperature was raised to 86 $^{\circ}C$ and then samples were allowed to digest for 45 min. Then 5 ml of hydrogen peroxide (H_2O_2) were injected to each digestion tube and digestion continued for 65 min. At this point 3 ml of 6M HCl was added to all tubes. The tubes were left in the digestion chamber for another 5 min to complete the digestion step. Digested samples were cooled for 45 min, followed by volume adjustment to 25 ml with distilled-deionized water at room temperature (22 °C) and then transfer to analysis tubes. Blanks were prepared in the same way but without sample addition. Six ml of digested solution was used each time to determine Fe concentration by F-AAS. Samples, standard working solutions, blanks and standard reference materials were measured by F-AAS under the same instrumental conditions (Table 3.3).

3.3.5. Statistical Analysis

The experiment was set up in a completely randomized design with four replications. Analysis of variance (ANOVA) was used to determine the concentration of Fe variation in

different lentil genotypes using the Mixed Model procedure (PROC MIX) of SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA). Average concentrations were separated by both genotype and sample size using Fisher's protected LSD procedure and level of significance was declared at $P < 0.05$ and 0.01. Contrast statistical analysis was performed using SAS covariance contrast (least squares mean) to compare the different lentil seeds sample sizes with one another.

3.3. Results and Discussion

3.3.1. Method validation

Quality of an analytical method, especially for quantitative analysis is established by its validation. Background knowledge of calibration linearity, accuracy, recovery percentage, precision and detection limit are the main criteria for assessment of methodology for quantitative analysis of micronutrients.

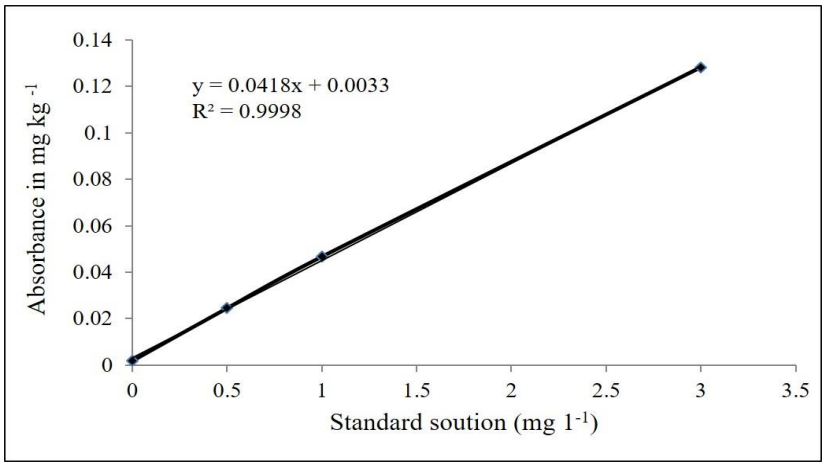

Figure 3.1. Calibration straight line for standard solutions containing 0, 0.5, 1.0 and 3.0 mg l^{-1} of Fe.

Four standard solutions of Fe concentration were employed to study the linearity of absorbance response. The calibration curves for different standard solutions were drawn after setting the parameters of F-AAS (Table 3.3) at optimum levels. A linear relationship was obtained

for Fe by plotting each standard solution concentration (0, 0.5, 1.0 and 3.0 mg l^{-1}) against absorbance of Fe (Figure 3.1).

The accuracy of the analytical method used in the study was assessed by preparing the same quantity of standard reference materials in a similar matrix followed by digestion and quantification of Fe by F-AAS. (Ghaedi et al., 2013) reported relative standard deviation for Fe concentration of about 4% and that recovery above 90% indicates that the analytical method is reliable. In this current study, mean recovery (% R) of Fe for three standard reference materials with certified values from NIST ranged from 90.3-101.1 %) (Table 3.3). Two standard reference materials (Bovine liver (NIST 1577a) and rice flour (NIST 1568a)) and yellow lentil used as laboratory standard. Four different sample sizes (0.1, 0.3, 0.5 and 0.7 g) of two different standard materials along with the laboratory standard (yellow lentil) were compared under the same instrumental conditions. The analysis of the 0.1 g samples was significantly different from the three larger sample sizes for Fe concentration, however, no significant differences were observed in rice flour and bovine liver Figure 3.2.

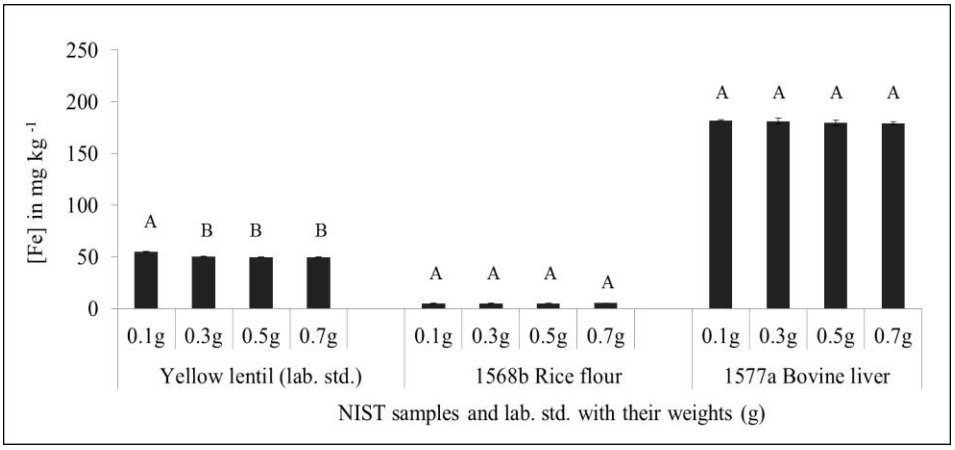

Figure 3.2. Comparisons of Fe concentration in four samples sizes of the lab check and two standard reference materials. Comparisons were made for each standard reference material separately for Fe. Letters above bars indicate significant differences at $P < 0.05$ among different sample weights.

Table 3.3. Accuracy of the method evaluated by comparing the Fe concentration (mg kg^{-1}) of three standard reference materials (certified values) to the average values obtained using the method developed in this study

Standard reference material	Certified values (mg Fe kg^{-1})	Average values (mg Fe kg^{-1})	Recovery (%)	Relative standard deviation (%)
Tomato leaves (NIST 1573a)	368.0±0.7	358.5	97.4	3.1
Durum wheat (NIST 8436a)	41.5±4	41.9	101.1	4.3
Rice flour (NIST 1568a)	7.4±4	6.7	90.3	5.1

Note: Average value of four estimated values of standard reference materials using a similar matrix.

The internal reproducibility and repeatability were measured under same instrumental settings to evaluate the precision of this analytical method. Repeatability of this method was assessed by analysing four different samples (each 0.3 g of CDC Robin) with two replications prepared individually on the consecutive days with the same equipment by the same operator. The relative standard deviation of four different samples prepared by the same operator was 0.3%, indicating acceptable repeatability of this method of analysis.

The internal reproducibility of the method was estimated by analysing two different lots of samples prepared on in four consecutive days by different operators. Four samples from lot 1 were analysed on four consecutive days by the same operator (day-to-day fluctuation) under the same instrumental conditions. The relative standard deviation for day-to-day fluctuation was 1.5% for Fe concentration. Four samples from lot 2 were analysed on two consecutive days by another operator (analyst-to-analyst fluctuation). The relative standard deviation for analyst-to-analyst

fluctuation was 1.5% for Fe (Table 3.4). Both relative standard deviations for day-to-day and analyst-to-analyst fluctuations showed good reproducibility of this analytical method.

Table 3.4. Reproducibility in the determination of Fe concentration with two different lots of samples preparations by two analysts

		Lot 1
Analyst	Day	Fe (mg kg^{-1})
A	1	68.7
A	2	70.2
A	3	71.8
A	4	68.6
Mean		69.8
R.S.D.* (%)		1.5
		Lot 2
Analyst	Day	Fe (mg kg^{-1})
A	5	70.0
A	6	71.2
B	7	68.0
B	8	71.2
Mean		70.1
R.S.D*. (%)		1.5

Note. Fe concentration is the mean of two digested solutions run through the F-AAS *R.S.D.-Relative Standard Deviation

3.3.2. Seed amount optimization for Fe analysis in lentil seeds

Weights of 100 seed samples of each lentil genotype were reported in Table 3.5. Based on the weight of 100 seeds, wild lentil species were subdivided into large-seeded (>1 g per 100 seeds) and small-seeded (<1 g per 100 seeds species (Figure 3.3 (a); 3.3 (b)). Large differences for Fe concentration were observed in both wild and cultivated lentil genotypes (Table 3.5). For the wild

lentil genotypes, Fe concentration ranged from 52-78 mg kg^{-1}. *Lens lamottei* (IG 110813) of the secondary gene pool had the highest Fe concentration and was significantly ($p < 0.05$) different from all other wild genotypes (Figure 3.3 (a & b). However, seeds of *Lens odemensis* (IG 72760) from the tertiary gene pool had the lowest concentration of Fe (Figure 3.3 (a & b)).

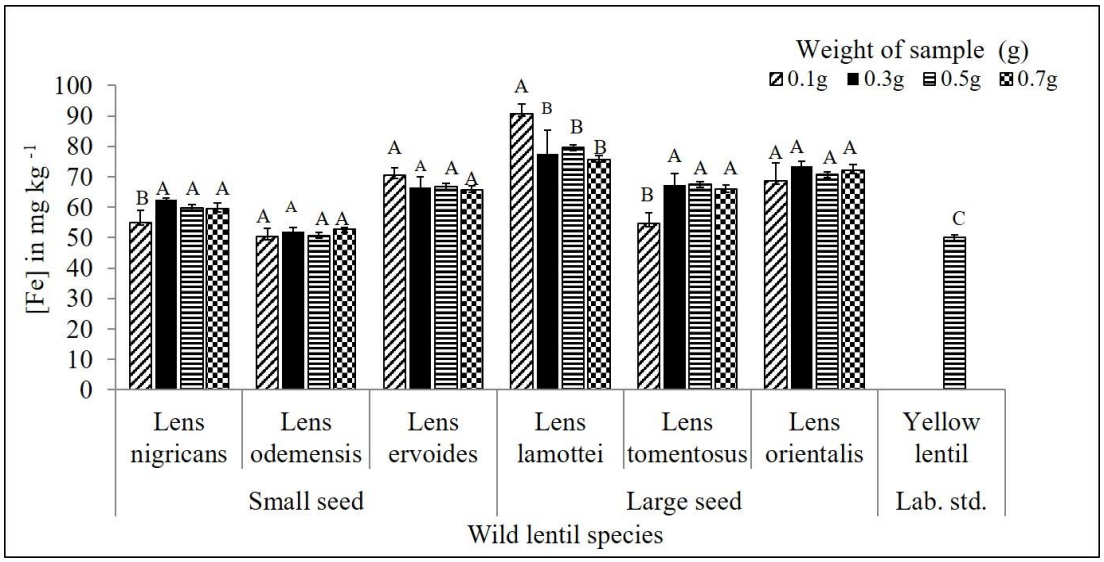

Figure 3.3a. Fe concentration in 4 sample sizes of six wild lentil species. Comparisons were made for each *Lens* species separately. Different letters above bars indicates significant differences at $P < 0.05$ among different sample weights.

For wild lentils, statistical analysis among different seed sample sizes showed 0.1 g of seed samples of *Lens lamottei, L. nigricans* and *L. tomentosus* were significantly different for Fe concentration in comparison to the three larger sample sizes (0.3, 0.5 and 0.7 g). However, other species did not show significant differences in Fe concentration among four different sample sizes (0.1, 0.3, 0.5 and 0.7 g). Therefore, estimation of Fe concentration using the same digestion matrix 0.3 g of seeds from wild lentil species was more precise and reliable. This would help to reduce seed expenses, analysis time and cost rather than analysing Fe concentration separately. Sample

sizes of 21-26 seeds of the larger seeded wild lentils (*Lens lamottei, L. tomentosus* and *L. orientalis*) and 44-61 seeds of small seeded wild lentil (*Lens nigricans, L. odemensis* and *L. ervoides*) were sufficient for reliable determination of Fe concentration in wild lentil using by F-AAS (Table 3.5).

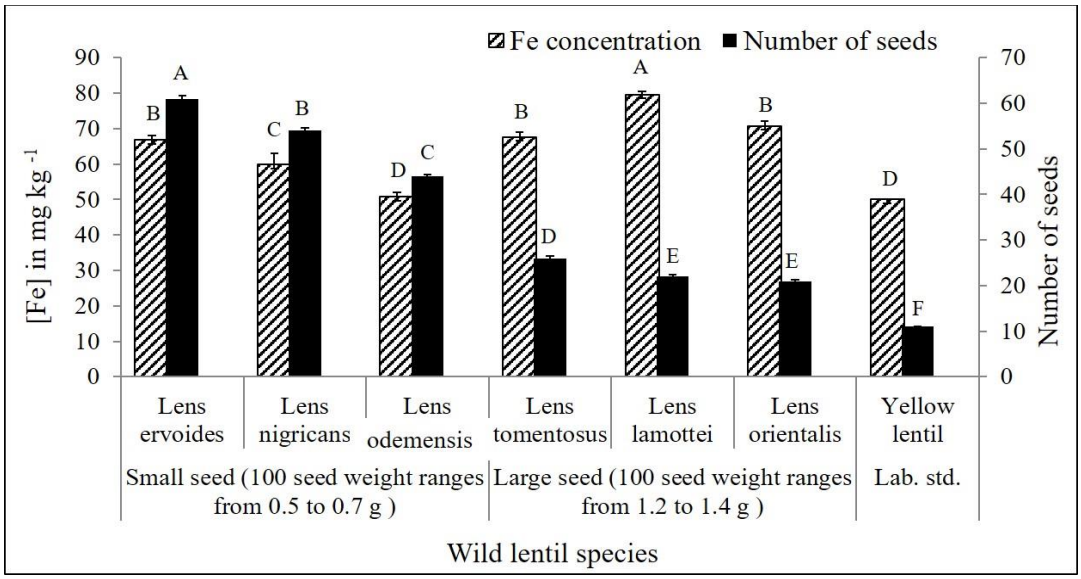

Figure 3.3b. Fe concentration (primary vertical axis) and mean number of lentil seeds (secondary vertical axis) in 0.3 g of different wild lentil species. Different letters above bars indicate significant differences at $P < 0.05$ among different wild lentil species.

Significant Fe concentration differences were also observed in different cultivated lentil genotypes. In the six cultivated lentil genotypes, Fe concentration ranged from 54 - 73 mg kg^{-1}. The small red genotype (CDC Maxim) had the highest Fe concentration and was significantly different ($p < 0.05$) from other cultivated lentil genotypes. The green cotyledon genotype (CDC QG-2) had the lowest concentration of Fe (Figure 3.4(a & b)). Average Fe concentration in different sample sizes of different market classes are shown in Table 3.5.

Table 3.5. Hundred seed weight (g), mean number of seeds in 0.3 g samples, and mean concentration of Fe in genotypes of six wild lentil species and in genotypes of cultivated lentil market classes

Lentil species	Genotype/market class	100 seed weight (g)	Mean number of seeds in 0.3 g	Mean Fe concentration (mg kg^{-1})
Lens orientalis	IG 72611	1.4	21	73
Lens tomentosus	IG 72643	1.2	26	67
Lens lamottei	IG 110813	1.3	22	78
Lens odemensis	IG 72760	0.7	44	52
Lens ervoides	IG 72815	0.5	61	66
Lens nigricans	IG 116024	0.5	54	62
Lens culinaris	CDC Robin/extra small red	2.9	18	67
Lens culinaris	CDC Maxim/small red	3.9	12	73
Lens culinaris	CDC KR-1/large red	5.4	9	63
Lens culinaris	CDC Viceroy/small green	3.1	15	67
Lens culinaris	CDC Greenland/large green	6.9	7	57
Lens culinaris	CDC QG-2/green cotyledon	3.2	17	54

In most cases, the smallest seed sample size (0.1 g) of different cultivated lentil genotypes had significantly higher Fe concentration than all other sample sizes. Contrast statistical analysis among different seed sample sizes from cultivated lentil genotypes revealed that 0.5 g of whole lentil seed was more reliable than 0.3 g seed sample size for precise estimation of Fe concentration. This is likely due to lower number of seeds in the 0.3 g of seed sample size which captures less seed variability than 0.5 g seed sample size of cultivated lentil genotypes. Samples of 7-18 seeds

(0.5 g) were reliable for precise estimation of Fe concentration in cultivated lentil genotypes (Table 3.6).

Table 3.6. Mean seed Fe concentration (mg kg^{-1}) in four sizes of lentil seed samples (0.1, 0.3, 0.5 and 0.7 g) of wild and cultivated lentil genotypes

	Wild lentil species		
Seed sample size (g)	Fe		
	df	Mean concentration (mg kg^{-1})	Pr > F
0.1	92	65	<.0001
0.3	92	67	<.0001
0.5	92	66	<.0001
0.7	92	65	<.0001
	Cultivated lentil genotypes		
Seed sample size (g)	Fe		
	df	Mean concentration (mg kg^{-1})	Pr > F
0.1	92	63	<.0001
0.3	92	62	<.0001
0.5	92	58	<.0001
0.7	92	60	<.0001

Note. Fisher's protected LSD procedure at $P < 0.01$

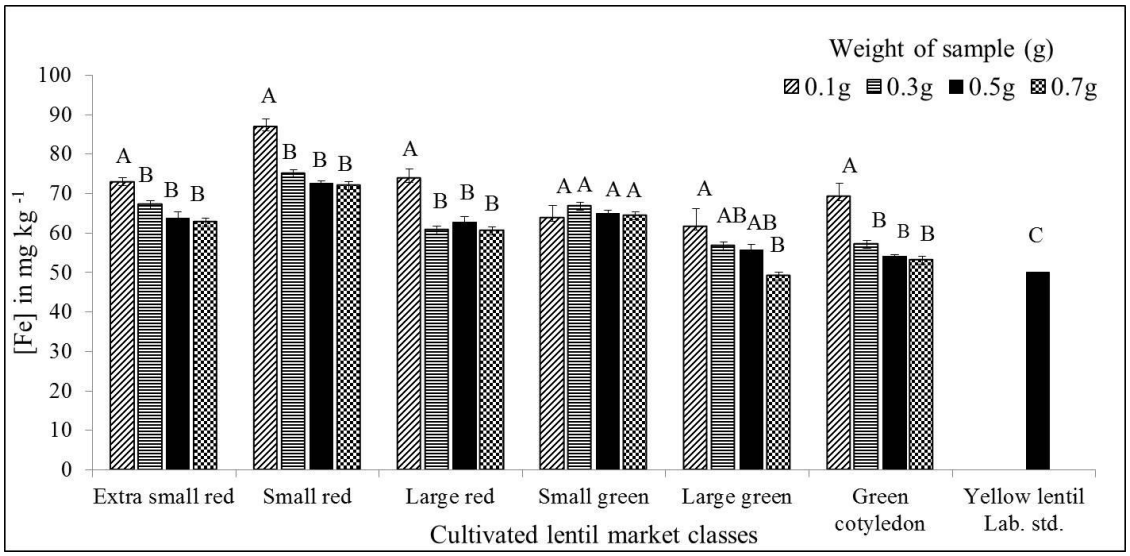

Figure 3.4a. Fe concentration in four t sample sizes of six cultivated lentil market classes. Comparisons were made for each market class separately. Letters above bars indicate significant differences in Fe concentration at $P < 0.05$ among different sample weights.

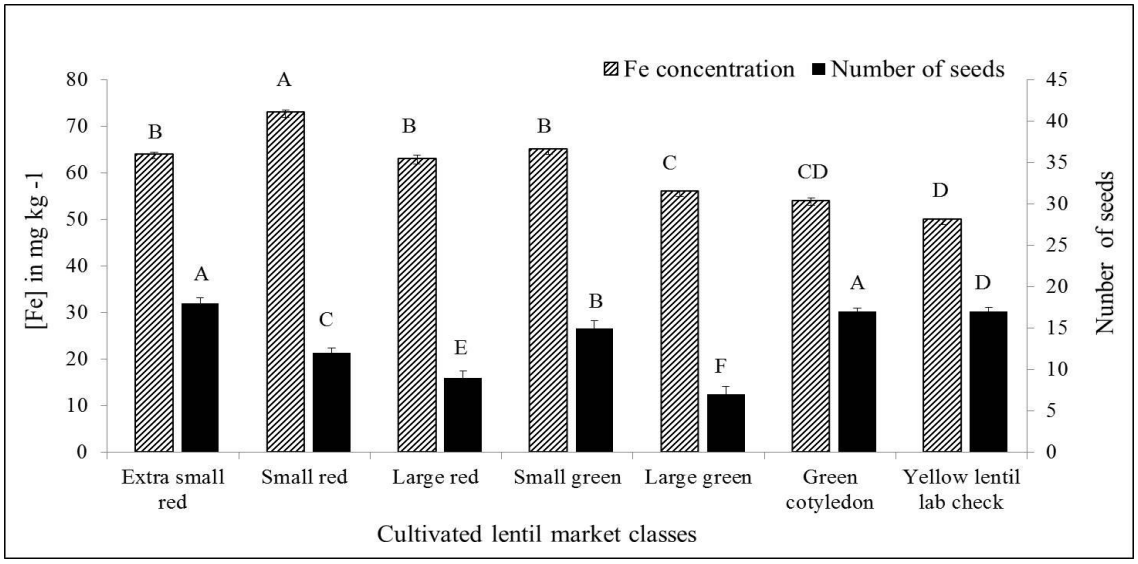

Figure 3.4b. Fe concentration (primary vertical axis) and mean number of lentil seeds (secondary vertical axis) in 0.5 g of six market classes of cultivated lentil. Different letters above bars indicate significant differences in Fe concentration at $P < 0.05$ among different cultivated lentil market classes.

3.4. Conclusions

The method reported here for measuring Fe concentration in whole lentil seed samples indicated that it was possible to accurately determine the amount of Fe in lentil seeds directly by F-AAS without using much seed. Samples as small as 0.3 g of wild and 0.5 g of cultivated lentil seeds provided sufficient minimum sample sizes of lentil seeds for precise and repeatable estimation of Fe from the same seed sample. Since sample preparation described does not require grinding, this procedure is rapid and simple, and therefore useful for routine analysis. In future, genotypes with contrasting Fe concentration could be used to conduct experiments for better understanding of Fe accumulation and homeostasis in lentils, and to investigate methods for developing cultivars with Fe concentration in lentil seeds. These results can be used to minimize the amount of valuable and rare seed used for micronutrient analyses of seed samples of wild lentil species and their interspecific hybrids.

Prologue to Chapter 4

In Chapter 3, we determined the optimum amount of whole lentil seeds required to measure seed Fe concentration using F-AAS for both cultivated and wild lentil species. This result was helpful for planning a subsequent study involving a larger number of wild lentil accessions, including unadapted genotypes that produce very small numbers of seeds, but are important from the standpoint of using them in long term breeding efforts for increasing seed iron Fe concentration through biofortification.

The results from this study clearly showed that small samples of seeds were sufficient to measure Fe concentration. This allowed us to conduct additional studies that estimated the variation of seed Fe concentration during different reproductive growth stages (harvest) of lentil, and the genotype by harvest interaction that can influence the seed Fe concentration in lentil. This study was conducted at the Crop Development Centre of the University of Saskatchewan and is described in Chapter 4.

CHAPTER 4

SEED IRON CONCENTRATION AT THREE GROWTH STAGES IN THREE ENVIRONMENTS FOR SEVEN LENTIL SPECIES

4.1. Introduction

Modern agriculture is increasingly dependent on the use of genetic resources, including landraces and crop wild relatives, to continue to make genetic gains in productivity. The genetic base of current commercial cultivars of many crops has been narrowed due to the high selection pressure during cultivar development. Development of new and improved cultivars with higher yield and resistance or tolerance to biotic and abiotic stresses will increasingly require deliberate efforts to increase useful variability in the available gene pool. The pool of wild relatives and landraces of any crop represents untapped potential reservoirs of genes that influence desirable qualitative and quantitative traits. Among these, micronutrient concentration in crop seeds is gaining prominence due to its increasing importance in human health and nutrition. Fe is an important essential micronutrient for biological systems of both plants and animals. Several processes are involved in acquisition of Fe for plant growth and for storage in seeds that are used as food for humans and animals.

Fe homeostasis in plants is a dynamic process involving proteins and small organic molecules that are essential for the uptake and transport of Fe from soil to different plant organs, and ultimately, for storage of Fe in seeds (Briat et al., 2010). Ferritin is one of the most common forms of non-heme Fe and legume seeds are known as a traditional source of plant ferritin (Zielińska-Dawidziak, 2015). Many environmental factors influence ferritin gene expression which ultimately influences plant ferritin storage in seeds (Briat et al., 2010).

Lentil is an indeterminate plant, and its vegetative growth is continuous under favorable conditions during reproductive stage (Saskatchewan Pulse Growers, 2017). The cultivated and wild species have wide variation in seed size, seed appearance, maturity and many other physiological and morphological traits. The genus *Lens* has one domesticated species (*Lens culinaris*) and six wild species (*L. orientalis, L. tomentosus, L. odemensis, L. lamottei, L. ervoides,* and *L. nigricans*) (Cubero et al., 2009; Wong et al., 2015). Substantial phenotypic variation for plant morphological characteristics is present among species (Cristóbal et al., 2014). Like many other dynamic plant characteristics, Fe accumulation in seeds might vary among reproductive growth stages within and between the species of *Lens.* In this study, we hypothesized that (1) the indeterminate growth habit of lentil influences the duration of seed development during the growing season, and this can influence seed Fe concentration in lentil and that (2) Fe accumulation in lentil seeds among the *Lens* species is influenced by genotype × harvest interaction. The following experimental objectives were considered in the design of experiments that could test the hypotheses.

- To estimate the variation in Fe accumulation in lentil seeds during growth stages of indeterminate growth
- To determine the genotype × harvest timing interaction that influences the Fe accumulation in the seeds of the seven lentil species.

4.2. Materials and Methods:

4.2.1. Selection of lentil genotypes

Fourteen lentil genotypes, including two genotypes from each of the six wild lentil species and two widely grown local cultivated commercial cultivars genotypes, were selected for this study (Table 4.1). All wild genotypes were obtained from the germplasm collection at Crop

Development Centre, University of Saskatchewan, Canada, and were selected based on their previous use in the lentil breeding program for development of intraspecific and/or interspecific RILs for inheritance studies of several traits of agronomic interest.

Table 4.1. Selected twelve wild and two cultivated species from the genus *Lens* used to determine the Fe concentration of seeds that mature at different times

***Lens* species**	**Genotypes**
Lens culinaris	CDC Maxim, CDC Greenstar
Lens orientalis	IG 72611, IG 72643
Lens tomentosus	PI 572390, IG 72613
Lens lamottei	IG 110810, IG 110813
Lens odemensis	IG 72760, IG 72623
Lens ervoides	L01-827A, IG 72815
Lens nigricans	IG 136681, IG 116024

4.2.2. Location and year

This study was conducted in Saskatoon at three University of Saskatchewan locations i.e. Crop Science Field lab (CSFL), in 2014, and at CSFL and the Sutherland (STH) farm in 2015.

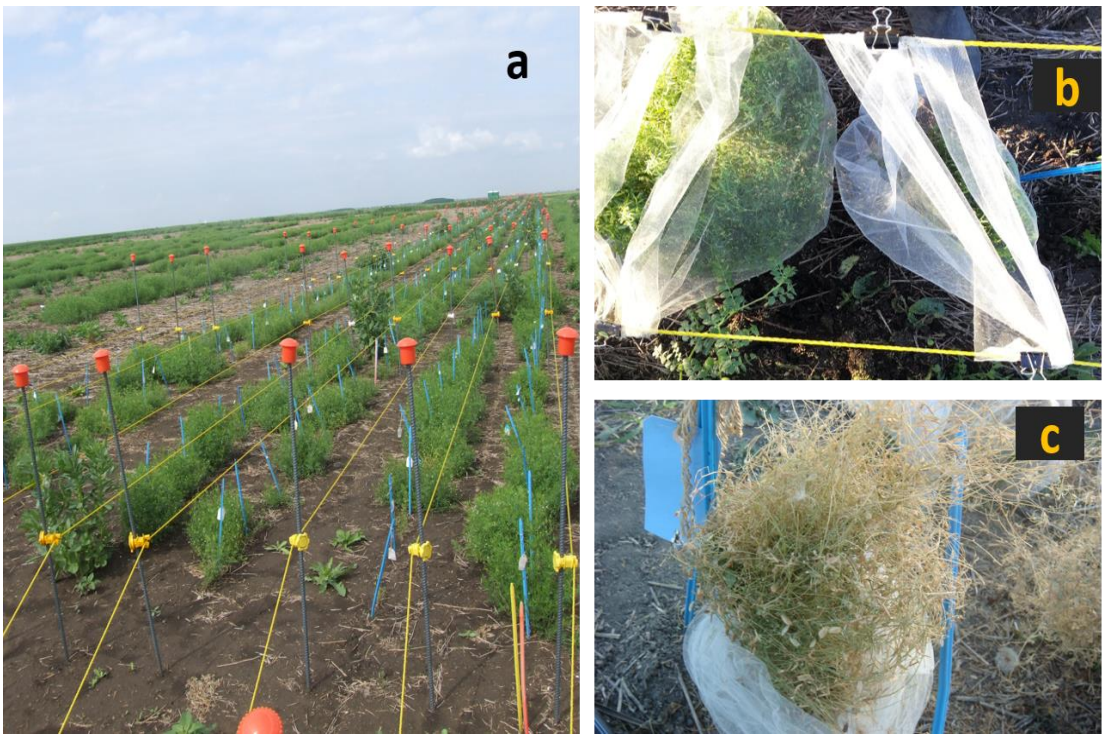

Figure 4.1. Images showing (a) field view of plants at mid-season growth stage in hill plots, (b) plants inside mesh bags used for seed collection, and (c) seed harvesting techniques for wild species genotypes used for the Fe accumulation study.

4.2.3. Seed harvest

Lentil plant growth of all species is indeterminate, and unlike cultivated species, the wild accessions have the dehiscent pod trait that causes seed dispersal at pod maturity. Collection of seeds requires extra care, using techniques to minimize seed loss (Figure 4.1). Every plant from each hill was covered with a mesh bag. The lower end of the mesh bag was tied at the bottom of the plant so that shattered seeds accumulated inside the bag. The top portion of the mesh bag was kept open and tied with nylon rope to hold the mesh bag in an upright position and to provide adequate sunlight and aeration (Figure 4.1).

The experiment was conducted using a randomized complete block design (RCBD) with three replicates in all three site years. Mature seeds from each plant of each genotype were harvested three times during the reproductive growth cycle (Figure 4.2). The first harvest was made after maturation of the 50% of the pods – all mature pods and shattered seeds were harvested. At 10-12 days after the 1st harvest, the 2nd harvest was done by collecting all the mature pods from the same plants. Again, at 10-12 days after the 2nd harvest, a 3rd harvest was made in a similar fashion. All the harvested seed was removed by hand and stored in separate paper envelopes for each genotype, each harvest and each replication. Seeds were stored at room temperature prior to estimation of seed Fe concentration.

Figure 4.2. Three seed harvests stage during lentil seed maturing period.

4.2.4. Seed Fe analysis

Seed Fe concentration (mg kg^{-1}) was analysed using F-AAS following the same procedures described in detail in Chapter 3 of this thesis.

4.2.5. Soil Fe status and weather conditions

The climate data for both years were collected from the Environment Canada (2017) website. The average monthly temperature (˚C) was similar across the three environments from May to August (Table 4.2). Total precipitation (mm) was 65.4 mm higher in 2014 (wetter than average) compared to 2015. Soil samples collected from different parts of each experimental site were analysed by ALS Laboratory Group Agriculture Services, Saskatoon, Canada (Table 4.3).

Table 4.2. Mean temperature (˚C) and total precipitation (mm) for the year 2014 and 2015 growing seasons (May-August) at Saskatoon area.

Climate data	May		June		July		August		Mean		Total	
	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015
Mean temperature (˚C)	10.1	10.1	14.1	17.2	18.3	19.4	17.9	17.4	15.1	16.0	--	--
Total precipitation (mm)	61.1	0.4	94.8	13.6	44.5	84.3	18.5	45.2	--	--	218.9	143.5

Table 4.3. Soil analysis from two field locations at Crop Science Field Lab (CSFL) and Sutherland (STH).

Location	Depth (inches)	Texture	pH	Salinity Rating	Organic Matter (%)	DTPA-extractable [Fe] (mg kg^{-1})
STH	0-6	Clay Loam	6.6	NS	3.6	58.5
CSFL	0-6	Loam	7.5	NS	3.7	45.1

NS, Non-saline

4.2.6. Statistical analysis

All statistical analysis was conducted using SAS version 9.4 (SAS institute Inc., Cary, NC, USA). Mean difference among the three seed harvests of each of the genotype and genotype × harvest interaction was analysed by using repeated statistical analysis and SAS Proc Mixed, respectively. Genotype, and genotype × harvest interaction was considered fixed factors, and replication was nested within location, which was considered a random factor. The statistical analysis was carried out separately for each site year.

4.3. Results

A wide range of variation for seed Fe concentration was observed among the genotypes (Table 4.4). Although seed Fe concentration was significantly ($p \leq 0.001$) different among the 14

genotypes in all the site-years, differences were not significant between harvest stages. The genotype × harvest interaction was significantly ($p \leq 0.001$) different only for the 2014 CSFL location. Unlike 2014, genotype × harvest interactions were not significantly different for the two locations in 2015. Across the three locations, comparing all the genotypes, the highest mean seed Fe concentration was observed in both the *Lens lamottei* accessions, IG 110813 (Fe conc. 97.3 mg kg^{-1}) and IG 110810 (Fe conc. 96.4 mg kg^{-1}) (Figure 4.3). The accession IG 110813 had the highest 123.3 mg kg^{-1} Fe in seeds harvested from the STH location in 2015. *Lens ervoides* had the lowest seed Fe concentration, irrespective of three site-years. The range of Fe concentration in both the cultivated species genotypes (CDC Maxim & CDC Greenstar) from all the locations was 66.3 - 76.8 mg kg^{-1}. Fe concentration was higher in the seeds produced in 2015 (both CSFL and Sutherland) compared to seeds from 2014, for most of the genotypes.

Table 4.4. Analysis of variance with F- values and significance level for Fe concentration (mg kg^{-1}) of 14 genotypes evaluated at three different environments in 2014 (CSFL) and 2015 (CSFL and Sutherland).

Effect	Degrees of freedom	F Value		
		CFSL - 2014	CFSL - 2015	Sutherland - 2015
Genotype	13	22.84^{**}	4.74^{**}	27.97^{**}
Harvest	02	4.24^{NS}	0.56^{NS}	1.46^{NS}
Genotype × Harvest	26	2.76^{*}	0.52^{NS}	1.08^{NS}

Note: **- *significant at $p \leq 0.001$;* *- *significant at $p \leq 0.05$;* ^{NS} - *not significant*

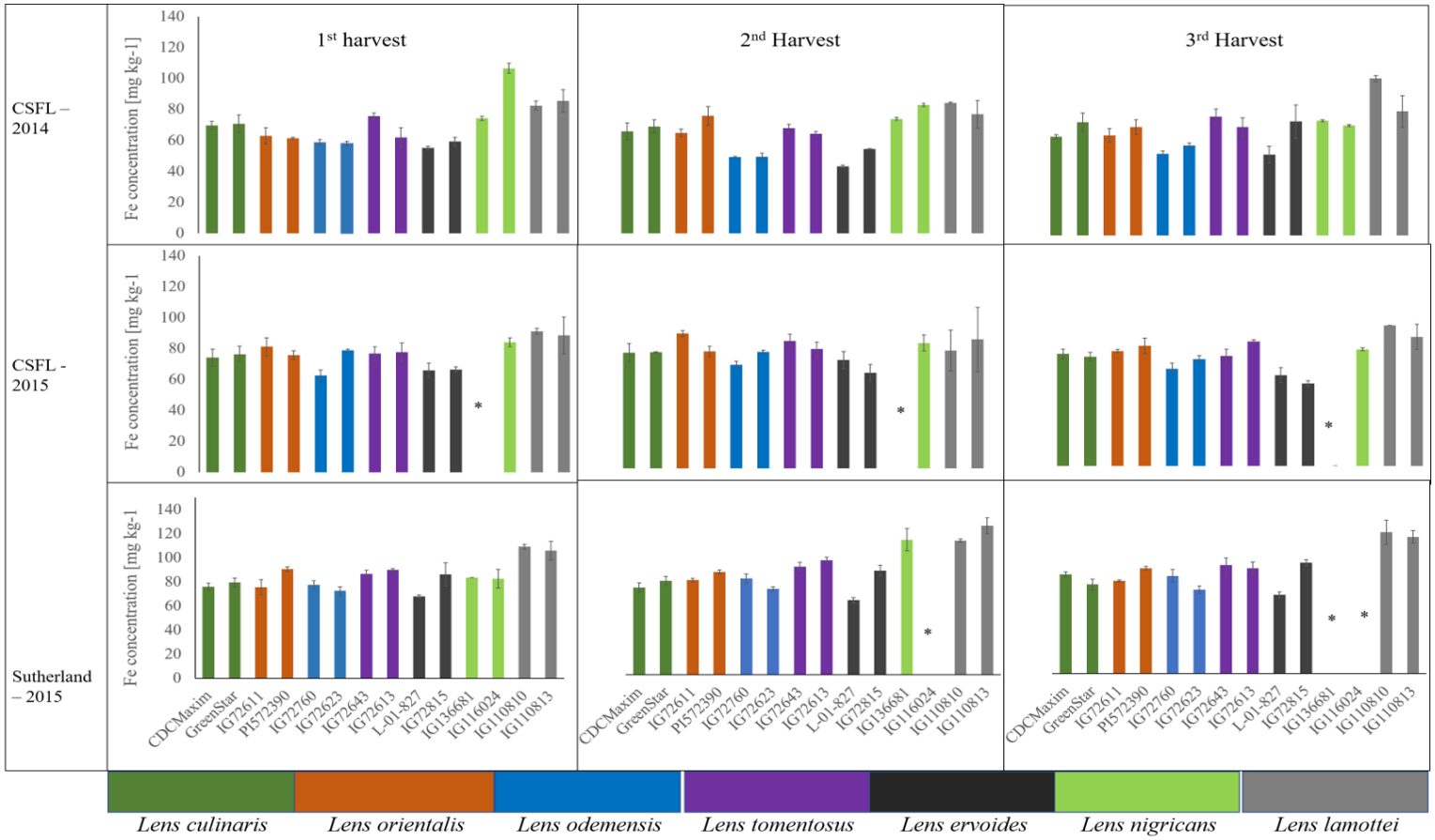

Figure 4.3. Average iron concentration (mg kg^{-1}) in seeds of seven lentil species harvested at three maturity stages and grown in three different environments: Crop Science field lab (CSFL) in 2014, Saskatoon; CSFL in 2015 and Sutherland in 2015. ***** - No seeds harvested.

4.4. Discussion

Significant differences were observed for seed Fe concentration among lentil genotypes but not for the harvest date and genotype × harvest interaction. In 2014 (one location), significant differences in seed Fe concentration were observed among the three harvests, but there were no differences in 2015. This could be due to the substantial difference in precipitation and temperature experienced between 2014 and 2015 (Table 4.2). Total in season precipitation in 2014 was higher than 2015, and the relatively wet year might have influenced the soil properties, such as pH and DTPA-extractable [Fe] (mg kg^{-1}) in both locations (Table 4.3). A similar result was reported by Kundu, (2016), where Zn uptake was studied in the same environments. Clérisse et al., (2017) reported that Fe concentration (ppm) in common bean was highly correlated with soil moisture regime. Nchimbi-Msolla & Muhamba, (2010) reported a significant ($p \leq 0.05$) location and genotype × environment for leaf and seed Fe concentration of common bean. Although the mean temperature from May to August in 2014 and 2015 was similar, in June and July, the average temperature was higher in 2015, and this might also have influenced seed Fe concentration. Thavarajah et al., (2010) found seed Fe, Zn and PA concentrations in lentil seeds were significantly higher in an increasing temperature regime compared to a decreasing temperature regime.

4.5. Conclusion

Our study revealed that the trend of seed Fe accumulation was similar over the entire seed maturity stage, but that substantial variability was observed between genotypes. This variability can be exploited in future breeding programs.

Prologue to chapter 5

The findings from the chapter 4 showed that some wild accessions, especially those of *Lens lamottei* had relatively higher seed Fe concentration compared to the cultivated species. This species has to date not been reported to successfully hybridize with the *Lens culinaris.* Some of the cultivated and wild genotypes (*e.g. L. orientalis, L. ervoides*) that were used in this study were selected earlier for development of interspecific RILs at the Crop Development Centre, University of Saskatchewan, Canada. The RILs were found to be very diverse phenotypically and were used to study the inheritance of many agronomically important traits. There is no evidence, based on Fe concentration in seeds of the genotypes used as interspecific parents, that indicates that interspecific RILs would be suitable for Fe biofortification. However, the possibility that interspecific hybrids may result in transgressive segregation for Fe concentration in seeds cannot be ruled out, since one of the species clearly has higher seed Fe concentration. We therefore made plans to study available interspecific RILs to determine the inheritance of seed Fe concentration and the influence of genotype × environment interaction on phenotypic expression of seed Fe concentration. In the next chapter of the thesis, we describe the inheritance and genotype × environment interaction of seed Fe concentration using two interspecific RIL populations derived from crosses between *L. culinaris* and *L. ervoides*.

CHAPTER 5

G × E INTERACTION EFFECTS ON FE ACCUMULATION IN LENTIL INTERSPECIFIC RECOMBINANT INBRED POPULATIONS

5.1 Introduction

Cultivated lentil is grown in diverse environments, from low to high altitudes, and from tropical to temperate regions in many parts of the world. In some areas, lentil is consumed on a regular basis as a cheap source of protein and micronutrients. The micronutrient content of lentils can vary for many reasons, but the genetic background and the environmental conditions where lentil grows are the two major factors. The accumulation and distribution of micronutrients from soil to other plant parts, including the seeds, are most important. In breeding programs, phenotyping of traits that are the objective of the improvement strategy is usually the most important task influencing selection and development of superior genotypes with desirable characters. The phenotype of any trait is determined by genotypic and environmental components. Some environmental effects are predictable, and some can be unpredictable (Nleya et al., 2000). Predictable factors such as harvest management, storage humidity and temperature, handling processes etc. can be controlled, but the unpredictable components (mainly climatic factors) are very difficult to control and can mask the genotypic effects. The breeder may try to explore the individual contributions of genotype and environment arising from G × E interaction to calculate the genetic progress through selection (Hébert et al., 1995). Greater interaction between genotype and environment results in reduced stability of a genotype over diverse environments (Kumar et al., 2013). Zhu et al., (2008) reported that the phenotype, or the observable expression of a particular complex trait, can be influenced by numerous quantitative traits and their interactions with environment, and also by the interaction between QTLs and environment.

The present study was conducted to assess the inheritance of seed Fe concentration by screening two interspecific recombinant inbred populations (RILs) of lentil. These were developed at Crop Development Centre, University of Saskatchewan, Canada, by crossing *Lens culinaris* (Eston) with *Lens ervoides* accessions IG 72815 and L01-827a (Fiala et al., 2009; Tullu et al., 2013). A second objective was to investigate the genotype × environment interaction for these two interspecific RILs. To our knowledge, this is the first study that investigates the effect of genotype, environment and their interaction and the inheritance of seed Fe concentration of interspecific hybrids of lentil.

Hypothesis

The concentration of Fe in seeds of recombinant inbred lines of *Lens culinaris x Lens ervoides* interspecific hybrids and their parents is the same across environments

Objectives

(i) To determine the seed Fe concentration of interspecific RILs grown across a wide range of environments.

(ii) To estimate the inheritance, heritability and the effect of genotype × environment interaction on seed Fe concentration.

5.2. Materials and Methods

5.2.1. Genetic materials

Two interspecific recombinant inbred populations (RILs), LR-26 and LR-59 were developed at the Crop Development Centre (CDC), University of Saskatchewan. The LR-59 population was developed from a cross between Canadian lentil cultivar 'Eston' and *L. ervoides* accession, L01-827a (Fiala et al., 2009). The LR-26 population was developed from a cross

between 'Eston' and *L. ervoides* accession IG 72815 (Tullu et al., 2013). From the LR-26 and LR-59 populations, 134 and 50 RILs, respectively, were selected to evaluate seed Fe concentration.

5.2.2. Field trials

All the selected lines from both RIL populations were grown in three site-years in the Saskatoon area - Sutherland farm (52°15′N, 106°52′W) in 2015 and Crop Science Field Lab (CSFL) area (52°36′N, 106°62′W) in 2014 and 2015. The experiments were designed as hill plots in randomized complete block design (RCBD) with three replications. A total of 20 seeds from each genotype were sown in each hill using a tray hill planter. Each hill plot had four rows of 30 cm length with 30 cm spacing between rows and seeds were sown in ~3.8 cm deep. The trial of 2014 in CSFL was seeded on May 23. In 2015, seeds were sown on May 8 at CSFL and on May 22 at Sutherland. All seeds including lines from both the RILs and *L. ervoides* parent were scarified followed by storage at -20˚C for two days before seeding. In 2015, seeds were stored at 4˚C and 80% humidity after scarification to increase the field germination rate. The climate and soil data for both years are shown in the previous chapter (Tables 4.2 and 4.3).

5.2.3. Seed harvest and Fe analysis

Seed harvesting, and seed Fe analysis was done following the same procedures described section 4.2 of this thesis except that seeds were harvested at one time.

5.2.4. Statistical analysis

A combined statistical analysis was done using SAS 9.4 (SAS institute Inc., Cary, NC, USA). The analysis of variance (ANOVA) was used to express the variation of Fe concentration of both the LR-26 and LR-59 RILs in Table 5.1. Homogeneity analysis was performed to estimate the variance of both RILs grown at three environments. SAS Proc Mixed procedure was used with genotype, environment and the interaction of genotype × environment as fixed factors, and replicates were nested within each environment as a random factor for the estimation of variance

components. Phenotypic variance was estimated as $\sigma^2p = \sigma^2_g + \sigma^2_e + (\sigma^2_{ge}/e) + (\sigma^2_{er}/er)$, where σ^2g, σ^2_{ge} and σ^2_{er} were estimates of genotypic, genotype × year and residual error variances, respectively, where r = number of replications, and e = number of environments. Broad sense heritability (H^2) of seed Fe concentration was calculated as the ratio of genetic variance (σ^2g) to phenotypic variance (σ^2p) as described by Singh et al., (1993) and Ubayasena et al., (2010).

5.3. Results

A wide range of variation was observed for seed Fe concentration among the lines in both LR-26 and LR-59 RIL populations (Table 5.1). The genotype, environment and genotype × environment effects were highly significant ($p \leq 0.001$) for both population across the environments (Table 5.1). The mean Fe concentration (ppm) from all the locations for LR-26 and LR-59 RILs was 70.9 and 68.4, respectively. In both populations, 'Eston' was the common parent. It had significantly higher Fe concentration compared to the two *Lens ervoides* parents.

Among the three site years, the seed Fe concentration from the 2015 Sutherland location was higher than the CSFL locations (2014 & 2015) for both RILs. The range of Fe concentration in seeds of LR-26 was greater (47.0- 102.9 ppm) than that of LR-59 (46.9-93.0 ppm) (Table 5.2). The broad sense heritability for Fe concentration in LR-26 was higher ($H^2 = 0.66$) than LR-59 ($H^2 = 0.54$). A significant ($p \leq 0.01$) negative correlation was observed for seed Fe concentration and seed yield for both populations.

Table 5.1. Analysis of variance, variance components and broad-sense heritability for seed Fe concentration (ppm) for 134 lentil RILs of LR-26 and 50 lentil RILs of LR-59 evaluated at Sutherland farms in 2015 and Crop Science Field Laboratory, University of Saskatchewan in 2014 and 2015.

Effect	LR-26		LR-59	
	DF	F Value	DF	F Value
Genotype	133	33.2**	49	22.7**
Environment	2	440.1**	2	171.1**
Environment × Genotype	266	4.3**	98	4.1**
Variance components				
σ^2 Genotype	69.5 (27.1)		53.7 (23.2)	
σ^2 Environment	24.0 (9.3)		31.9 (13.8)	
σ^2 Genotype × Environment	21.3 (8.3)		30.3 (13.1)	
σ^2 Error	37.3 (14.5)		49.1 (21.2)	
σ^2 Phenotype	104.7 (40.8)		66.5 (28.8)	
H^2	0.7		0.5	

Note: **, significant at $p \leq 0.001$

Table 5.2. Minimum, maximum, mean seed Fe concentration (ppm) of parents, LR -26 s and LR-59 RILs grown at Crop Science Field Lab (CSFL), University of Saskatchewan in 2014 and CSFL and Sutherland in 2015.

	Seed Fe concentration (ppm)							
	LR-26				LR-59			
	2014	2015		2014-15	2014	2015		2014-15
	CSFL	CSFL	Sutherland	Mean	CSFL	CSFL	Sutherland	Mean
Minimum	43.6	47.6	49.9	47.0	46.2	42.7	51.9	46.9
Maximum	94.7	102.7	111.5	102.9	82.9	96.6	99.6	93.0
Mean	66.9	70.9	75.0	70.9	65.0	65.5	74.7	68.4
Parents								
Eston	60.1	61.0	74.4	65.2	63.9	70.1	71.2	68.4
IG 72815	56.5	52.4	64.2	57.7	--	--	--	--
L-01-827a	--	--	--	--	56.6	58.5	63.7	59.6
Mid-parent value	58.3	56.7	69.3	61.5	60.2	64.3	67.5	64

Frequency distribution for Fe concentration is presented in Figure 5.1 and 5.2 for both LR-26 and LR-59 RILs, respectively. Although the seed Fe concentration of the parents of both populations were significantly ($p \leq 0.05$) different, they were close numerically. In the LR-26 RIL population, however, 13 (8%) and 82 (61%) RILs had significantly lower and higher Fe concentration than the *L. culinaris* parent 'Eston', respectively (Appendix 1). Again, in LR-59, 19 (38%) and 11 (22%) RILs had significantly higher and lower Fe concentration than 'Eston', respectively (Appendix 2). These lines can be considered transgressive segregants.

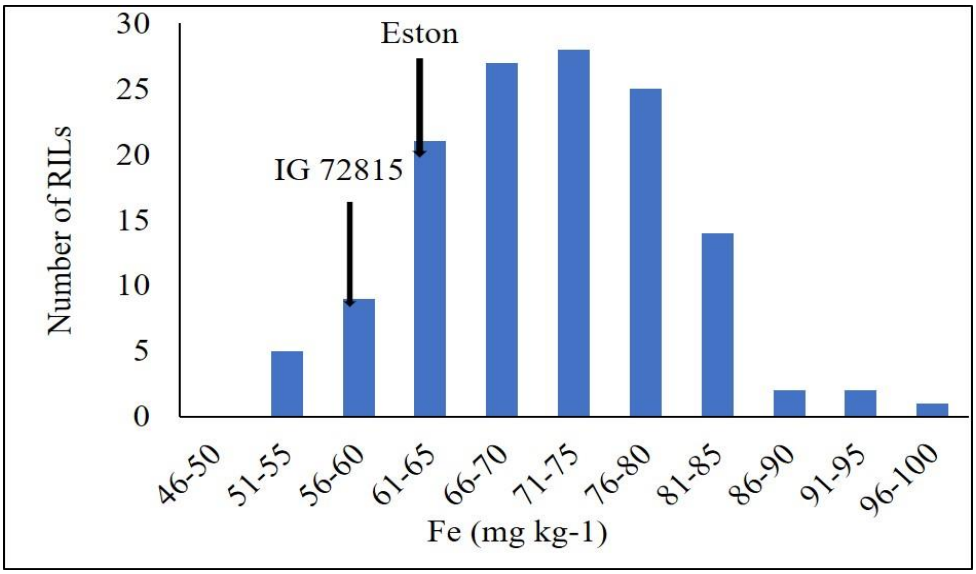

Figure 5.1. Frequency distribution of seed Fe concentration of 134 interspecific lentil recombinant inbred lines (RILs) derived from Eston × IG 72815 (LR-26) were grown in three different environments, Crop Science Field Lab (CSFL), University of Saskatchewan in 2014 and CSFL and Sutherland in 2015. RIL parents seed Fe concentration was indicated by arrows.

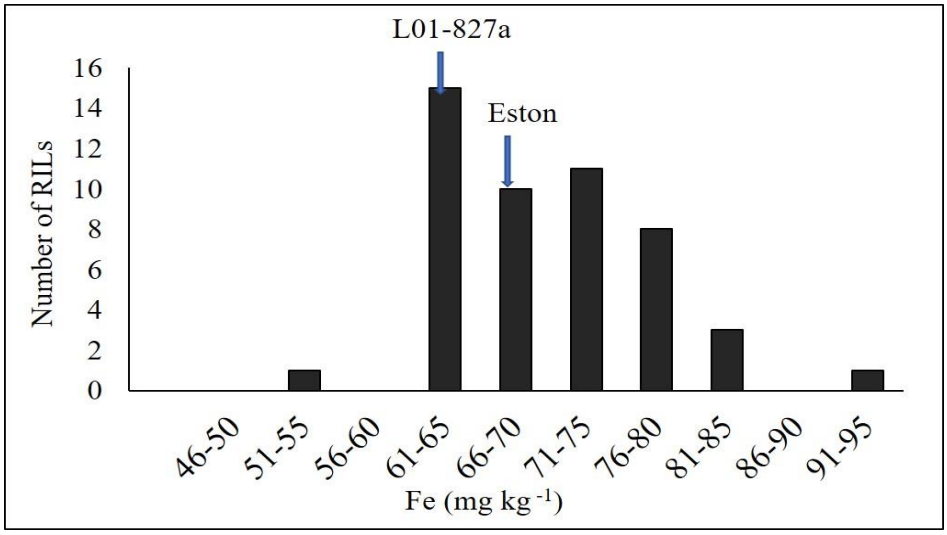

Figure 5.2. Frequency distribution of seed Fe concentration of 50 interspecific RILs derived from Eston × L01-827a (LR-59) grown in three different environments - Crop Science Field Lab (CSFL), University of Saskatchewan in 2014 and CSFL and Sutherland in 2015. RIL parents seed Fe concentration is indicated by arrows.

5.4. Discussion

Significant genotype × environment interaction was observed in both RIL populations. The soil pH and existing Fe status might have influenced the Fe accumulation. There was a substantial difference for precipitation and temperature between 2014 and 2015 (Table 10). This difference could influence the soil properties, since soil analysis showed that pH and DTPA-extractable [Fe] (mg kg^{-1}) were different between years. In both populations, seedling emergence of some of the lines was not uniform, and this might have influenced the results. A wide range of phenotypic and genotypic variability was observed for above and below ground phenotypes in comparisons among different lentil species (Gorim & Vandenberg, 2017a). Non-allelic interaction, such as environmental effects may influence quantitative traits, thus influencing the phenotypic expression

of a trait (Saha et al., 2013). Moreover, chromosomal rearrangements after hybridization between two species can also influence the phenotypic expression of the trait (Baack & Rieseberg, 2007).

The variability in seed Fe concentration among the LR-26 lines was greater than for LR-59. It could be due to a lower number of RILs, which was almost three times higher in LR-26 (134 lines) than LR-59 (50 RILs). With increases of number of environments and the number of genotypes, the possibility of G × E interaction also increases (Baye et al., 2011). Although the Fe concentration difference between the two parents of both the population was small, a large number of transgressive segregants were found. This could be due to the fact that *L. ervoides* parents with contrasting phenotypes for many traits related to root systems, such as total root length, total root surface area, root length per unit volume of soil, total root volume, the mean root diameter, root volume, fine root distribution in different soil horizon (Gorim & Vandenberg, 2017b). The wide variation among the RILs in both the population can be used in future genetic studies. Mapping populations also can be developed using diverse lines for seed Fe concentration to identify QTLs linked to Fe concentration.

A continuous frequency distribution in LR-26 indicated that the Fe uptake, transport or storage in seeds might be quantitatively inherited. Evidence for multiple genes related to inheritance of Fe concentration was reported in some studies (Upadhyaya et al., 2016; Diapari et al., 2014; Wang et al., 2008). Mineral accumulation in higher plants or in different plant parts are controlled by many genes with major or minor effects. For instance, a study of an interspecific cross between wild and cultivated species of common bean showed quantitative inheritance of Fe concentration (Guzmán-Maldonado et al., 2003). Inheritance of mineral uptake in *Arabidopsis thaliana* was found to be both quantitative and oligogenic (Vreugdenhil et al., 2004). A number of candidate genes related to mineral uptake was also reported. Blair et al., (2009) reported 13 QTLs

for Fe concentration after studying an inter-genepool RIL population of common bean using a quantitative trait locus mapping approach. High variability could be due to the difference of loci segregation in wild and cultivated species of common bean, as suggested by (Blair and Izquierdo, 2012).

5.5. Conclusion

A wide range of variation for seed Fe concentration was observed among the LR-26 and LR-59 RILs based on an evaluation in three environments. This variation can be used in future breeding programs to broaden the gene pool of lentil. In both populations, broad sense heritability was higher than 50, even though the effect of G, E, and G × E were significantly different. A number of RILs with high seed Fe concentration from both the populations should be selected for future studies. New RIL populations can be developed using selected RILs from both the populations. For instance, LR-59-81 was reported to be highly resistant to both races of *C. truncatum* (Fiala et al., 2009). This line was selected for its anthracnose resistance and crossed with CDC Redberry (cultivated lentil variety) to develop an interspecific RIL (LR-64) to further study the genetic control of resistance to different races of *C. truncatum* (Vail & Vandenberg, 2010). In the current study, 21 LR-26 lines (LR-26-4, LR-26-7, LR-26-181, LR-26-206, LR-26-47, LR-26-49, LR-26-228, LR-26-233, LR-26-99, LR-26-105, LR-26-111, LR-26-267, LR-26-280, LR-26-123, LR-26-125, LR-26-288, LR-26-132, LR-26-136, LR-26-298, LR-26-301, LR-26-165) and and six LR-59 lines (LR-59-1, LR-59-5, LR-59-81, LR-59-15, LR-59-106, LR-59-122) had relatively high (> 80ppm) seed Fe concentration after evaluation in three site years. These lines can be used in future breeding programs. Selection of RILs with higher (> 80 ppm) and lower (< 60 ppm) to develop new RILs can give more diverse populations that can be used for inheritance studies to identify QTL for seed Fe concentration in lentil.

Prologue to Chapter 6:

Results from Chapter 5 suggested that genotype × environment interaction had a significant effect on phenotypic expression of seed Fe concentration. It was also observed that Fe concentration was quantitatively inherited. Understanding the QTL responsible for seed Fe concentration is an appropriate approach, and linkage analysis and association mapping are two commonly used approaches that can help to identify marker-trait associations for Fe uptake, transport and storage in seeds. In the next chapter the association mapping approach will be discussed to determine the marker-trait association for seed Fe concentration from a diverse set of germplasm of cultivated lentil.

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Copyright for use of this manuscript (# 2) in this thesis was obtained and is reported in Appendix 13.

CHAPTER 6

MARKER-TRAIT ASSOCIATION ANALYSIS OF IRON CONCENTRATION IN LENTIL (*LENS CULINARIS* MEDIK.) SEEDS

6.1. Introduction and objectives

Lentil (*L. culinaris* Medik. subsp. *culinaris*) is a self-pollinated, herbaceous, diploid crop with 2n=2x=14 chromosomes and a haploid genome size of 4,063 Mbp (Arumuganathan & Earle, 1991). Lentils are considered a potential whole food that can provide micronutrients such as Fe, Zn, Cu, and Se (Thavarajah et al., 2009). They are also a good source of macronutrients, micronutrients and phytochemicals, which have potential health benefits for humans (Dueña et al., 2002).

The phenotype or the observable expression of a particular complex trait may be influenced by a number of quantitative traits and their interactions, the environment and also the interaction between QTLs and environment (Zhu et al., 2008). Two different strategies, linkage analysis and association mapping have been used to dissect complex traits. The latter one is the more promising tool that employs modern genomic technologies to exploit natural diversity and to assess the historical and evolutionary recombination events that occur at the population level (Nordborg & Tavaré, 2002; Zhu et al., 2008). Association mapping, also known as population mapping, makes use of a diverse set of individual lines, such as breeding populations, land races, or random mating populations of wild species (Singh and Singh, 2015). Yu & Buckler, (2006) reported three advantages of association mapping over linkage analysis - much higher mapping resolution, greater allele number and broader reference population, and reduced research time.

Association mapping studies to identify marker-trait association for micronutrient uptake or accumulation in seeds have been reported for some legume crops, most frequently for soybean.

Some examples were described in Chapter 2. For lentil, Fedoruk, (2013) observed associations for four quantitative traits - seed diameter, seed plumpness, seed thickness and days to flower. Thirty different associations were observed for the seed traits, but none for days to flower.

To our knowledge, the research presented here may be the first study using lentil genetic sequences to explore the genetic information controlling seed Fe accumulation in lentil seeds. This information might help with deployment of marker-assisted selection. The goal of this research was to understand the genetic basis of seed Fe concentration in lentil using association mapping to determine marker-trait associations using a single nucleotide polymorphism (SNP) array derived from cultivated lentil sequences.

6.2. Materials and Methods:

6.2.1. Plant samples and locations

A total of 138 diverse genotypes of *Lens culinaris* were evaluated for the lentil association mapping study by the Crop Development Centre (CDC) of University of Saskatchewan, Canada. These genotypes included cultivars and breeding lines developed at the Crop Development Centre of University of Saskatchewan, Canada, and landraces and germplasm obtained from the USDA gene bank at Washington State University, Pullman, USA and from the International Center for Agricultural Research in the Dry Areas (ICARDA). The field experiment was conducted in 2013 and 2014 at two locations (SPG and Sutherland farms) near Saskatoon (52°08´N 106°41´W). The origin of all genotypes was described by (Fedoruk, 2013). The soil at the two locations is Brown and Dark Brown chernozem that contains 1-17 % soil organic carbon and has a C:N ratio less than 17 (Agriculture and Agrifood Canada, 2013). Thirty seeds of each genotype were grown in 1 m^2 microplots in randomized complete block design with six and three replications in 2013 and 2014,

respectively. All seeds from each plot were harvested, cleaned, dried to13% moisture, and then stored at room temperature.

6.2.2. Digestion of lentil seed samples

A five gram sample of dry seeds from each plot was rapidly washed with distilled water followed by air drying to remove all soil and other micro-particles attached to the seed surface. Seed samples were ground into fine flour (<0.5 mm) using a UDY cyclone sample mill (UDY Corporation, 201 Rome Court, Fort Collins CO). Fe concentration was estimated from a 0.5g ground sample by digestion in a 30 mL digestion tube following a HNO_3-H_2O_2 digestion as described in section 3.2.

6.2.3. Data analysis

Statistical analysis (ANOVA) for seed Fe concentration was performed using a mixed effect model in PROC ANOVA, SAS version 9.4. The two locations were combined to determine the location × genotype effect. The two years of data were analysed separately due to differences of number of replications between them. Fe concentration was considered as the dependent variable. Replications were considered random and genotype, year and location as fixed effects. Broad sense heritability (H^2) of the Fe concentration was calculated as the ratio of genetic variance (σ^2g) to phenotypic variance (σ^2p). Phenotypic variance was estimated as $\sigma^2p = \sigma^2_g + \sigma^2_l + (\sigma^2_{gl}/l) + (\sigma^2_{er}/lr)$, where σ^2g, σ^2_{gl} and σ^2_{er} are estimates of genotypic, genotype by locations and residual error variances, r is the number of replications, and l is the number of locations.

6.2.4. Genotyping

Leaf samples were collected from all 138 genotypes grown in the field. The DNA extraction was completed using a CTAB extraction method (Doyle & Doyle, 1990). The samples were genotyped using the Lc1536 Golden Gate SNP OPA described by (Fedoruk, 2013). All genotypic information is stored in the KnowPulse web portal (http://knowpulse.usask.ca/portal/project/Lc1536-Golden-Gate-Assay). Of the 1243 polymorphic SNP markers, 1150 markers were used for association mapping analysis after considering minor allele frequency (MAF) < 0.05 were excluded.

6.2.5. Phylogenetic tree construction

A phylogenetic tree was constructed from Nei's distance matrix using UPGMA and the resulting tree was visualized using iTOL v. 3.0 (Letunic & Bork, 2011).

6.2.6. Population structure and kinship calculations

STRUCTURE v. 2.3.4 (Pritchard et al., 2000) was used to calculate the most probable number of sub-populations (K). Five independent runs were done for each K ranging from 1 to 10 with both a burn-in time and Markov Chain Monte Carlo (MCMC) replication number of 500,000. Selection of the best K value was based on the procedure presented in Evanno et al., (2005) by submitting the results for each K to the STRUCTURE HARVESTER website, which returned the L(K) and ΔK value (Earl & vonHoldt, 2012). A Q-matrix (Q) was obtained from the membership probability of each variety and was used for further association mapping. The relatedness among the genotypes was estimated using kinship matrix (K) that was derived from SPAGeDi software (Hardy & Vekemans, 2002) as described by Loiselle et al., (1995). Principal component analysis (PCA) was performed to analyze genetic structure of the lentil population using GenAlEx v6.5 (Peakall & Smouse, 2012).

6.2.7. Association analysis

Association analysis was performed using the TASSEL (Trait Analysis by Association, Evolution, and Linkage) software program v. 5.2.31 (Bradbury et al., 2007) to test the marker trait association between SNP markers and seed Fe concentration. Association studies are sensitive to population structure that can create spurious association between markers and traits and can increase the false positive rate (Cappa et al., 2011). To reduce this problem, population structure (Q) and Kinship (K) were used as described by (Fedoruk, 2013). The Q and K were used as covariates in a Mixed Linear Model (MLM) that is widely accepted as a useful tool for the association (Yu & Buckler, 2006; Zhao et al., 2007). In MLM, genotypes, phenotypes, principal component analysis (PCA), population structure (Q), and kinship matrix (K) were incorporated. Both MLM (PCA + K) and MLM (Q + K) models were used. The PCA matrix was calculated using TASSEL.

6.3. Results

6.3.1. Phenotypic variability of genotypes

A wide range of variability was observed for seed Fe concentration (ppm) among the genotypes. In both the years, Fe concentration and variability were higher in SPG than Sutherland (Table 6.1). In 2013, two genotypes, ILL 1337 and PI 299215 had higher Fe concentration in SPG, but the highest mean Fe concentration was found from CDC Redwing. In 2014, PI 431705 had maximum Fe concentration in SPG but mean highest Fe concentration was found from PI 299215.

A significant location effect was observed for seed Fe concentration only in 2013, while significant genotype by location interaction (G × L) was observed in both years (Table 6.2). This result led us to analyze the association for each site and year separately. Broad sense heritability

(H^2) was calculated for Fe concentration (Table 6.2) using different variance components and higher heritability (< 50) was found for both the years.

Table 6.1. Maximum, minimum and average amount of lentil seed Fe concentration of the association mapping samples obtained from two different locations in 2013 and 2014.

Year	Location	Seed Fe concentration (ppm)		
		Average	Maximum	Minimum
2013	SPG	80.1	101.7	49.5
	Sutherland	74.1	92.2	54.9
	SPG & Sutherland	77.1	93.7	54.9
2014	SPG	75.1	100.3	41.5
	Sutherland	75.3	93.9	51.7
	SPG & Sutherland	75.2	92.9	46.6
2013 &2014	SPG & Sutherland	76.1	92.9	53.1

6.3.2. Phylogenetic tree construction

The phylogenetic tree revealed that the majority of accessions in one of the groups were Canadian breeding lines and cultivars (Figure 6.1). The second group had a mixture of ICARDA breeding lines and accessions from the collection held by the USDA gene bank.

6.3.4. Relative kinship estimation

The kinship estimation revealed a complex familial relationship among the 138 accessions, which complements the known pedigree history. About 72 % of the pairwise kinship estimates were zero or close to zero, suggesting that these accessions are unrelated. The remaining estimates ranged from 0.1 to 1.2 (Figure 6.2), with an exponentially decreasing number of pairs falling into higher estimate categories.

Table 6.2. F values from the analysis of variance (ANOVA) and variance components with broad sense heritability estimates of Fe concentration in lentil seeds grown at SPG and Sutherland farms in 2013 and 2014.

	2013		2014	
Source of variation	Df	F Value	Df	F Value
Genotype (G)	137	27.42***	137	16.84***
Location (L)	1	312.07***	1	1.12^{NS}
G × L	137	2.95***	137	2.22***
Error	690		176	
Total	965		451	
Variance components	2013		2014	
$\sigma^2 l$	13.06		0.01	
$\sigma^2 g$	79.80		81.98	
$\sigma^2 gl$	11.50		12.88	
$\sigma^2 e$	41.27		33.12	
σ^{2p}	145.62		127.99	
H^2	0.55		0.64	

Note: Df, degree of freedom; G, L, and G X L are genotype, location and genotype by location interaction respectively; $\sigma^2 g$, $\sigma^2 l$, $\sigma^2 gl$ and $\sigma^2 e$ are estimates of genotypic, location, genotype by location interaction, and error variance, respectively. *** indicates significant difference at $P \leq 0.001$. ns, nonsignificant.

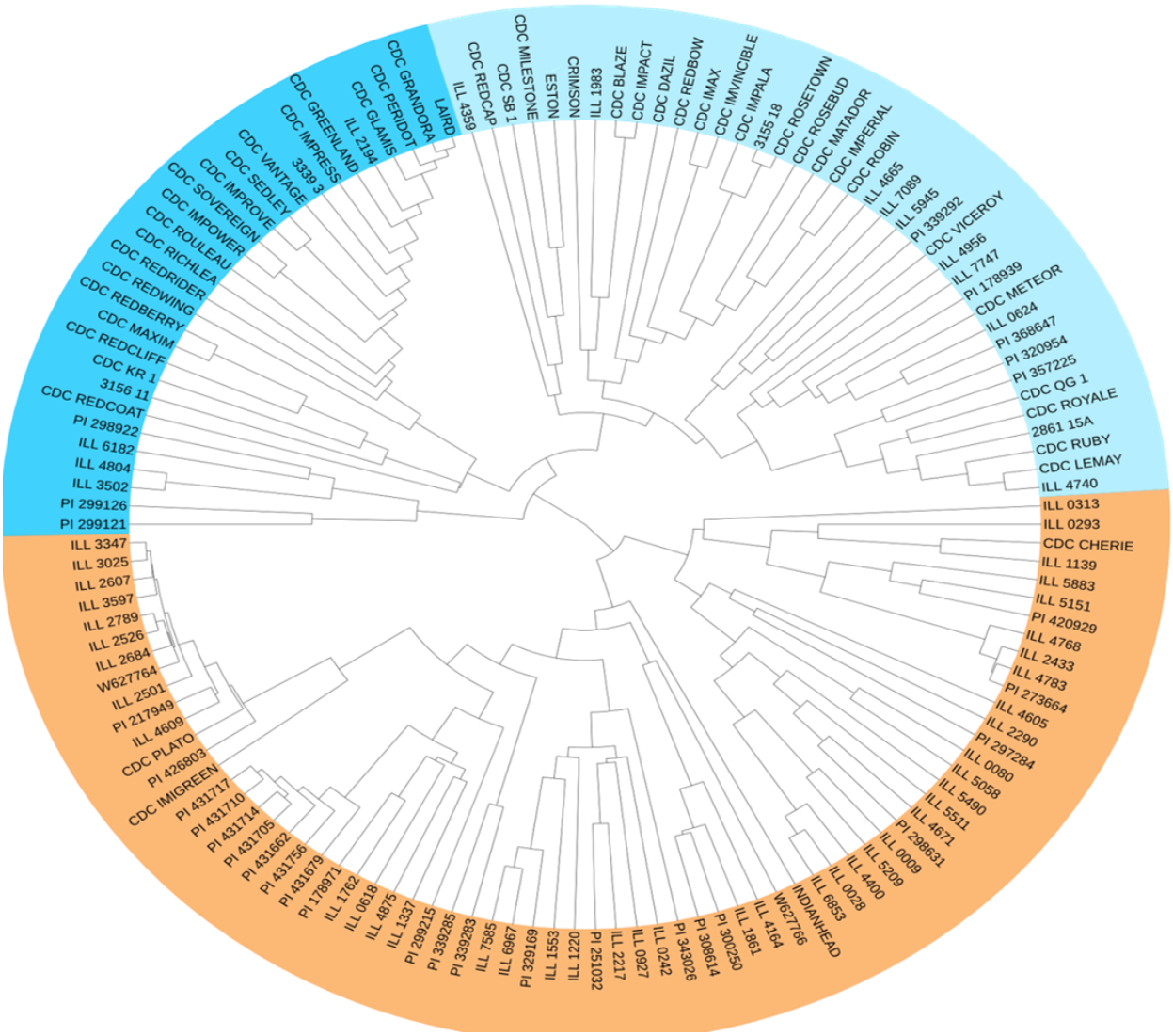

Figure 6.1. Dendrogram showing relationships among individuals from 138 lentil accessions based on 1150 SNP markers. Sandy brown and blue coloration reflects genotypes belonging to two different groups; dark and light sky blue are sub-groups.

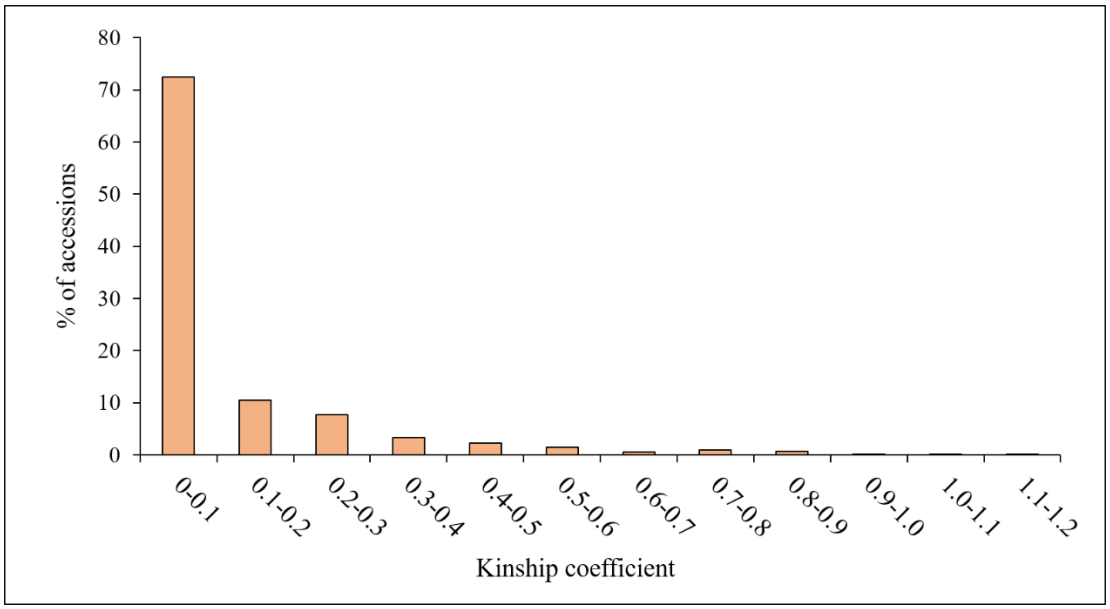

Figure 6.2. Frequency of the relative kinship between pairs of individuals.

6.3.5. Marker trait associations

Both MLM (Q+K) and MLM (PCA+K) association tests of seed Fe concentration identified 9 significantly linked SNP markers with a significance level of at least $-\log10\ P \geq 3.06$ (Table 6.3). Two of these SNP markers had a strong association ($-\log10\ P \geq 4.36$) with seed Fe concentration. Significant SNP markers found in two or more datasets (site and year) are regarded as more reliable than those significant only in a single dataset. Most significant markers revealed associations across multiple environments (sites and years) as well as with two different MLM methods (Q and PCA). LcC25737p350 and LcC24316p626 had a strong association with seed Fe concentration at both SPG and Sutherland across 2013 and 2014. LcC06625p437 was highly significant for Fe in 2014 only (Table 6.3). In general, the percentage of phenotypic variation explained by each marker (R^2) ranged from 9 to 21 % for the trait (seed Fe concentration). The results also indicated that five out of nine SNP markers associated to seed Fe concentration are located on lentil chromosome 5.

Table 6.3. Single-nucleotide polymorphism markers associated with Fe concentrations in seeds of lentil using mixed linear model [population structure (Q) and principal component analysis (PCA)] models for Saskatchewan Pulse Growers (SPG) and Sutherland (STH) farm locations, Saskatchewan, Canada, in 2013 and 2014.

Year	Location	Marker name	Chromosome. Number	Position	Q Matrix		PCA Matrix	
					P value	R^2 (%)	P value	R^2 (%)
2013	SPG	LCC25737P350	5	1206	3.44E-04	10	4.02E-04	8
		LCC24316P626	5/6	1177	6.52E-05	17		
		LCC01329P253	1/2/6	185	6.91E-04	9		
	Sutherland	LCC11104P161	5	861	4.51E-04	13	4.00E-04	12
		LCC24316P626	5/6	1177	5.53E-04	12		
	Both locations	LCC24316P626	5/6	1177	6.32E-05	17		
		LCC25737P350	5	1206	9.53E-04	8		
2014	SPG	LCC01714P78	5	230	7.72E-04	12		
		LCC10829P367	7	849	--	--	5.60E-04	9
		LCC01908P896	3	251	--	--	7.09E-04	10
	Sutherland	LCC25737P350	5/6	1206	7.20E-05	12	6.65E-05	11
		LCC06625P437	5/7	654	7.00E-05	15	9.60E-05	13
		LCC07856P82	4	724	1.02E-04	14	4.52E-04	10
		LCC21183P306	5	1131	2.23E-04	14	6.71E-04	10
		LCC01908P896	3	251	8.79E-04	12	3.79E-04	11
		LCC03534P135	1	1177	6.94E-04	12	--	--
		LCC01329P253	1/2/6	185	5.48E-04	10	--	--

	LCC24316P626	5/6	414	4.44E-04	13	--	--
	LCC17753P341	2	1049	9.09E-04	12	--	--
Both locations	LCC06625P437	5/7	654	5.76E-05	15	9.22E-05	13
	LCC25737P350	5	1206	1.81E-04	11	1.49E-04	10
	LCC01908P896	3	251	3.24E-04	13	1.15E-04	13
	LCC07856P82	4	724	4.32E-04	12	5.42E-04	10
	LCC21183P306	5	1131	8.07E-04	12	7.29E-04	10
	LCC06877P157	5	668	3.23E-04	13	--	--
	LCC01329P253	1/2/6	185	5.97E-04	10	--	--
	LCC07588P354	1/6	709	6.72E-04	12	--	--
	LCC01714P78	5	230	2.14E-04	14	--	--
All locations and years	LCC25737P350	5	1206	2.97E-04	10	3.89E-04	8
	LCC01908P896	3	251	2.11E-04	13	4.01E-04	11
	LCC06625P437	5/7	654	4.45E-04	11	5.95E-04	10
	LCC24316P626	5/6	1177	1.56E-04	14	--	--
	LCC01329P253	1/2/6	185	7.52E-04	9	--	--
	LCC17953P450	6	1061	8.20E-04	11	--	--

6.4. Discussion

Association mapping (AM) is a tool that helps to detect the association between both phenotypically and genotypically characterized heritable traits and their genotypic polymorphisms (Oraguzie & Wilcox, 2007). It is now widely used due to availability of high throughput genotyping technologies that use large numbers of markers to detect the polymorphisms more

precisely than linkage mapping. In this study, we conducted association mapping analysis to detect genetic markers associated with seed Fe concentration in a diverse array of cultivated lentil germplasms.

The genotypes studied here were representative of lentil germplasm grown around the world. Seed Fe concentration was highly variable, ranging from 53.1 to 92.9 ppm across genotypes, sites and years (Appendix 3). This variation can be used by lentil breeders to develop new genotypes with the potential to accumulate higher amounts of Fe for storage in seeds. The average Fe concentration was 7% higher, but not statistically significant in Canadian accessions (79 ± 6.2 ppm) compared to the international accessions (74.1 ± 10.5 ppm), and indication that better adaptation could result in higher Fe accumulation. Thavarajah et al., 2011) also reported that Canadian lentils are rich in Fe (73-90 ppm). The highest Fe concentration was found in PI 299215 from Chile and the lowest in ILL 2607 and ILL 3025 from India. The same genotypes were also evaluated for seed Zn concentration and its association with markers (Khazaei et al., 2017; Kundu, 2016). A strong positive correlation was also observed for seed Fe and Zn concentration suggesting that similar mechanisms exist for both micronutrients.

A total of nine SNP markers associated with seed Fe concentration in lentil were identified and two of them were highly associated. Compared to other pulses (Diapari et al. 2015; Upadhyaya et al. 2016) we found relatively few markers for seed Fe concentration, which may indicate a lower level of genetic variability is present in the germplasm used in the study. Alternatively, a greater number of molecular markers may be needed to sample the variability inherent in a large genome like that of lentil.

Population structure can elevate the likelihood of false positive associations in marker trait association studies (Khazaei et al., 2016). In this study, both Q matrix and PCA were used to generate an overall picture of population disparity (Lander & Schork, 1994; Zhao et al., 2007) and to summarize relatedness in genome patterns (Yang et al., 2011), respectively. The program STRUCTURE was also used to calculate the possible number of sub populations (K). In this study, we used both MLM (Q + K) and MLM (PCA + K) models to identify marker trait association for seed Fe concentration to minimize both false positive and false negative association (Khazaei et al. 2017). Results for both the models had slight differences. Moreover, all highly significant association were observed for PCA + K model.

Iron movement from soil to the whole plant level, including seeds, involves several processes. Fe uptake, transport and accumulation in plants are regulated by metal transporters (Vasconcelos et al., 2014). Most of the studies that identify transporters were conducted using the model genomes of *Arabidopsis thaliana* and *Medicago truncatula*. In this study, we identified a number of candidate genes (Appendix 4) that contain SNP markers associated to seed Fe concentration. For instance, the sequence of marker LcC 06625p437 was found within a cytosolic Fe-S cluster assembly factor NUBP1-like protein mRNA of *Medicago truncatula* chromosome 1 (Bernard et al., 2013). This protein may regulate Fe uptake and storage in plants. Another marker LcC24316p626 was found in *Medicago truncatula* transmembrane protein that are known to play important roles in Fe-uptake from root and cell to cell transportation of Fe in plants (Dubeaux et al., 2015).

6.5. Conclusion

This study provides insight into the genetic basis of variability in seed Fe concentration in a diverse set of lentil genotypes. The identified SNP markers could be used in conjunction with the high Fe accessions to breed for increased levels of seed Fe concentration in lentil. These markers can be validated in future on a broad range of wild and cultivated lentil populations. The identified and selected markers could then be used for marker-assisted selection to further increase of Fe concentration in lentil seeds with desi qualities that are desirable for end-users.

Prologue to Chapter 7

In the last few chapters including Chapter 6, we have discussed the results of experimental work in the area of diversity of seed Fe concentration in lentil. Chapter 6 revealed that a few markers are associated with seed Fe concentration, and some candidate genes that might be associated with uptake, transport and accumulation of Fe in lentil seeds. Validation of the markers will be required to confirm the association with increased Fe concentration in lentil seeds. The biofortification process will require long term, sustained research effort to develop reliable methods to achieve the goal of increasing uptake and storage of higher concentrations of Fe in seeds.

We hypothesised that an alternative approach, fortification of dehulled lentil (dal), may quickly provide higher amounts of bioavailable Fe in lentil dal as a strategy for alleviating Fe malnutrition. We conducted a series of experiments to develop a protocol for Fe fortification of dehulled lentil. In the next chapter, the development of fortification technology for dehulled red lentil is described.

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CHAPTER 7

IRON FORTIFICATION OF LENTIL (*LENS CULINARIS* MEDIK.) TO ADDRESS IRON DEFICIENCY

7.1. Introduction and objectives

Lentil (*Lens culinaris* Medikus) is an important legume crop, cultivated for food and feed since prehistoric times. As a source of dietary protein, lentil can be combined with cereals to prepare human diets and animal feeds that provide a balance of essential amino acids and essential micronutrients such as iron, zinc and selenium (Podder et al., 2013; Thavarajah et al., 2011). Lentil is a good source of non-heme iron, ranging from 73-90 mg kg^{-1} (Thavarajah et al., 2009). The crude protein content ($N \times 6.25$) of Western Canadian lentil is reported to range from 25.8-27.1% (Wang & Daun, 2006). Lentil also is considered to be a starchy legume as it contains 27.4-47.1% starch, with a significant level of amylose (23.5-32.2%) (Hoover et al., 2010; Huang et al., 2007). Although lentil is a good source of intrinsic Fe, the bioavailability/absorption is low (DellaValle et al., 2015). These authors reported that the mean Fe absorption from lentil dal was 2.2%, which was significantly lower than the 23.6% observed for a similar amount of Fe given as ferrous sulphate to women with poor Fe status. Low bioavailability may be due to the presence of phytic acid and polyphenols in the lentil dal (DellaValle et al., 2015; Lynch et al., 1984).

Iron (Fe) is the most abundant element in the earth′s crust and is an essential micronutrient for both plants and animals. In plants, Fe deficiency affects key metabolic processes such as the electron transfer system for photosynthesis and respiration (Li et al., 2013). Iron deficiency in humans refers to a condition in which an insufficient amount of bioavailable Fe results in Fe

deficiency anemia (Bermejo & Garcia-Lopez, 2009). This deficiency has become a major nutritional disorder, widespread in both developing and developed countries (Detzel & Wieser, 2015). The major consequences of Fe deficiency are reduction of physical activity, fitness and work capability, a reduced ability to maintain body temperature, a lowered resistance to infection, and an increase in mortality during pregnancy and in newborns (Boccio & Iyengar, 2003). According to Food and Agriculture Organization (FAO) and World Health Organization (WHO) recommendations, the estimated daily average Fe requirements for females and males 19–50 years of age are 29.4 mg and 10.8 mg, respectively, based on 10% bioavailability (WHO & FAO, 2006).

Several strategies are used around the world to address micronutrient malnutrition. Micronutrient supplementation, dietary diversification, biofortification, food fortification, nutrition education, public health interventions and food safety measures are approaches that can solely, or in combination, be applied to address micronutrient deficiency in a target population (Northrop-Clewes, 2013). Supplementation is an effective means of providing immediate benefits to "at risk groups" but not for other household or community members (Dary et al., 2002) since it requires supplemental Fe consumption on a long-term basis, in tablet form for example. Dietary improvement through supplementation requires a change in dietary behavior, and this process also requires changes in food supply and availability that may require a long time to achieve success (Northrop-Clewes, 2013). Also, public health intervention can help prevent micronutrient malnutrition, but micronutrient malnutrition can also be associated with a high prevalence of microbial infection that causes a variety of different diseases. Food fortification can overcome this limitation due to its sustainability in improving the dietary quality of a targeted group or population without changing dietary habits. Food fortification is a potentially cost-effective way to add

micronutrients to processed foods in a way that can rapidly mitigate micronutrient malnutrition (WHO & FAO, 2006).

A successful Fe fortification program was first reported in Canada in 1944, when the government began fortifying wheat flour with Fe along with thiamine, riboflavin and niacin (Northrop-Clewes, 2013). A remarkable reduction in child mortality was observed from 102/1000 live births in 1944 (first year) to 61/1000 in 1947 in Canada (Nilson & Piza, 1998). During the twentieth century, Fe fortification became mandatory in several developing countries, including Bolivia, Chile, Colombia, Costa Rica, Ecuador, Guatemala, Indonesia and others (Darnton-Hill & Nalubola, 2002). In every country, either wheat or maize flour was chosen as the food vehicle. The requirements for selecting an appropriate food vehicle for fortification were established by FAO in 1995 (Darnton-Hill & Nalubola, 2002). In 1980, the FDA (U. S. Food and Drug Administration) established a "Food Fortification Policy" that was guided by six basic principles (Dwyer et al., 2015). The WHO has recommended Fe compounds and concentration for fortification of wheat flour in 13 countries (Pachón et al., 2015). To optimize iron bioavailability and maintain the organoleptic attributes that influence consumer acceptability of fortified foods, selected food vehicles and Fe fortificants need to be well matched. The food vehicle should be safe, widely accepted by the target consumers, have good storage capability after fortification, and the added Fe should be stable with high bioavailability (Martínez-Navarrete et al., 2002).

Fortifying lentil with suitable Fe fortificants is a research area with potential application to reduce Fe deficiency. We hypothesized that it would be possible to increase the amount of bioavailable Fe in dehulled (decorticated) pulses (dal) such as lentil, in a biologically and culturally meaningful way, to a level that could prevent Fe deficiency in humans. Our experimental approach had two main objectives, first, to determine the most suitable iron fortificant and the appropriate

dose of Fe for dehulled lentil based on ease of fortification, and second, to determine the optimal processing technology to fortify iron in dehulled lentil based on current processing practices. To fulfill the first objective, research was focused on selection of the appropriate genotype and product type of dehulled lentil and identifying the best form of Fe solution with which to fortify dehulled lentil products. The Fe fortificants, ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$), NaFeEDTA (ethylenediaminetetraacetic acid iron (III) sodium salt) and ferrous sulphate monohydrate ($FeSO_4 \cdot H_2O$), are acceptable fortificants that have potential for fortifying dehulled lentil seed (WHO & FAO, 2006). The second objective was fulfilled by conducting studies to help standardize the protocol for lentil fortification. These included assessments of the appropriate dose of Fe solution, selection of the most appropriate fortification method in the context of changes in organoleptic properties and storage capability, assessment of the best temperature for drying lentil after the addition of fortificants, and the effect of fortification on boiling time.

7.2. Materials and methods

The procedure followed for development of a lentil fortification protocol is shown in Figure 7.1, and is discussed below.

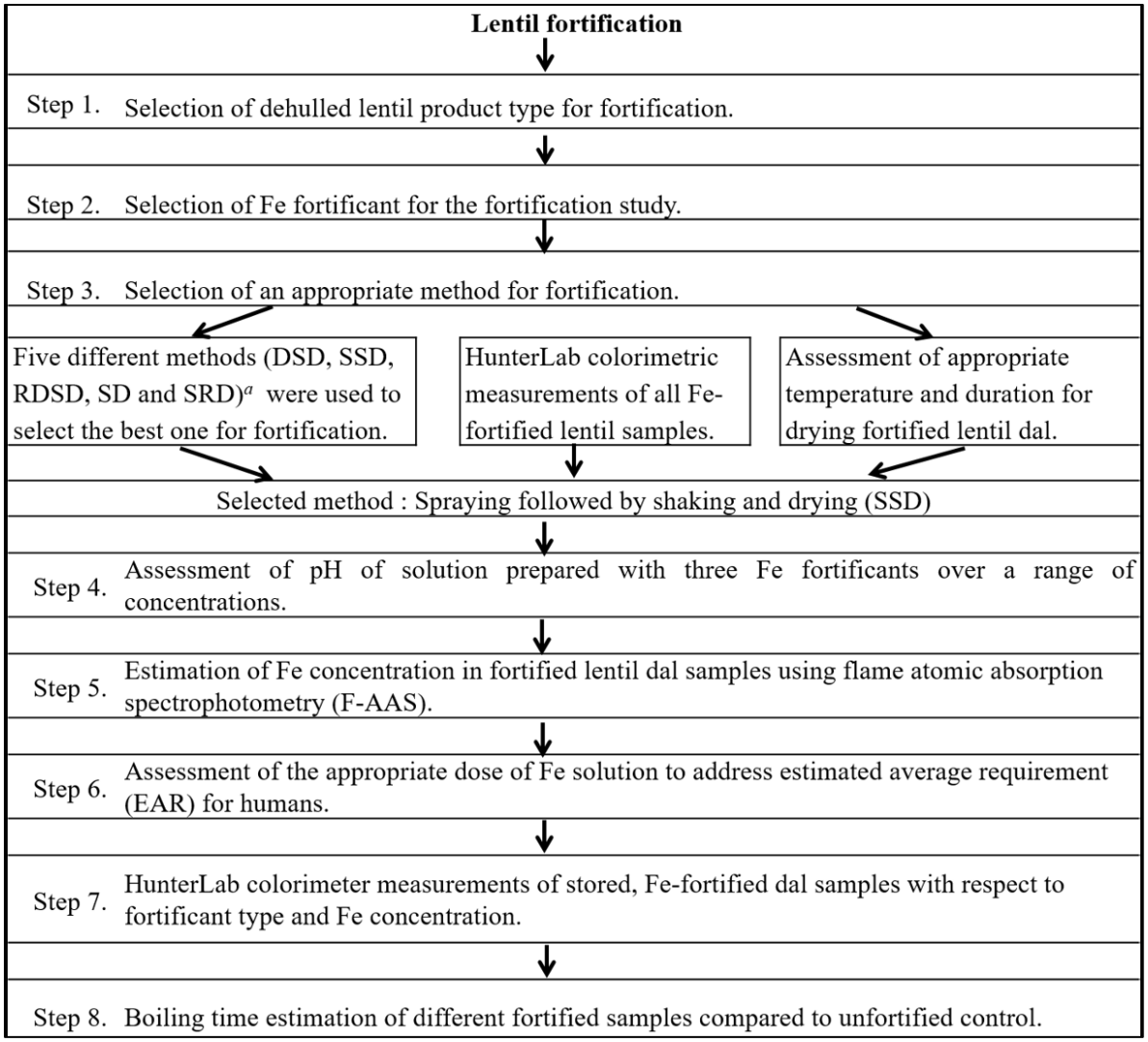

Figure 7.1. Flow chart for development of a lentil fortification protocol. [a]Oven dried, soaked and oven dried (DSD); sprayed followed by shaking and drying (SSD); rinsed, oven dried, soaked, and oven dried (RDSD); directly soaked in Fe solution (SD) and rinsed, soaked, and oven dried (SRD).

7.2.1. Selection of Lentil Genotype and Dehulled Lentil Product Type

Fifteen red cotyledon lentil cultivars/genotypes were analyzed to estimate the concentration (ppm) of Fe in seeds (data not shown). One widely grown and popular cultivated red lentil cultivar, CDC (Crop Development Centre) Maxim, developed at the Crop Development Centre, University

of Saskatchewan, Saskatoon, SK, Canada, was selected for fortification studies due to its high Fe concentration (75–90 ppm) compared to other red lentil cultivars grown in Saskatchewan (Martínez-Navarrete et al., 2002).

Four different types of dehulled lentil products are usually available in the red lentil market: polished football (dehulled, unsplit), polished splits, unpolished football and unpolished splits (Figure 7.2a). The Fe concentration in each product type was measured to determine the range of variability in Fe concentration. The product types then were used in a fortification study and samples of 200 g of each product type were mixed with 20 mL of NaFeEDTA solution (1600 ppm Fe) with four replications. The best product type in relation to uniformity of absorption of Fe solution, drying time and concentration of Fe in the fortified product was selected. The statistical analysis was conducted using SAS version 9.4 (SAS Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) was used to compare the Fe concentration of unfortified and fortified red lentil product types. The least significant difference (LSD) was calculated and the level of significance set at $P < 0.05$.

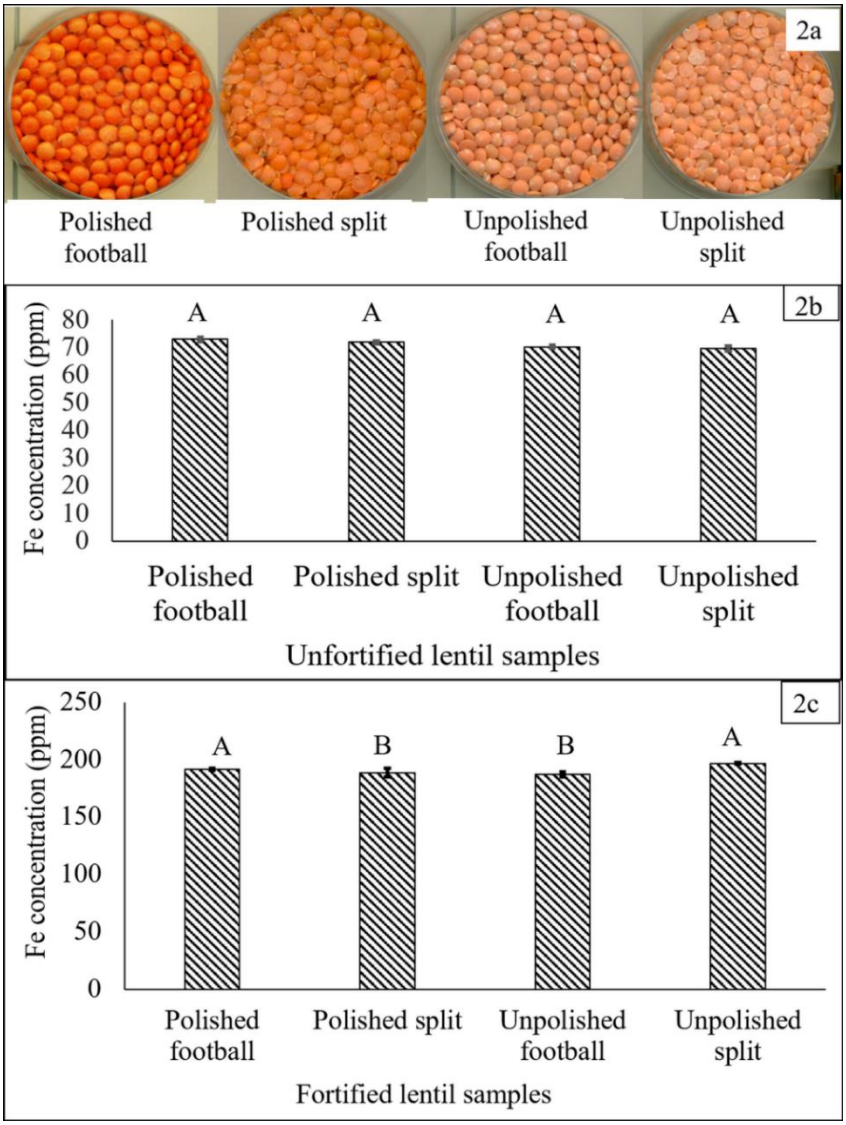

Figure 7.2. (a) Four dehulled, red lentil product types; (b) Fe concentration (ppm) in four dehulled, unfortified, red lentil product types; and (c) Fe concentration (ppm) in red lentil product types fortified with $FeSO_4.7H_20$ solution (1600 ppm Fe). Different letters within each figure represent significant differences ($P < 0.05$).

7.2.2. Selection and Evaluation of the Most Suitable Fe Fortificant for Lentil

The selection of the most appropriate Fe fortificant is challenging due to possible interactions between the food product and the Fe compound. Three water-soluble Fe compounds, $FeSO_4 \cdot 7H_2O$, NaFeEDTA and $FeSO_4 \cdot H_2O$ were selected from a list of iron fortificants published in the WHO

and FAO document "Guidelines on Food Fortification with Micronutrients" (Allen et al., 2006). The $FeSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot H_2O$ were supplied by Crown Technology, Inc., Indianapolis, IN, USA, and NaFeEDTA by Akzo Nobel Functional Chemicals, LLC, Chicago, IL, USA. The three fortificants were food grade and were selected on the basis of their relative bioavailability, interaction with the food vehicle and cost of fortification (Northrop-Clewes, 2013).

7.2.3. Selection of an Appropriate Method of Fortification

Techniques Used for Lentil Fortification

An experiment was designed to determine the most appropriate method for fortifying dehulled, polished, football lentil dal with a Fe solution prepared with $FeSO_4 \cdot 7H_2O$, one of the three Fe fortificants studied. Five methods were used to fortify lentil dal with $FeSO_4 \cdot 7H_2O$ solution (1600 ppm Fe) at 10 mL fortificant solution/100g dal. The 1600 ppm Fe concentration was selected with the aim that this concentration may provide a major part of the recommended daily allowances (RDAs) for humans. However, each method to fortify lentil dal is described below.

Method 1 (Dry-Soak-Dry). Lentil dal was oven dried at 80 °C for 10 min, soaked in 10 mL of fortificant solution for 2 min, and then dried again at 80 °C to obtain a moisture content of 14%.

Method 2 (Spray-Shake-Dry). Lentil dal was sprayed with fortificant solution using a 473 mL clear, fine-mist spray bottle (SOFT 'N STYLE, Product Code VO-302564, SKS Bottle and Packaging, INC., Watervliet, NY, USA), shaken using a Barnstead Thermolyne M49235 Bigger Bill Orbital Shaker (Sigma-Aldrich Corp., St. Louis, MO, USA) at 400 rpm for 10 min to mix the solution with the dal sample, and subsequently dried to 14% moisture under a 250-watt electric heat lamp (NOMA incandescent, clear, 130 V heat lamp, Trileaf Distributors, Toronto, ON, Canada) which produced a temperature of approximately 70 °C at the surface of the fortified dal.

Method 3 (Rinse-Dry-Soak-Dry). The third method consisted of rinsing 100 g dal samples under a continuous flow of deionized water for 30 s followed by oven drying at 80 °C for 10 min. The dried sample then was soaked in the fortificant solution (10 mL fortificant solution/100 g lentil) for 2 min and then placed in the oven again for 15 min at 80 °C to reduce the moisture level to 14%.

Method 4 (Soak-Dry). Lentil dal was soaked in fortificant solution followed by oven drying at 80 °C to 14% moisture.

Method 5 (Soak-Rinse-Dry). Lentil dal was soaked in fortificant solution and then rinsed with deionized water for 30 s, followed by oven drying at 80 °C to 14% moisture.

HunterLab Colorimetric Measurements of Fe-Fortified Lentil Samples

The color of the Fe-fortified lentil sample from each of the five fortification methods was measured using a HunterLab instrument (Hunter Associates Laboratory Inc., Reston, VA, USA) to allow comparison with unfortified control samples. For each method, four samples were assessed. The dimensions L*, a* and b* were compared with those of the control sample, where L* indicates lightness (ranging from 0–100), a* indicates red (+) and green (−) and b* indicates yellow (+) and blue (−) with a range of +80 to −80 (Wrolstad & Smith, 2010). The L*, a* and b* values were analyzed using ANOVA in SAS 9.4.

Assessment of Appropriate Temperature and Duration for Drying Fortified Lentil Dal

Electric heat lamps of three power levels (100, 200 and 250 watts) (Trileaf Distributor) were used to dry fortified football dal after spraying with fortificant solution. The distance between the bulb and the lentil dal surface was 15 cm. Samples of 100 g of dal were fortified with 10 mL of $FeSO_4 \cdot 7H_2O$ solution (1600 ppm Fe concentration). The maximum temperature (°C) in the middle

of the fortified dal sample during drying with the three bulb types and shaking using a Barnstead Thermolyne M49235 Bigger Bill Orbital Shaker (Sigma-Aldrich Corp.) was assessed using a thermometer (VWR Scientific, Chicago, IL, USA). The time to achieve 14% moisture for each sample was recorded for each treatment method. Both temperature and drying time were assessed three times and the mean temperature and drying time were calculated.

7.2.4. Estimation of Fe Concentration in Fortified Lentil Dal Samples by Flame Atomic Absorption Spectrophotometry (F-AAS)

The iron concentration in the fortified lentil dal was analyzed by flame atomic absorption spectrophotometry (F-AAS, Nova 300, Analytic Jena AG, Konrad-Zuse-Strasse, Neu-Ulm, Germany). Each sample was sub-sampled, and 0.5 g was digested in a 30-mL digestion tube with HNO_3-H_2O_2 using an automatic digester (Vulcan 84, Questron Technology, Ontario, CA, USA). All chemicals (nitric acid (70%), hydrogen peroxide (30%) and hydrochloric acid (37%)) used for digestion were of analytical grade. The digestion was repeated twice, with three technical replications per repeat. In the digestion chamber, a total of 72 samples were digested in each run, along with eight standards (yellow lentil laboratory check) and four blanks. Samples were first digested with HNO_3 at 90 °C for 45 min, followed by addition of 5 mL of 30% H_2O_2 and then further digested for another 65 min. The solutions were then reduced with 3 mL of 6 M HCl, followed by heating at 90 °C for 5 min prior to cooling to room temperature. All sample solutions were then diluted with deionized water to a volume of 25 mL. Six mL of each of the digested samples was then used to determine the Fe concentration as described previously (Diapari et al., 2014). The Fe concentration values were analyzed using ANOVA in SAS 9.4 to determine differences for Fe concentration among the fortified lentil samples within each of the three

fortificants at concentrations ranging from 100–3200 ppm. The LSD was calculated, and the level of significance set at $P < 0.001$.

7.2.5. Assessment of the Appropriate Dose of Fe Solution

A total of 51 different solutions of the three fortificants (17 solutions of each fortificant with Fe concentrations of 100, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400, 2600, 2800, 3000 and 3200 ppm) were prepared to fortify dehulled lentil dal samples. Ten mL of each fortificant solution at each Fe concentration was added to a 100-g dal sample and processed using the SSD (Spray–Shake–Dry) method described earlier. Twenty-five Fe solutions were prepared using the three Fe fortificants at eight concentrations (200, 400, 800, 1200, 1600, 2000, 2800 and 3200 ppm of Fe plus deionized water as the control) to assess the effect of increasing fortificant concentration on the pH of the solutions, which was measured three times for each solution using a pH meter (Oakton H_2O proof BNC pH tester, Cole-Parmer Scientific Experts, Montreal, QC, Canada). Data were analyzed using SAS 9.4.

7.2.6. HunterLab Colorimeter Measurements of Stored Fe-Fortified Dal Samples

The initial color of Fe-fortified lentil dal samples was measured using a HunterLab (Hunter Associates Laboratory Inc., Reston, VA, USA) instrument. Twenty-seven samples (nine concentrations of each of the three Fe fortificants) and one control (unfortified lentil dal) with four replications were scored for their L*, a* and b* values. Samples of each treatment were stored individually at room temperature (25 °C) for one year in clear plastic bags (Ronco, Toronto, ON, Canada), similar to methods traditionally used to store dal products. After six months and one year of storage, the L*, a* and b* values of the lentil dal again were measured to determine if any color change had occurred. The one-year storage period was considered an approximate maximum

storage period from processing to consumption by dal consumers. The L*, a* and b* values were analyzed using ANOVA in SAS 9.4.

7.2.7. Boiling Time Estimation of Fortified Lentil Dal Samples

Three fortified dal samples ($FeSO_4 \cdot 7H_20$, NaFeEDTA and $FeSO_4 \cdot H_2O$ at 1600 ppm Fe concentration) and one unfortified control were used to determine if differences existed in boiling time between fortified samples and the control. Two hundred fifty grams of each of the lentil dal samples were cooked in 1L of deionized water containing 5 g of NaCl on a single burner gas stove at 104 °C. The boiling time was recorded as the point when >90% of the dehulled lentils were softened to the point that the mixture with water produced a thickened soup, a method of preparation like that commonly used in the South Asian Region (Kohinoor et al., 2010). This study was replicated three times and data were analyzed using SAS 9.4.

7.2.8. Relative Fe Bioavailability and Phytic Acid Content of Fortified Lentils

Lentil dishes were prepared for four different samples, including Fe-fortified lentil and the control (unfortified lentil). Both fortified and control samples were rinsed with 18 MΩ deionized water. A traditional Bangladeshi lentil dish (dal) was prepared in stainless steel cookware using a traditional Bangladeshi recipe (Kohinoor et al., 2010), where salt, turmeric powder, onion, canola oil and deionized water were used as ingredients at a 15:75:5:3:2 ratio. The prepared dish was cooled to room temperature for 2 h, frozen at −80 °C for 24 h, freeze dried using a FreeZone 12 Liter Console Freeze Dry System with Stoppering Trays (Labconco, model 7759040, Kansas City, MO, USA) for 72 hours stored at room temperature (DellaValle & Glahn, 2014). Ten grams of freeze-dried dal from each dish was finely grounded and sent to the USDA-ARS Robert Holley Center for Agriculture and Health (Ithaca, New York, USA) to assess iron concentration and

bioavailability using an in vitro digestion/Caco-2 cell culture bioassay (Glahn, 2009). Total Fe concentration from the cooked lentil samples was measured using a standard HNO_3-$HClO_4$ method and atomic absorption spectrophotometry (Diapari et al., 2014). The phytic acid (total phosphorus) test kit (Megazyme International, County Wicklow, Ireland), a simple, quantitative, colorimetric and high throughput method (DellaValle & Glahn, 2014; McKie & McCleary, 2016), was used for the measurement and analysis of phytic acid in the four cooked lentil samples used for the bioavailability assessment. The ANOVA was conducted using SAS 9.4 to determine differences in iron concentration, relative iron bioavailability and phytic acid concentration among the cooked fortified lentil dishes. The LSD was calculated, and the level of significance set at $P < 0.001$.

7.3. Results and Discussion

7.3.1. Selection of Dehulled Lentil Product Type for Fortification

Prior to fortification, no significant differences in Fe concentration existed among product types (70-73 ppm Fe) (Figure 7.2b). After fortification with 1600 ppm of Fe, significant differences in Fe concentration were observed among product types (Figure 7.2c). The highest Fe concentrations were observed in fortified unpolished split (196.7 ppm) and polished football (191.5 ppm) dal. Polished football dal, which is typically polished with water and/or vegetable oil after milling, performed best in the context of uniformity of mixing with the fortificant solution and drying in the shaker - when placed in the shaker, the polished football dal moved more and agitated more quickly in the mixing trays. This helped to distribute the heat over the surface of the dal, hence it dried more uniformly and did not stick to the tray surface when wet. Selection of dehulled lentil rather than whole lentil was important, because removal of the seed coat has a

significant effect on reducing the levels of polyphenolic compounds, thereby increasing Fe bioavailability (DellaValle et al., 2013).

For commercial-scale fortification, any of the four lentil product types potentially could be fortified. Consumer demand and the relative cost and availability of the various processing techniques would be important considerations. Successful fortification to produce fortified food depends on the interactions among the food vehicle, fortificant and the fortification technique. Dehulled lentil dal is available in three colors-red, yellow and green. Red cotyledon lentil was selected for fortification since it is the most widely consumed form of lentil dal, with wide acceptability in South Asia and the Middle East (Erskine, 2009). Consumers from some countries in these regions consume lentil as an essential component of their typical daily diet. Yellow and green lentil dal samples also were fortified, and no significant differences were observed for final Fe concentration when fortified with similar concentrations of Fe fortificants (data not shown). Hence, any of red, yellow or green lentil dal could be fortified with the Fe fortificants.

7.3.2. Selection and Evaluation of the Most Suitable Fe Fortificant for Lentil

The success of food fortification programs is based on the chemistry between food vehicles and the fortificant selected to fortify foods (Mellican et al., 2003). Different food vehicles may contain different moisture levels and oxidizing agents that can react with fortificants and develop rancidity, metallic taste, off-color or degradation of vitamins, all factors that can influence bioavailability (Hurrell & Cook, 1990; Hurrell, 1997).

NaFeEDTA was shown previously to be two to four times more effective for increasing absorption of dietary Fe in humans compared to $FeSO_4$ and ferrous fumarate (Hurrell et al., 2000). It also was reported that Fe absorption was increased by using a mixture of $FeSO_4$ and NaFeEDTA, instead of NaFeEDTA alone (Thuy et al., 2003). In another study, NaFeEDTA was proven to be a

promising cost effective, water-soluble and highly bioavailable Fe fortificant that improved the Fe status of Vietnamese woman when consumed for 6 months (10 mg Fe for 6 days/week) (Thuy et al., 2003). These authors also reported that the prevalence of Fe deficiency and Fe deficiency anemia were reduced from 62.5% to 32.8% and from 58.3% to 20.3%, respectively.

The effect of NaFeEDTA-fortified wheat flour on urinary zinc extraction was studied and no effect was found in children (Amalrajan et al., 2012) Another study revealed no significant negative effects of NaFeEDTA-fortified bread (bread made with 100 g of NaFeEDTA-fortified wheat flour that contained 5 mg of Fe and was consumed as a single meal per day) consumption on Zn and Ca metabolism, and that NaFeEDTA might increase Zn absorption and Fe bioavailability from the low bioavailability diets (Davidsson et al., 1994). In another study, NaFeEDTA was shown to have no influence on absorption or urinary excretion of Mn (Davidsson et al., 1998). NaFeEDTA-fortified fish sauces also increased significantly the amounts of Hb and serum ferritin when provided to iron-deficient, anemic school children in Cambodia (Longfils et al., 2008).

The review of the safety and efficacy of different dietary strategies for improving Fe status revealed that there are no reported data that demonstrate specific adverse effects of iron-fortified food items (Prentice et al., 2017). Moreover, the daily dose of Fe is much lower from fortified food than on supplementation (Eichler et al., 2012). The joint FAO/WHO Expert Committee on Food Additives (JECFA) summarized data on the basis of acute and chronic toxicity, reproduction, carcinogenicity, genotoxicity and teratogenicity of EDTA and its salts, such as NaFeEDTA (FAO and WHO, 2007). The Committee also evaluated biochemical and toxicological aspects of using NaFeEDTA as a fortificant and stated that: (i) Fe from NaFeEDTA is released from the chelate to the common non-heme iron pool before Fe absorption; (ii) a very small fraction (1–2%) of

NaFeEDTA is absorbed intact and is rapidly and completely excreted via the kidneys in the urine; (iii) dietary Fe fortification with NaFeEDTA does not increase the risk of iron accumulation in iron-replete individuals, and has no negative influence on the absorption of other micronutrients, such as Zn; and (iv) NaFeEDTA has low oral toxicity and does not induce gene mutations when tested with bacterial and mammalian cells in vitro. In addition, considering the cost of fortificant, NaFeEDTA is more expensive compared to $FeSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot H_2O$, but its extra cost can be offset by its higher bioavailability in phytate-rich foods such as lentil (Northrop-Clewes, 2013).

7.3.3. Selection of Appropriate Methods for Fortification

Techniques Used for Lentil Fortification

Significant variation in Fe concentration was found among the five methods used to fortify lentil dal. The highest concentrations of Fe were found with the DSD (lentil dal oven dried, soaked, followed by oven drying) and SSD (lentil dal sprayed with fortificant solution followed by shaking and drying) methods (Figure 7.3). Although the highest Fe absorption into the lentil seed was observed with DSD, the discoloration (increased darkness) of the final product may cause concern in the context of expected consumer preferences and longer fortification time (Figure 7.4). The homogeneity of Fe concentration was tested by randomly selecting six samples from the mixing tray. All samples contained similar amounts of Fe (215–220 ppm) after fortification.

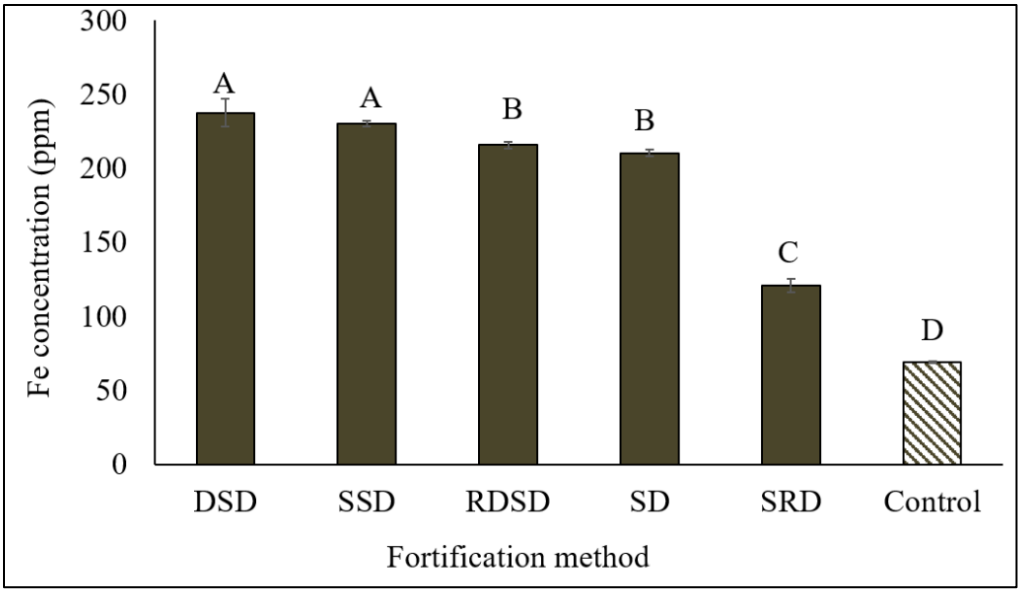

Figure 7.3. Iron concentration in polished football lentil dal fortified with FeSO4.7H2O solution (1600 ppm Fe) at 10 mL/100 g lentil dal using five different techniques. DSD = lentil dal oven dried for 10 minutes followed by soaking in fortificant solution and drying at 80 °C; SSD = lentil dal sprayed with fortificant solution followed by shaking and drying; RDSD = lentil dal rinsed, oven dried, followed by soaking in fortificant solution and then drying; SD = lentil dal directly soaked in fortificant solution followed by drying; SRD = lentil dal soaked in fortificant solution followed by rinsing with deionized water and drying. Different letters within the figure represent significant differences ($P < 0.05$).

HunterLab Colorimetric Measurements of Fe-Fortified Lentil Samples

The HunterLab results indicated significant variation for all three scales (L*, a* and b*), indicating off-color development due to fortification (Figure 7.4b-1, 7.4b-2 and 7.4b-3). The highest values for all three scales were found in the unfortified control lentil dal sample. The lowest L* value was found for the DSD sample, whereas the lowest a* and b* values were found for the

samples produced by the SD, RDSD and DSD methods. The L*, a* and b* values ranged from 46.3–52.8, 25.3–33.1 and 36.6–44.6, respectively. The shortest processing time was required with the SSD method (Figure 7.4b-4), which also generated off- color but significantly less compared to the SD, RDSD and DSD methods.

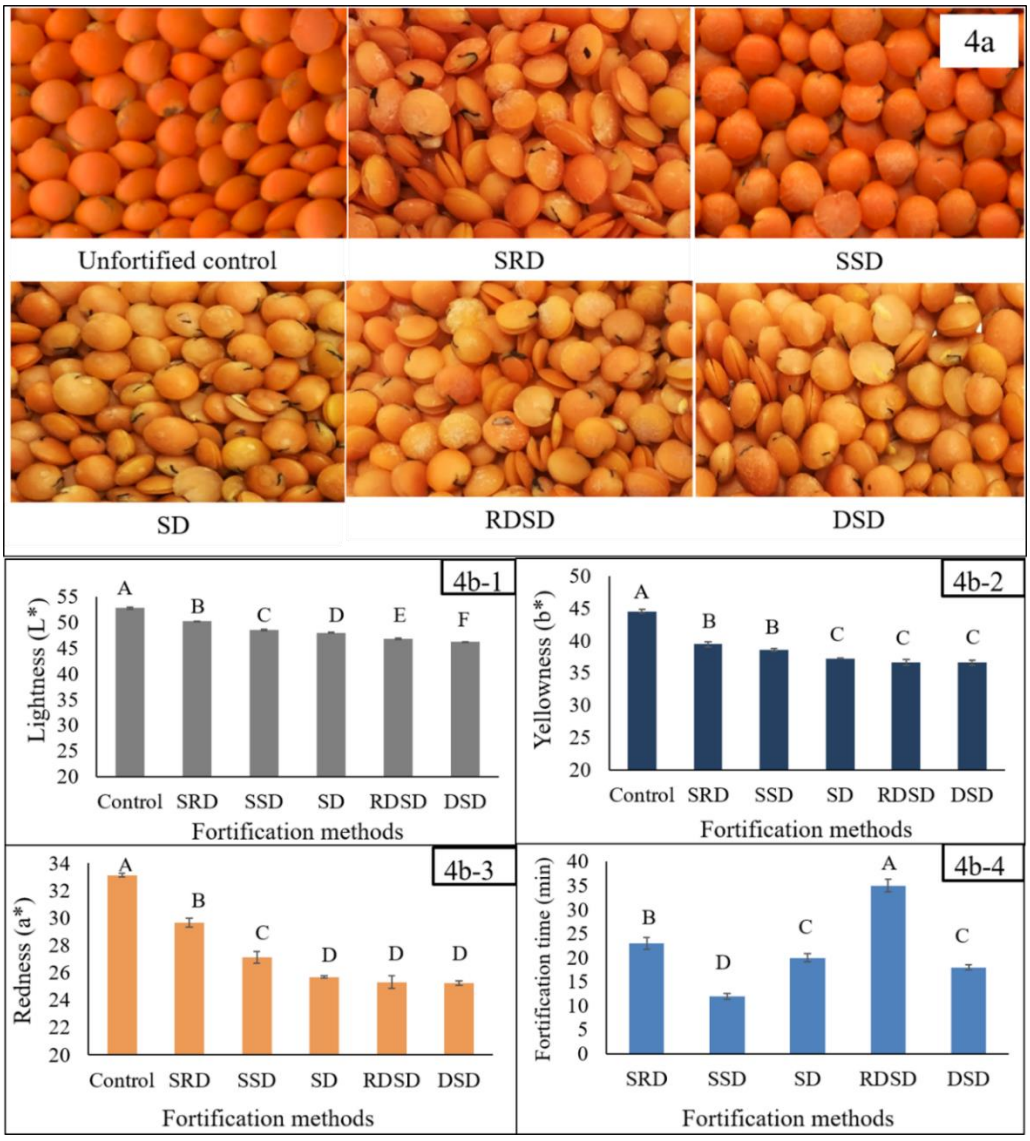

Figure 7.4. (a) Fe-fortified lentil developed by five different fortification methods: SRD = lentil dal soaked in fortificant solution followed by rinsing with deionized water and drying; SSD = lentil dal sprayed with fortificant solution followed by shaking and drying; SD = lentil dal directly

soaked in fortificant solution followed by drying; RDSD = lentil dal rinsed, oven dried, followed by soaking in fortificant solution and then drying; DSD = lentil dal oven dried for 10 minutes followed by soaking in fortificant solution and drying at 80 °C; (b1–b4) Effect of different fortification methods on changes in lightness (L*), yellowness (b*) and redness (a*) score of Fe-fortified lentil dal and on the fortification process. Different letters within each figure represent significant differences ($P < 0.05$).

Assessment of Appropriate Temperature and Duration for Drying Fortified Lentil Dal

Temperature has been shown to have a significant effect on the drying time required to achieve a level of moisture suitable for safe storage (Hayma, 2003). The results from the assessment of appropriate temperature and duration for drying fortified lentil dal showed that with an increase in temperature caused by raising the light bulb wattage, there was an increase in the temperature (°C) of both the aluminum foil tray used for fortification and the fortified lentil seed. An inverse relationship was observed between total drying time and temperature (Figure 7.5). The temperature used to dry fortified lentil dal should be optimized to avoid off-color development, as a relationship between temperature and off-color development in fortified foods has been observed (Hurrell, 1997). Using the 250-watt bulb, the temperature rose to 75 °C, which dried the fortified lentil dal in the shortest time (12–14 min). The moisture content of the fortified dal was approximately 14%, which is similar to the moisture content (%) of dehulled lentil dal (13–14)% that is commercially available in the local market (McVicar et al., 2017). During fortification, lentil dal was treated with fortificant solution and then heat was applied to dry the product. This process might reduce the level of phytate and phenolics level to some extent, and enhance the bioavailability of both Fe and Zn (Oghbaei & Prakash, 2016).

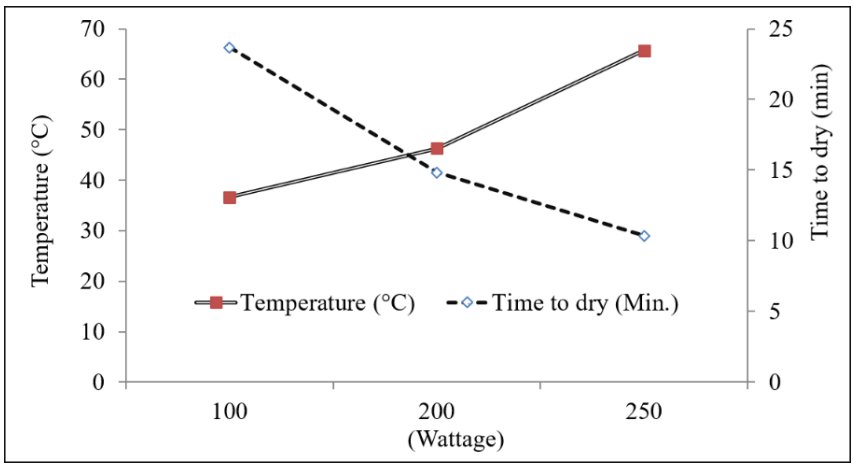

Figure 7.5. Effect of increasing light bulb wattage on temperature (°C) and drying time (min) of fortified lentil samples.

7.3.4. Assessment of the pH of Solutions Prepared with Three Fe Fortificants over a Range of Concentrations

Measurement of pH over a range of concentrations of the Fe fortificants showed an inverse relationship between pH and an increase in the concentration of Fe in the solution. The pH values of the three fortificant solutions were lower (<5) than that of deionized water (6.7). The rate of decrease of pH with an increase in Fe concentration was highest for $FeSO_4 \cdot H_2O$, followed by $FeSO_4 \cdot 7H_2O$ and NaFeEDTA (Figure 7.6). The pH of the fortificant solution would have an effect on the solubility of Fe (Mehansho, 2006). Both pH and redox potential influence the oxidation state of Fe, and both the Fe^{+2} and the Fe^{+3} form are used for fortification. Both have unfilled orbits that can react with electron-rich components, thus influencing organoleptic attributes and bioavailability (García-Casal & Layrisse, 2001). The oxidation-reduction reactions (redox potential) in fortified foods, due to the addition of Fe that can react with phenolic compounds, cause off-color development (Oghbaei & Prakash, 2016). Ferrous ion oxidizes to the ferric form as redox potential increases, but remains constant at a lower redox potential (Hurrell, 1997;

Mehansho, 2006). The solubility of $FeSO_4$ in 0.1 M HCI was reported to decrease by 74% with changes in pH over the range of 2–6, but remained constant for NaFeEDTA (García-Casal & Layrisse, 2001). In this study, an increase in $FeSO_4$ concentration resulted in a faster rate of pH reduction in comparison to NaFeEDTA. Moreover, to obtain a similar amount of soluble Fe at a specific pH, more $FeSO_4$ is required than NaFeEDTA. This may cause a major change in the organoleptic characteristics of lentil dal. This study showed that NaFeEDTA would be a better choice than $FeSO_4$ for fortification of lentil dal.

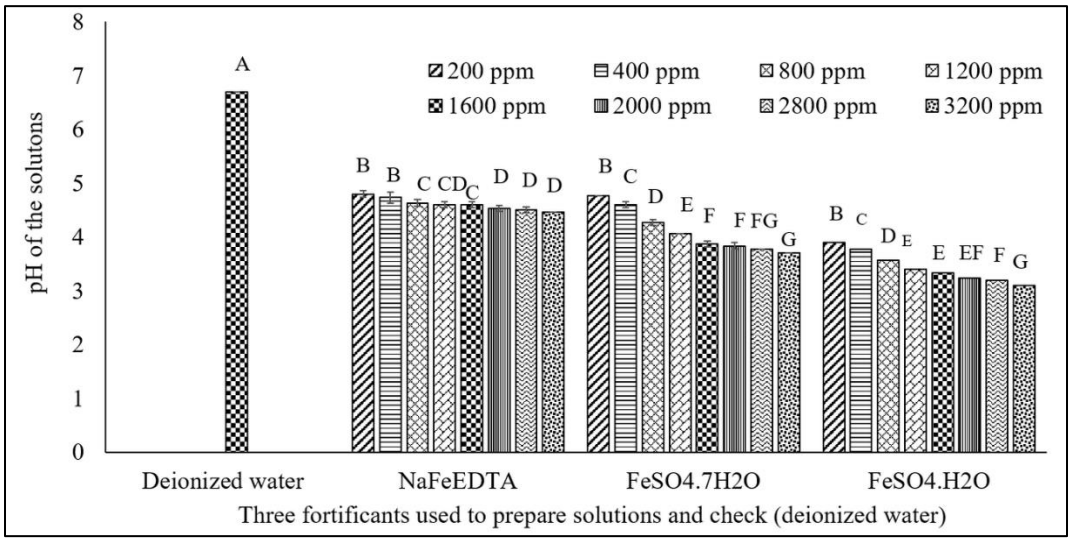

Figure 7.6. pH of Fe solutions prepared with three fortificants (NaFeEDTA, $FeSO_4.7H_2O$, and $FeSO_4.H_2O$) ranging in concentration from 200-3200 ppm. Different letters within each figure represent significant differences ($P < 0.05$).

7.3.5. Estimation of Fe Concentration in Fortified Lentil Dal Samples using F-AAS

The concentration of Fe in fortified lentil dal increased with an increase in Fe concentration in the fortificant solution (Table 7.1). Off-color development also increased gradually with an increase in the Fe concentration of the fortificant (Table 7.2).

Table 7.1. Fe concentration (ppm) in polished football lentil dal samples prepared using three fortificants ($FeSO_4.7H_2O$, NaFeEDTA and $FeSO_4.H_2O$) at concentrations ranging from 100-3200 ppm.

Fe Concentration in Fortificant Solution (ppm)	Fe Concentration in Fortified Lentil Dal		
	$FeSO_4 \cdot 7H_2O$	NaFeEDTA	$FeSO_4 \cdot H_2O$
Control	69.0 ± 0.9 [a]	69.0 ± 0.9 [a]	65.6 ± 0.8 [a]
100	76.0 ± 1.9 [a]	83.7 ± 2.5 [a]	71.8 ± 0.7 [b]
400	132.5 ± 3.2 [b]	113.2 ± 4.2 [b]	108.6 ± 1.1 [c]
800	147.9 ± 4.7 [c]	182.9 ± 5.8 [c]	151.4 ± 2.8 [d]
1200	157.8 ± 4.3 [c]	185.3 ± 5.6 [c]	185.0 ± 6.6 [e]
1600	203.6 ± 3.9 [d]	205.3 ± 2.8 [d]	207.5 ± 3.9 [f]
2000	217.5 ± 8.2 [d]	274.7 ± 5.6 [e]	261.8 ± 3.9 [g]
2400	246.6 ± 9.3 [e]	309.7 ± 10.0 [f]	322.3 ± 3.7 [h]
2800	286.7 ± 6.0 [f]	346.7 ± 5.2 [g]	363.5 ± 6.2 [i]
3200	349.0 ± 1.8 [g]	326 ± 3.1 [h]	381.7 ± 3.6 [j]

[a] Mean ± SD. Mean scores for Fe concentration followed by different letters within columns are significantly different ($p < 0.001$).

Table 7.2. Lightness (L*), redness (a*) and yellowness (b*) scores of fortified lentil samples prepared using $FeSO_4.7H_2O$, NaFeEDTA and $FeSO_4.H_2O$ at concentrations ranging from 100–3200 ppm after six months and after one year of storage.

Fe Concentration (ppm)	Lightness (L*)			Redness (a*)			Yellowness (b*)		
	Initial	After 6 months	After one year	Initial	After 6 months	After one year	Initial	After 6 months	After one year
$FeSO_4.7H_2O$ fortified samples									
Control	50.6 ± 0.4 a	50.8 ± 0.2 a	51.0 ± 0.2 a	31.5 ± 0.2 a	31.3 ± 0.2 a	30.6 ± 0.6 a	41.6 ± 1.0 a	41.2 ± 1.0 a	40.3 ± 1.0 a
200	49.9 ± 0.6 ab	50.6 ± 0.6 a	52.0 ± 0.5 b	29.7 ± 0.8 b	29.4 ± 0.8 b	28.8 ± 0.8 b	40.5 ± 0.1 b	38.9 ± 0.1 b	37.9 ± 0.1 b
800	49.6 ± 0.2 b	50.3 ± 0.1 a	51.5 ± 0.0 b	27.4 ± 0.3 c	26.8 ± 0.2 c	25.8 ± 0.3 ac	37.8 ± 0.3 c	36.4 ± 0.3 c	34.6 ± 0.3 c
1600	46.2 ± 0.5 c	46.9 ± 0.5 b	48.5 ± 0.4 c	24.6 ± 0.7 d	24.9 ± 0.6 d	25.5 ± 1.2 c	36.4 ± 0.1 d	33.9 ± 0.1 d	34.0 ± 0.1 c
2400	43.9 ± 0.2 d	44.5 ± 0.1 c	45.8 ± 0.2 c	22.6 ± 0.2 e	22.2 ± 0.1 e	21.3 ± 0.2 d	32.0 ± 0.3 e	31.2 ± 0.1 e	30.0 ± 0.4 d
3200	42.1 ±0.6 e	42.7 ± 0.6 d	43.9 ± 0.6 d	21.3 ± 0.8 f	34.4 ± 0.9 f	20.3 ± 1.2 d	30.0 ± 0.2 f	29.7 ± 0.7 f	28.6 ± 0.3 e
NaFeEDTA fortified samples									
Control	50.5 ± 0.4 a	50.8 ± 0.2 a	50.8 ± 0.2 a	31.5 ± 0.2 a	31.3 ± 0.3 a	30.6 ± 0.6 a	41.6 ± 0.3 a	41.2 ± 0.1 a	40.3 ± 0.7 a
200	50.4 ± 0.1 a	51.0 ± 0.2 a	51.0 ± 0.2 a	31.6 ± 0.7 a	31.1 ± 0.8 a	30.3 ± 0.8 a	41.9 ± 0.1 a	41.5 ± 0.1 a	40.6 ± 0.3 a
800	50.1 ± 0.2 a	50.6 ± 0.6 a	50.6 ± 0.6 b	31.1 ± 0.3 a	30.5 ± 0.2 a	29.0 ± 0.5 b	40.6 ± 0.9 b	39.3 ± 0.4 a	36.9 ± 0.8 b
1600	48.8 ± 0.1 b	52.0 ± 0.5 b	52.0 ± 0.5 b	29.4 ± 0.3 b	29.1 ± 0.2 b	28.6 ± 0.4 b	38.9 ± 0.2 c	38.2 ± 0.2 b	36.6 ± 0.5 b

2400	47.5 ± 0.2 c	50.3 ± 0.1 c	50.3 ± 0.1 c	27.5 ± 1.3 c	27.0 ± 1.2 c	26.1 ± 1.1 c	36.3 ± 0.7 d	35.8 ± 0.6 c	34.6 ± 0.6 c
3200	46.4 ± 0.5 d	51.5 ± 0.0 d	51.5 ± 0.0 c	27.8 ± 0.4 c	27.4 ± 0.4 c	26.5 ± 0.4 c	36.9 ± 0.7 d	36.4 ± 0.8 c	35.2 ± 0.9 c
$FeSO_4 \cdot H_2O$ fortified samples									
Control	50.5 ± 0.4 a	50.5 ± 0.4 a	50.8 ± 0.2 a	51.2 ± 0.3 a	31.5 ± 0.2 a	31.3 ± 0.2 a	30.6 ± 0.6 a	41.6 ± 0.3 a	41.2 ± 0.1 a
200	51.1 ± 0.5 a	51.1 ± 0.5 a	51.3 ± 0.3 b	51.7 ± 0.3 b	30.0 ± 0.7 b	29.9 ± 0.7 a	29.8 ± 0.7 b	39.9 ± 0.1 b	39.6 ± 0.1 b
800	49.3±0.7 b	49.7 ± 0.7 b	50.4 ± 0.5 b	27.9 ± 0.3 c	27.6 ± 0.4 c	27.1 ± 0.4 a	37.3 ± 0.9 c	36.9 ± 0.4 c	36.5 ± 0.8 c
1600	46.9 ± 0.7 c	47.3 ± 0.4 c	48.1 ± 0.2 c	25.4 ± 0.3 d	25.4 ± 0.3 d	25.4 ± 0.4 c	34.6 ± 0.2 d	34.6 ± 0.2 d	34.6 ± 0.5 d
2400	44.4 ± 0.6 d	44.7 ± 0.4 d	45.4 ± 0.4 d	23.3 ± 0.7 e	22.8 ± 0.7 e	21.9 ± 0.9 d	32.2 ± 0.7 e	31.9 ± 0.6 e	30.2 ± 0.6 e
3200	42.6 ± 0.3 e	42.6 ± 0.3 e	42.7 ± 0.5 e	22.7 ± 0.7 e	22.1 ± 0.7 e	21.1 ± 0.8 d	31.5 ± 0.7 e	30.9 ± 0.8 f	29.8 ± 0.9 f

[a] Mean ± SD. Mean scores for lightness (L*), redness (a*) and yellowness (b*) score followed by different Roman letters within columns are significantly different ($p < 0.001$).

7.3.6. Assessment of the Appropriate Dose of Fe

Consideration of the appropriate dose of Fe is important for optimizing the amount of fortificant required to provide a major part of the estimated average requirement (EAR) for available Fe. The WHO has suggested suitable iron compounds to fortify specific food vehicles (WHO & FAO, 2006). For instance, NaFeEDTA was suggested to fortify high extraction wheat flour, sugar, soy sauce, and fish at different rates. The bioavailability of Fe depends on the levels of various compounds present in the food vehicle, e.g. phytate, dietary fiber, tannins and other polyphenols (DellaValle & Glahn, 2014; Hurrell & Egli, 2010). These components can reduce the absorption of micronutrients, e.g. Fe, Zn. Moreover, Fe of plant origin is exclusively non-heme

Fe, which is less bioavailable than the heme Fe from animal sources (Hurrell & Egli, 2010; Minihane & Rimbach, 2002). In this study, lentil dal fortified with three different fortificants showed an increase in Fe concentration as the Fe concentration increased in the fortificant solution. Lentil seed may exhibit a wide range in Fe concentration (DellaValle et al., 2015). According to the FAO and WHO, EARs for iron having 10% bioavailability are 29.4 and 10.8 mg Fe day^{-1} for females and males, 19–50 years of age, respectively (WHO & FAO, 2006). Therefore, 50 g of unfortified dehulled lentil could provide approximately 3.5 mg of Fe, based on the Fe concentration in the control lentil dal sample. The bioavailability may decrease if the dal is prepared with spices or condiments and is eaten with other foods such as rice, bread or vegetables, which may contain phytate, polyphenols or other components that reduce the absorption of Fe. To obtain a major portion of daily Fe from food fortificants, an optimum dose should be recommended. In this study, it was shown that lentil dal fortified with 1600 ppm of Fe could provide approximately 130–140 ppm of Fe per 100 g of lentil. Therefore, 50 g of fortified lentil could provide approximately 10 mg of Fe (6.5–7 mg of Fe from the fortificant + 3.5 mg from the lentil). This could provide a major portion of the EAR. Currently, 30–45 mg kg^{-1} ferrous sulphate and 250 mg kg^{-1} NaFeEDTA are used to fortify wheat flour and soy/fish sauce, respectively (WHO & FAO, 2006).

7.3.7. HunterLab Colorimeter Measurements of Stored Fe-Fortified Dal Samples

Color attributes influence the acceptability of a food product to consumers. The L*, a* and b* scores were significantly decreased with an increase in Fe concentration provided by any of the fortificants. Significant variation in color was observed among lentil dal samples fortified with the three fortificants at any concentration. Samples fortified with NaFeEDTA had higher L*, a* and

b* scores, similar to those of the control, indicating less off-color development when compared to dal samples fortified with $FeSO_4 \cdot 7H_2O$ or $FeSO_4 \cdot H_2O$ (Figure 7.7).

The usual expectation for any Fe-fortified food product is that it does not exhibit any off-color. The dark color of the micropylar area of fortified lentil dal possibly could be used as an indicator to help consumers distinguish between fortified and unfortified lentil dal, where the micropylar region is white. The L*, a* and b* color values for the fortified lentil dal samples showed some inverse relationships with the progress of storage time (Table 2). Lightness (L*) increased slightly, but a* and b* decreased in all of the fortified lentil dal samples over time. Initially, just after fortification, the L* value ranged from 50.6 (unfortified control) to 42.2 (fortified with 3200 ppm of $FeSO_4 \cdot 7H_2O$), which was similar to the samples fortified with $FeSO_4 \cdot H_2O$ (42.6). The range was narrower for the L* value of samples fortified with NaFeEDTA (50.6 to 46.4) (Table 7.2). For all three fortificants, after 6 months and one year of storage of fortified lentil dal, there was an increasing trend in L*, but a decreasing trend for the a* and b* values (Table 7.2). The non-significant differences in the L*, a* and b* scores for the unfortified and fortified lentil samples provides assurance that the minor changes observed will not influence consumer acceptability. The L*, a* and b* values for fortified lentil dal, prepared with the three fortificants at 1600 ppm of Fe, showed numerical decreases, but these were not significant for the three storage periods, except for the L* and b* scores for the $FeSO_4.7H_2O$-fortified and the NaFeEDTA-fortified samples, respectively (Figure 7.8). These small changes may be caused by the presence of very small amounts of lipid (1.52%–2.95%) (Zhang et al., 2014) that could increase the likelihood of lipid oxidation and result in off-color development over time.

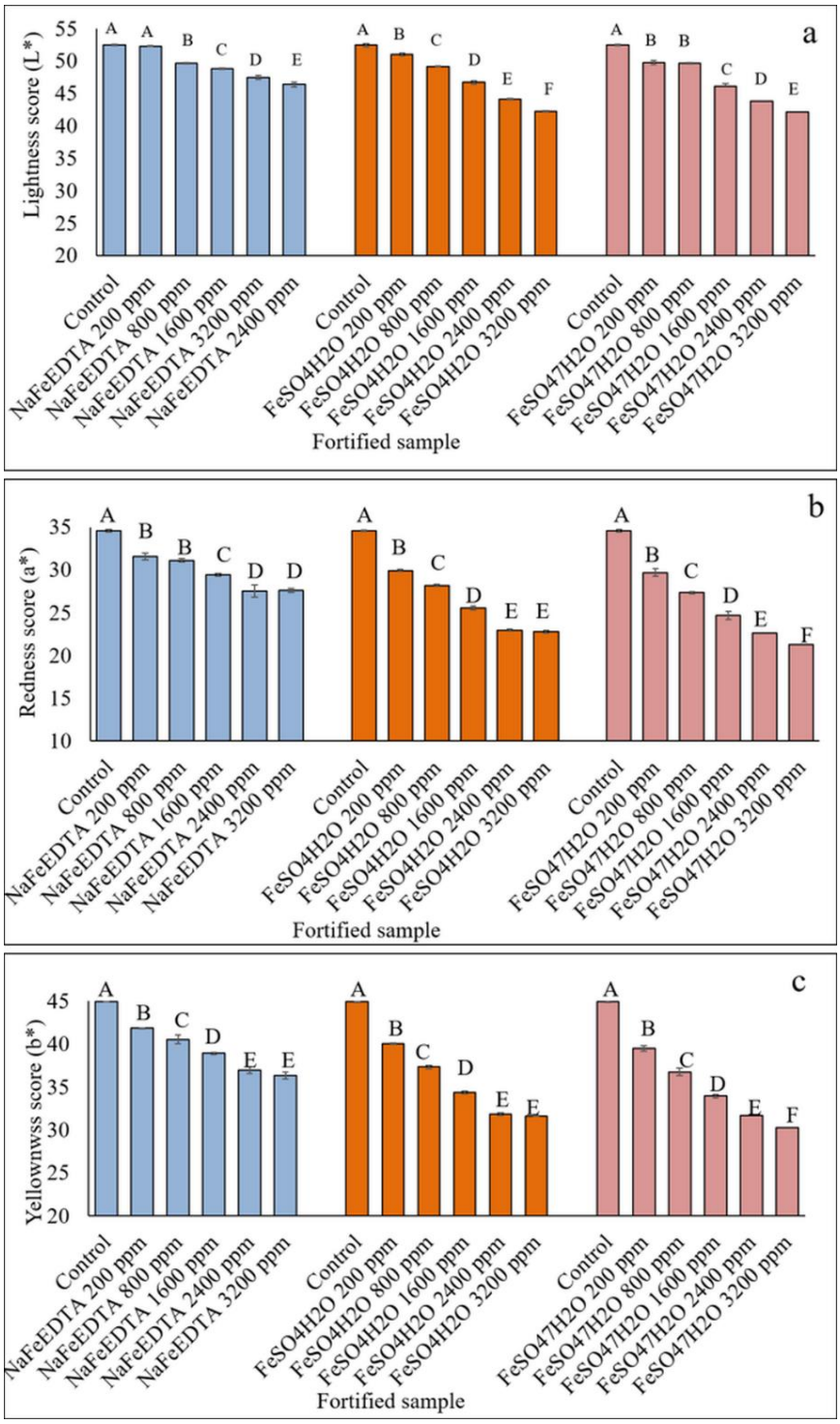

Figure 7.7. Effect of increasing Fe concentration on lightness (L*), redness (a*) and yellowness scores (b*) of lentil dal samples fortified with $FeSO_4.7H_2O$, NaFeEDTA and $FeSO_4.H_2O$ at five

different concentrations ranging from 200-3200 ppm. Different letters within each figure represent significant differences ($P < 0.05$).

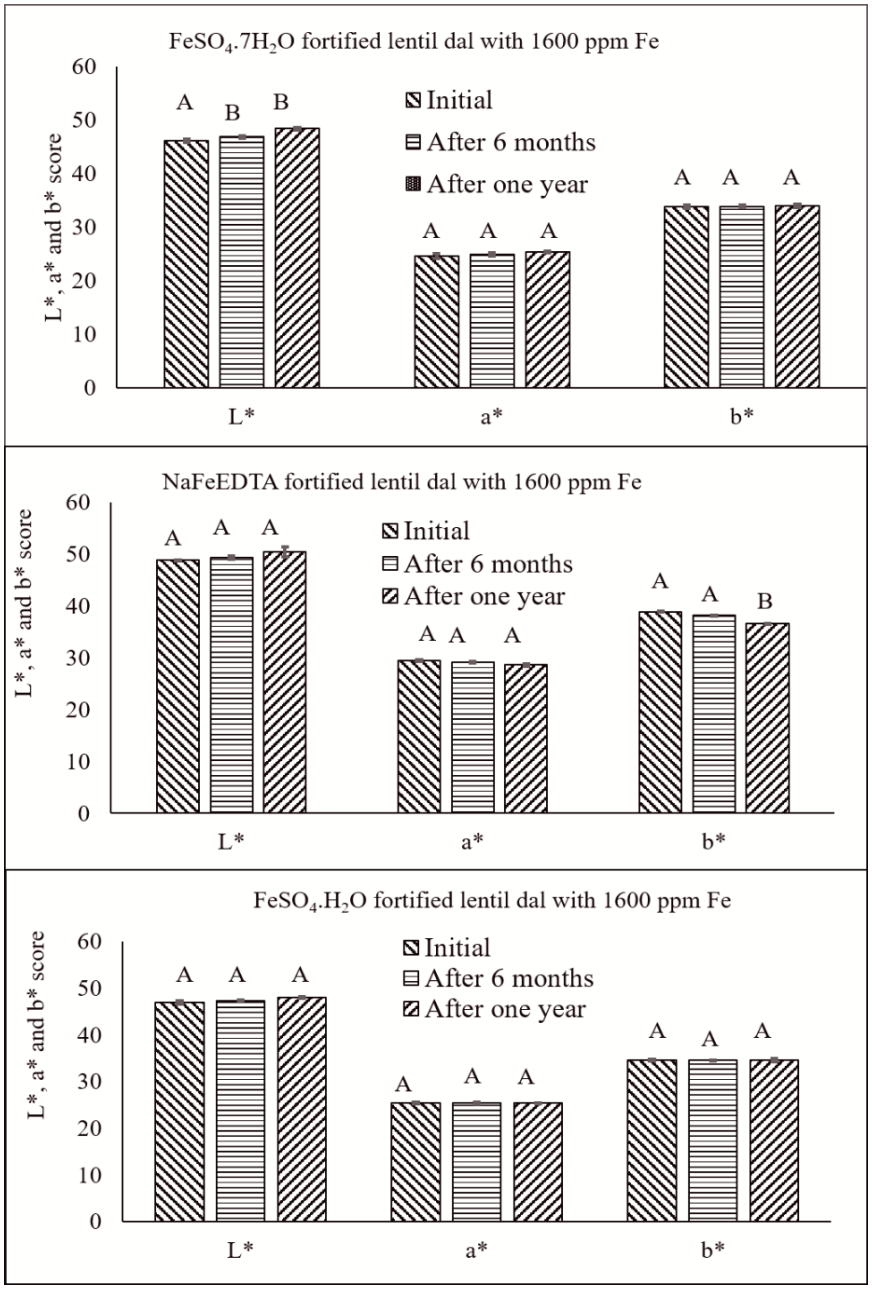

Figure 7.8. Effect of storage time on changes in L*, a* and b* score of football lentil samples fortified with 1600 ppm of Fe using $FeSO_4.7H_2O$, NaFeEDTA and $FeSO_4.H_2O$. Different letters within each figure represent significant differences ($P < 0.05$).

7.3.8. Boiling Time Estimation of Fortified Samples Compared to the Unfortified Control

The boiling time of lentil dal is important and may influence consumer acceptability due to energy and time consumption during cooking. Compared to unfortified lentil dal, the fortified lentil dal should take equal or less time to cook, and have similar texture, taste and appearance after cooking. Among the four samples that were cooked to determine the variability in boiling time, all had similar cooking times (Figure 7.9). Fortification had no significant influence on the boiling time of $FeSO_4 \cdot 7H_2O$-, $FeSO_4 \cdot H_2O$- or NaFeEDTA-fortified samples compared to the control.

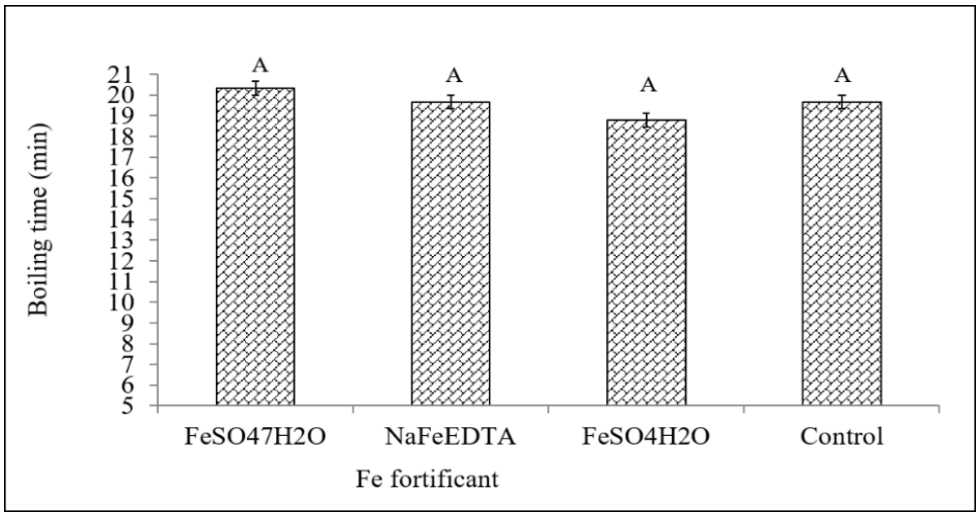

Figure 7.9. Effect of the three fortificants solution used to prepare three fortified lentil samples ($FeSO_4.7H_20$, NaFeEDTA and $FeSO_4.H_2O$) on boiling time compared with one unfortified control sample. Different Roman letters within each figure are significantly different ($p < 0.05$).

7.3.9. Iron Concentration, Relative Fe Bioavailability and Phytic Acid Concentration of Fortified Lentils

Significant differences were observed among fortified and unfortified lentil samples in Fe concentration, relative Fe bioavailability and phytic acid concentration (Table 7.3). Similar iron and phytic acid concentrations were observed in FeSO4.7H_2O- and NaFeEDTA- fortified samples. The unfortified lentil samples were statistically different than the three fortified samples for all four measurements. The relative bioavailability was similar for all three fortified lentil dal samples. Iron concentration and relative Fe bioavailability ranged from 68.7 to 238.5 ppm and 68.3 to 104.9, respectively. The relative Fe bioavailability of the three cooked fortified lentil dal samples was 1.4 to 1.5 times higher than that of unfortified cooked lentil sample (control). Phytic acid concentration ranged from 7.2 to 8.0 mg g^{-1}.

Table 7.3. Mean iron (Fe) concentration (ppm), relative bioavailability [ng ferritin (mg protein)-1] and phytic acid concentration (mg g-1) of four cooked freeze-dried lentil samples.

Cooked Lentil Sample	Fe Concentration (ppm)[a]	Ferritin Formation [ng Ferritin (mg Protein)$^{-1}$][a]	Relative Fe Bioavailability (% Control Lentil)[a]	Phytic Acid (mg g^{-1})[a]
Unfortified dehulled lentil	68.7 ± 0.3 [a]	12.7 ± 1.0 [a]	68.3 ± 14.8 [a]	8.0 ± 0.1 [a]
NaFeEDTA fortified (1600 ppm Fe)	230.8 ± 8.5 [b]	17.4 ± 2.7 [b]	100.5 ± 7.5 [b]	8.0 ± 0.2 [a]
$FeSO_4 \cdot H_2O$ fortified (1600 ppm Fe)	220.5 ± 2.1 [c]	17.6 ± 2.2 [b]	104.9 ± 16.7 [b]	7.2 ± 0.1 [c]
$FeSO_4 \cdot 7H_2O$ fortified (1600 ppm Fe)	238.5 ± 4.7 [b]	21.2 ± 1.9 [b]	103.4 ± 10.4 [b]	7.4 ± 0.1 [b]

[a] Mean ± SD. Mean scores for Fe concentration, bioavailability [ng ferritin (mg protein)-1], relative Fe bioavailability (% control lentil) and phytic acid (mg g-1) followed by different letters within columns are significantly different ($P < 0.001$).

7.4. Conclusion:

Fortification of lentil dal is more complex than fortifying flour, beverages and most other food products due to the requirement to apply fortificant solution to the surface of the dal. Considering all of the results from the various experiments, it was concluded that lentil dal could be used as a vehicle for Fe fortification and that NaFeEDTA was the most suitable Fe fortificant for lentil dal. These results represent baseline data for the commercial production of Fe-fortified lentil dal. This research is unique in the context of lentil dal fortification, and will be followed by sensory evaluation to select the most appropriate fortificant after evaluation of overall acceptability. Results from sensory evaluation with both uncooked and cooked fortified lentil dal compared favorably with the control and will be described in a subsequent manuscript. Community-based efficacy and effectiveness studies with fortified lentil in the target populations will be required. The bioavailability of fortified lentil in a large-scale human trial also could be evaluated to obtain an empirical estimate of the amount of Fe required to provide a major portion of the EARs for Fe in regions where Fe deficiency exists.

Prologue to chapter 8

In the previous study, the colorimetric analysis with fortified lentil revealed that Fe fortification of dehulled lentil influenced and changed organoleptic attributes that can influence consumer acceptability. Sensory evaluation by consumers can help with selection of the most acceptable product considering changes of organoleptic characteristics of food or food products. In the following chapter the results of research from sensory evaluation by consumers will be described in consideration of the potential effect of Fe fortification on changes of appearance, odour, taste, texture and overall acceptability of fortified lentils.

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CHAPTER 8

SENSORY ACCEPTABILITY OF IRON-FORTIFIED RED LENTIL (*LENS CULINARIS* MEDIK.) DAL

8.1. Introduction

Interest in the consumption of low-calorie foods or vegetarian dishes is increasing throughout the world. This includes grain legumes (pulses), which play important roles in human health by providing energy, dietary fibre, protein, minerals and vitamins (Gramatina et al., 2012). The two most widely consumed grain legumes, soybean and peanut, contain substantial amounts of edible oil. Most other grain legumes, including lentil (*Lens culinaris* Medik.), consist primarily of protein and carbohydrate, which includes dietary fibre (5.1-26.6%) (Grusak, 2009). Global annual lentil production reached ~4.9 million tonnes in 2014 (FAOSTAT, 2017). Overall, about 56% of the lentil produced in the world is consumed in Asia (Kumar et al., 2013), where it is considered a staple food. The consumption of lentil is increasing because it is fast cooking and an inexpensive source of protein compared to animal protein.

Improvement of the micronutrient content of staple crops, including lentil, is a means to mitigate Fe deficiency in the human diet. Several approaches have been explored to improve the Fe status of food crops, including fortification, biofortification and genetic transformation. Fortification of foods with micronutrients and vitamins is considered one of the most effective ways to prevent human nutritional deficiencies (Bishai & Nalubola, 2002). Iron is an essential micronutrient in the human body, but more than two billion people, particularly in the developing world, are anaemic, many due to Fe deficiency. Fortification of food with Fe has become a suitable and recommended approach to prevent and eradicate Fe deficiency (Mehansho, 2002). Food

fortification is also mandatory now for various micronutrients and vitamins by legislation in 84 countries (Food Fortification Initiative, 2015).

Fortification with Fe may cause organoleptic changes in food products, resulting, for example, in a metallic aftertaste, unacceptable flavour, undesirable colour changes or degradation of vitamins (Mehansho, 2006). Sensory evaluation helps to determine the factors that affect the flavor of foods or drinks and the acceptability to the preferences of consumers (de Melo et al., 2009) and has become important as a means of assessing market acceptability. A series of techniques are used to measure the human response to foods and reduce the bias effects of brand identity and other information that may impact stakeholder perception (Lawless & Heymann, 2010). The Institute of Food Technologists and the American Society for Testing and Materials define sensory evaluation as a scientific method used to evoke, measure, analyze and interpret responses to products as perceived through sight, smell, touch, taste and sound (Stone & Sidel, 2004).

The aim of this study was to investigate the sensory properties of both uncooked and cooked, Fe-fortified, dehulled red lentil (dal) as determined by panelists that were familiar with lentil-based meals. The goal was to use this information to select the most appropriate Fe fortificant from the consumer point of view. In our previous Fe-fortification study, dehulled red lentil (dal) was fortified with three different Fe-fortificants ($FeSO_4 \cdot 7H_2O$, NaFeEDTA and $FeSO_4 \cdot H_2O$) with three different fortificant Fe concentrations (Podder et al., 2017). After a series of experiments, we identified an appropriate method and optimal dosages for Fe concentration in lentil. Fortified red lentil samples have a distinguishable appearance compared to unfortified lentil. Any change in the organoleptic properties of fortified lentil can be evaluated by consumers, and their remarks can provide valuable information to guide food scientists or product developers with respect to

commercial food production. The key sensory attributes in this context are appearance, taste, odour, texture and overall acceptability. To the best of our knowledge, this is the first report of a sensory evaluation by panelists of Fe-fortified lentil. In this study, uncooked and cooked samples were evaluated by lentil consumers in two locations, in Saskatoon, Canada (with panelists originally from five South Asian countries) and in Dhaka, Bangladesh (local panelists). Preferences in both locations were compared to determine if the groups had different sensory perceptions.

8.2. Materials and Methods

8.2.1. Ethical review

The study was approved by the Research Ethics Office, University of Saskatchewan, Canada (certificate number, BH 14-320) and by the Ethical Review Committee of the James P. Grant School of Public Health, BRAC University, Bangladesh (ethics approval reference number-56).

8.2.2. Preparation of uncooked and cooked lentil samples

Commercial red lentil dal in the unsplit form (known as football type) was fortified with three different fortificants ($FeSO_4 \cdot 7H_2O$, NaFeEDTA and $FeSO_4 \cdot H_2O$), each at three different fortificant Fe concentrations (800, 1600 and 2800 ppm). This resulted in a total of nine uncooked fortified lentil samples plus an unfortified control sample (Figure 8.1). The fortification procedure was reported in our previous article (Podder et al., 2017) which provides details on choices of suitable fortificants, appropriate methods for lentil fortification, colorimetric study results of Fe fortified lentil, assessment of proper dose of Fe fortificant, fortification duration, shelf-life and boiling time of fortified lentil, as well as the Fe bioavailability and phytic acid concentration of

Fe-fortified lentil. It also showed that 50 g of Fe-fortified dehulled lentil dal fortified with 1600 ppm of Fe could provide approximately 11-12 mg of Fe, including the intrinsic Fe present in the unfortified lentil and Fe added from the fortificants. This amount of Fe can meet the WHO and FAO recommendation of 29.4 and 10.8 mg Fe day^{-1} for females and males, respectively, considering 10% bioavailability (WHO & FAO, 2006).

Figure 8.1. Images of the uncooked lentil samples, including the unfortified control (left-most column) and samples fortified with $FeSO_4 \cdot 7H_2O$ (top row), NaFeEDTA (middle row) and $FeSO_4 \cdot H_2O$ (bottom row) at fortificant Fe concentrations of 800, 1600 and 2800 ppm.

For sensory evaluation of cooked lentil, sub-samples of the unfortified lentil and of lentil treated with each fortificant at a concentration of 1600 ppm Fe were used to prepare a typical South

Asian lentil dish (Figure 8.2). The recipe (Kohinoor et al., 2010) used to prepare the dish involved cooking 500 g of each of the four lentil samples (unwashed) for 25 min in 2.5 L of deionized water. The result was a semi-thick soup, a south Asian traditional lentil dish to which 20 g of table salt, 10 g of turmeric powder, 30 mL of canola oil and 100 g of chopped onion were added for the last five minutes of cooking. Food samples were prepared in the food sensory laboratory of the University of Saskatchewan and the Food Processing Laboratory of International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) in Dhaka, Bangladesh. Samples were cooled to room temperature, and then portioned in cups with lids. Four cooked samples were served at room temperature in a single tray.

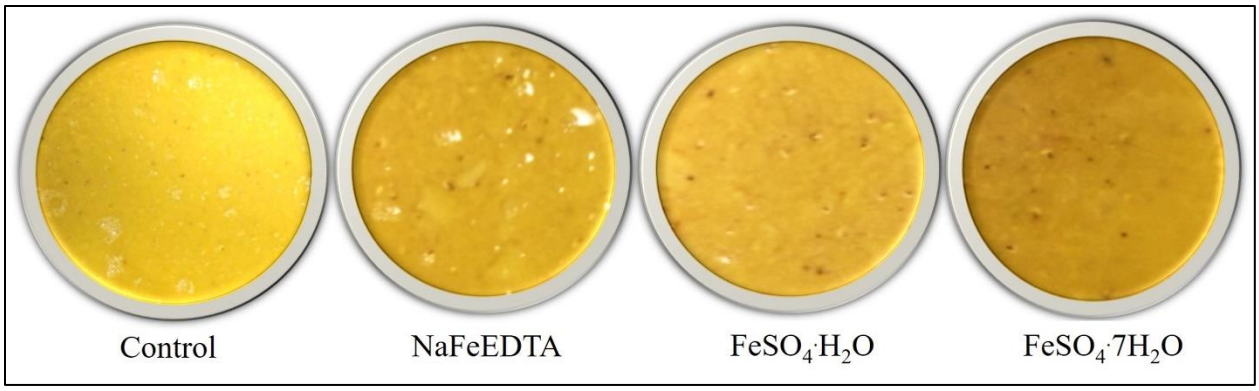

Figure 8.2. Four cooked dal samples including the control (left) and samples prepared using each of the three fortificants ($FeSO_4 \cdot 7H_2O$, $FeSO_4 \cdot H_2O$ and NaFeEDTA) at a fortificant Fe concentration of 1600 ppm.

8.2.3. Selection of panelists

The sensory evaluation was performed in two locations. In Canada, 45 untrained panelists (aged 18-57 years) were recruited from staff and students at the University of Saskatchewan. All panelists were originally from South Asia, specifically Bangladesh (18), India (15), Nepal (5), Sri

Lanka (5) and Pakistan (2). The region has a long tradition of lentil consumption in a form similar to that used in this study. The sensory evaluation was conducted twice with these participants. In Bangladesh, 101 untrained panelists (aged 18-60 years) were recruited, all of whom lived in Dhaka, Bangladesh and were employed at the James P. Grant School of Public Health (JPGSPH), BRAC University.

Consent (verbal and written) was obtained from all participants. Participants were excluded if they were less than 18 or over 60 years of age or if they were suffering from a cold, fever or gum inflammation. Other exclusion criteria included those taking medicines for treatment of cancer or thyroid, neurologic or psychiatric ailments. Anyone with an allergy to lentil, with Fe deficiency or who was pregnant was excluded due to potentially altered taste perception. In Bangladesh, panelists who had used *Paan/Jarda* (a preparation combining betel leaf with betel nut and tobacco) within one hour of the sensory evaluation were excluded due to potential residual psychoactive effects.

8.2.4. Sensory evaluation and data collection

In Saskatoon, participant consent and sensory evaluation forms were provided to all participants to start the evaluation. The consent form described the purpose of the sensory evaluation studies, food preparation procedures, potential risks of evaluation, confidentiality of each participant's evaluation, and procedures should a participant choose to withdraw from the evaluation. In Bangladesh, the data collection procedure was similar, except that another structured questionnaire was supplied to the evaluators to collect data on socio-economic indicators such as monthly income, education and household status. In both locations, lentil consumption frequency, purchasing frequency and place of purchase were recorded when possible to assist in determining

the lentil consumption pattern among panelists. The sensory evaluation form comprised three parts. First, some general information on participants was recorded as coded information, including participant, age, sex, date and sample code. The second part comprised questions using a nine-point hedonic scale (1 = dislike extremely to 9 = like extremely) to describe the appearance, odour and overall acceptability of the uncooked samples, and the appearance, odour, taste, texture and overall acceptability of the cooked lentil samples. In the third part, any additional opinions of participants were documented (verbatim), whether positive or negative. Participants were requested to carefully read and then sign the consent form prior to starting their evaluation. For the Bangladesh location, all forms and questionnaires were translated into Bangla (the most commonly spoken language). This ensured that the meaning of questions was not altered; back-translation to English also was performed by the investigators. Furthermore, data in Bangladesh were collected by 11 research assistants in face-to-face interviews (as opposed to participants filling out their own forms in Saskatoon).

In Saskatoon, data collection was completed in a single day (9:30 a.m. to 3:30 p.m.) for each replication in seven individual booths at the University of Saskatchewan Food Sensory Laboratory. All booths were well illuminated with white light and separated from each other to avoid any communication among participants. In Bangladesh, sensory evaluation also was completed in a single day (9:30 a.m. to 2:30 p.m.). A total of 11 partitioned booths were constructed for data collection and all tests were performed under uniform white light conditions. The study investigators were present for the purpose of overall supervision and monitoring. In both locations, participants received cooked lentil dal prepared from approximately 17 g of uncooked lentil from each of the four lentil samples. If they consumed all four samples, participants would have ingested a total of 11.40 mg of Fe (10.25 mg from the total of the three fortified samples +

1.15 mg from the unfortified check). The tolerable upper intake level of iron per day for adults (19+ years) is 45 mg/day (U. S. Department of Health and Human Services, 2016), and thus we did not expect any side effects; moreover, the added fortificants were of food grade and have been approved by the FAO.

Sensory evaluation was conducted in single sessions to avoid reporting bias. Uncooked lentil samples (50 g) were presented in white plastic containers labeled with three-digit codes. All of the uncooked samples (n =10) were displayed on a single tray, all at once and in random order. All uncooked samples (including the control) were assessed visually because this is the form of lentil presented to panelists in the markets or supermarkets where purchase decisions are made. Cooked lentil samples (~75 mL; n = 4) also were presented in random order in white plastic containers labeled with three-digit codes. Water was provided to allow participants to conduct oral rinsing before and after testing each of the dishes.

8.2.5. Panelists' consistency assessment for sensory data based on the Cronbach's alpha coefficient

Cronbach's alpha (CA) has proven to be the best approach for assessing the internal consistency reliability (ICR) of a sensory panel (Pinto et al., 2014). Its use is important for statistical expression of a panels' consistency and reliability in multi-item evaluations scores. Cronbach's alpha is a numeric expression ranging between 0 and 1 (Tavakol et al., Mohagheghi, & Dennick, 2008) with the resulting CA value considered an index of reliability (Tavakol & Dennick, 2011). Reliability estimates measure the index of measurement error by squaring the correlations (α values) and subtracting them from 1.00 (Kline, 1994). The value after subtraction shows the error variance in the score. We assessed the ICR of the sensory scores from 45 and 98

panelists in Saskatoon and Bangladesh, respectively, for the ten uncooked and four cooked samples. An acceptable CA value range, as reported in a variety of studies (Bland & Altman, 1997; DeVellis, 2003), is 0.70 to 0.95.

8.2.6. Data analysis

The data from the two repeats from Saskatoon were combined and mean data were used in the analysis. Among the 101 panelists in Bangladesh, three did not complete the sensory evaluation form and their data were excluded from the analysis. Statistical analysis of the sensory data was conducted using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). For the questions regarding sensory attributes (appearance, odour, taste and texture) and overall acceptability, one-way analysis of variance (ANOVA) was used to verify the differences between the samples (including the control). The least significant difference (LSD) was calculated and the level of significance set at $P < 0.05$. The Cronbach's alpha was analysed using IBM SPSS version 24 (IBM Statistics, Version 24, 2016). Data were analyzed separately for the two sites due to different panel sizes.

8.3. Results

8.3.1. Consumer demographics from Dhaka, Bangladesh and Saskatoon, Canada

Demographic data for the study participants are shown in Table 8.1. The mean ages of the panelists in Saskatoon and Bangladesh were 35 years (range 18 to 57) and 30 years (range 19 to 49), respectively. About 40% (Saskatoon) and 66.3% (Bangladesh) of the participants were in the 25 to 34-year age group. Almost half (45.5%) of the Bangladesh participants had a monthly income of 30,000 to 39,000 Bangladesh taka (BDT) (~500 to 650 CAD). In Bangladesh, 28.7% of panelists had post-graduate degrees, and 10.9% had completed an undergraduate degree. Half (49.5%) of

the panelists from Bangladesh had completed technical degrees (Master of Public Health, Master of Development Studies, MBA, CA, etc.) after completing their undergraduate degrees. The remaining 10.9% of panelists had completed secondary or elementary school. All Saskatoon panelists in the study had annual incomes ranging from 21,000 to 45,000 CAD and had completed at least an undergraduate degree.

8.3.2. Consumer attitudes toward lentil consumption

In both Saskatoon and Bangladesh, the majority of participants consumed lentil at least weekly (Table 8.2). The most common frequency of lentil purchase was monthly (63.3 and 66.7% of respondents in Bangladesh and Saskatoon, respectively). In Bangladesh, 37.6% of panelists bought lentil at local markets (where lentil is usually sold by scooping from open sacks), 13.9% at grocery stores (where lentil is usually sold in small packets of various sizes), and 15.8% at both. In Saskatoon, all panelists bought lentil from supermarkets. Approximately one-third of the Bangladesh panelists did not buy lentil, but were regular consumers, and ate lentil prepared by someone else (in homes or restaurants).

8.3.3. Sensory responses to the attributes of uncooked fortified lentil dal

Sensory scores obtained from panelists in both Saskatoon and Bangladesh for the ten uncooked samples are shown in Figure 8.3, along with their range, dispersion and outliers. Consumer responses varied significantly for all three attributes (appearance, odour, and overall acceptability) scored in both locations. For all attributes, the highest score was observed for unfortified control lentil samples, followed by NaFeEDTA-fortified samples at 800 ppm Fe, except in Bangladesh where NaFeEDTA-fortified lentil with 2800 ppm Fe received the highest score, but this score was not significantly different from that of NaFeEDTA-fortified lentil at 800 ppm Fe.

The lowest scores were obtained for the $FeSO_4 \cdot 7H_2O$- and $FeSO_4 \cdot H_2O$-fortified samples at 2800 ppm Fe at Saskatoon and Bangladesh, respectively. In both locations, the NaFeEDTA-fortified sample at the highest dose (2800 ppm Fe) scored similarly or higher to the $FeSO_4 \cdot 7H_2O$- and $FeSO_4 \cdot H_2O$-fortified samples fortified with the lowest Fe dose (800 ppm) for all three attributes.

Table 8.1. Panelist demographics for Bangladesh and Saskatoon study sites

Background characteristics		Saskatoon	Bangladesh
		Number (%)	Number (%)
Gender	Male	28 (62.2)	53 (54.1)
	Female	17 (37.8)	45 (45.9)
Age (in years)	18-24	7 (15.6)	12 (12.2)
	25-34	18 (40.0)	65 (66.3)
	35-44	10 (22.2)	16 (16.4)
	45+	10 (22.2)	5 (5.1)
Panelist's attitudes toward lentil consumption			
Observation	Consumer responses	Number (%)	Number (%)
How frequently do you eat lentil?	Every day	5 (11.0)	18 (18.4)
	Every week	27 (60.0)	55 (56.1)
	Every month	13 (29.0)	25 (25.5)
How frequently do you buy lentil?	Every week	9 (20.0)	6 (6.1)
	Every month	30 (66.7)	63 (64.3)
	Do not buy	6 (13.3)	29 (29.6)
From where do you buy lentil?	Local market	--	37 (37.8)
	Grocery store	45 (100.0)	16 (16.3)
	Both sources	--	16 (16.3)
	Do not buy	--	29 (29.6)

In general, the box plots for the control sample had a smaller range and less dispersion than those for the nine fortified samples. The box plot skewed either to the right (positive skew) or was neutral for nearly all samples fortified with NaFeEDTA, with the average score being significantly ($P < 0.05$) lower than that of $FeSO_4 \cdot 7H_2O$- and $FeSO_4 \cdot H_2O$-fortified samples. The boxplots for the NaFeEDTA-fortified samples (800 and 1600 ppm Fe) skewed either to the right (positive skew) or were neutral (except for the NaFeEDTA-fortified lentil sample fortified with 2800 ppm Fe) and their mean values were significantly different ($P < 0.05$), but closer to the control compared to samples fortified with $FeSO_4 \cdot 7H_2O$ or $FeSO_4 \cdot H_2O$ for all three attributes at both locations.

8.3.4. Sensory response to the attributes of cooked, fortified lentil dal

Significant variation in acceptability was observed for the four cooked lentil dal samples evaluated by panelists at both locations (Figure 8.4). In Saskatoon, the unfortified cooked sample received the highest mean score for all five attributes. In Bangladesh, the NaFeEDTA- and $FeSO_4 \cdot 7H_2O$ fortified samples received the highest scores for appearance and overall acceptability, respectively. Again, the NaFeEDTA- and $FeSO_4 \cdot 7H_2O$ fortified samples received the highest scores for both taste and texture. Odour was scored highest for the unfortified control and $FeSO_4 \cdot 7H_2O$ -fortified samples. The numerical differences between scores across all samples for the five attributes were small. Specifically, the box plots for cooked samples for both locations showed less dispersion and a narrower range of sensory scores for all attributes compared to those for the uncooked samples. All samples scored well (above 6.0) for all five attributes. In Bangladesh, there were no significant differences between scores for control and NaFeEDTA- and $FeSO_4 \cdot 7H_2O$ fortified samples for appearance, texture or overall acceptability. In Saskatoon, mean

values for the NaFeEDTA-fortified samples were consistently the closest to the mean value for the control sample. Both the $FeSO_4 \cdot 7H_2O$-and $FeSO_4 \cdot H_2O$-fortified samples were significantly different than the control for all attributes, with the exception of the overall acceptability of $FeSO_4 \cdot 7H_2O$-fortified samples.

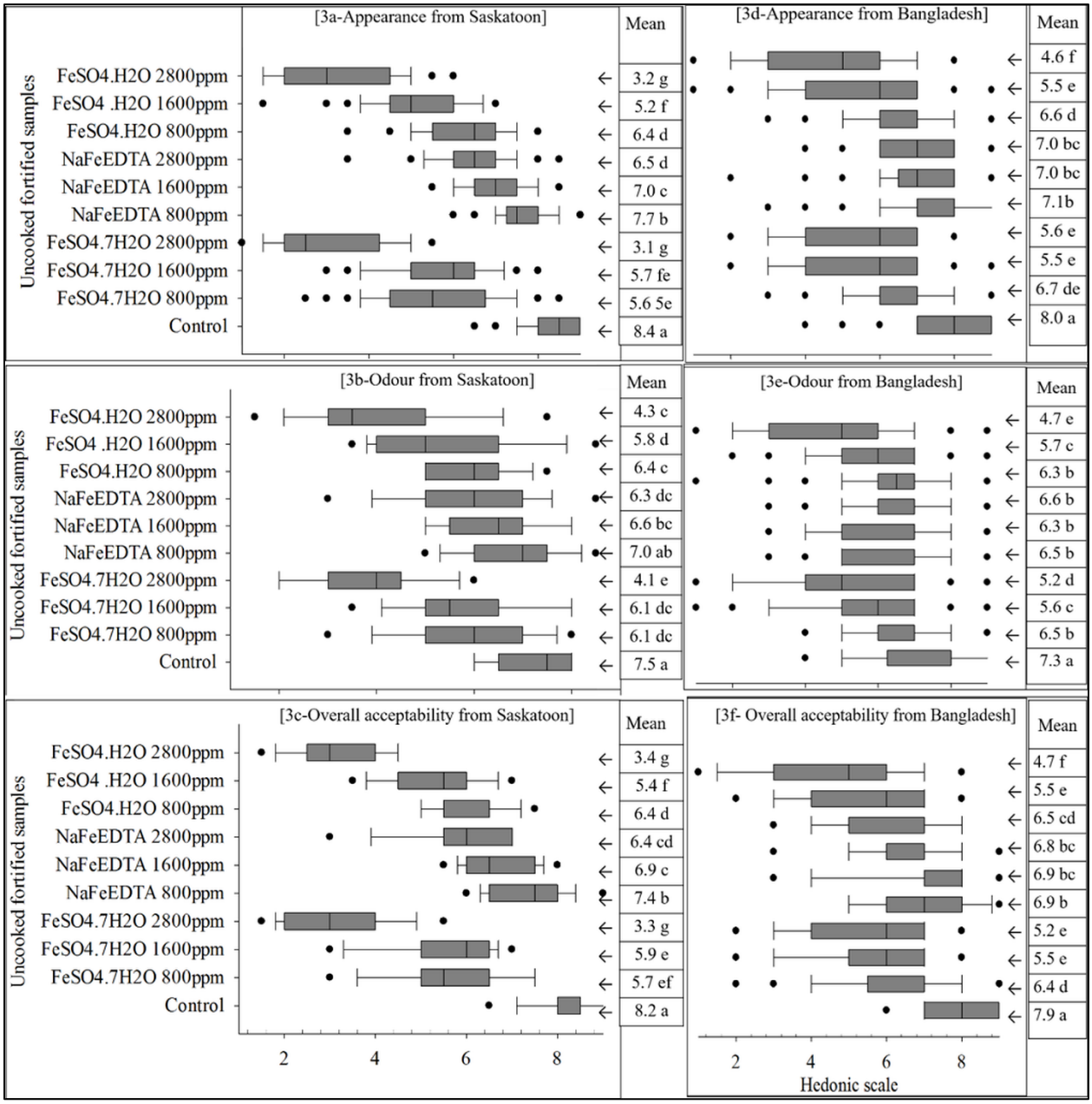

Figure 8.3. Box plot analysis of hedonic scores obtained for ten uncooked lentil dal samples (three fortificants × three Fe concentrations (800, 1600, and 2800 ppm) plus one unfortified control) evaluated for appearance (a,d), odour (b,e) and overall acceptability (c,f) by 45 and 98 panelists in Saskatoon (a to c) and Bangladesh (d to f), respectively. Different letters after mean values indicate significant differences between treatments ($P < 0.05$). A nine-point hedonic scale (1 = dislike extremely to 9 = like extremely) was used.

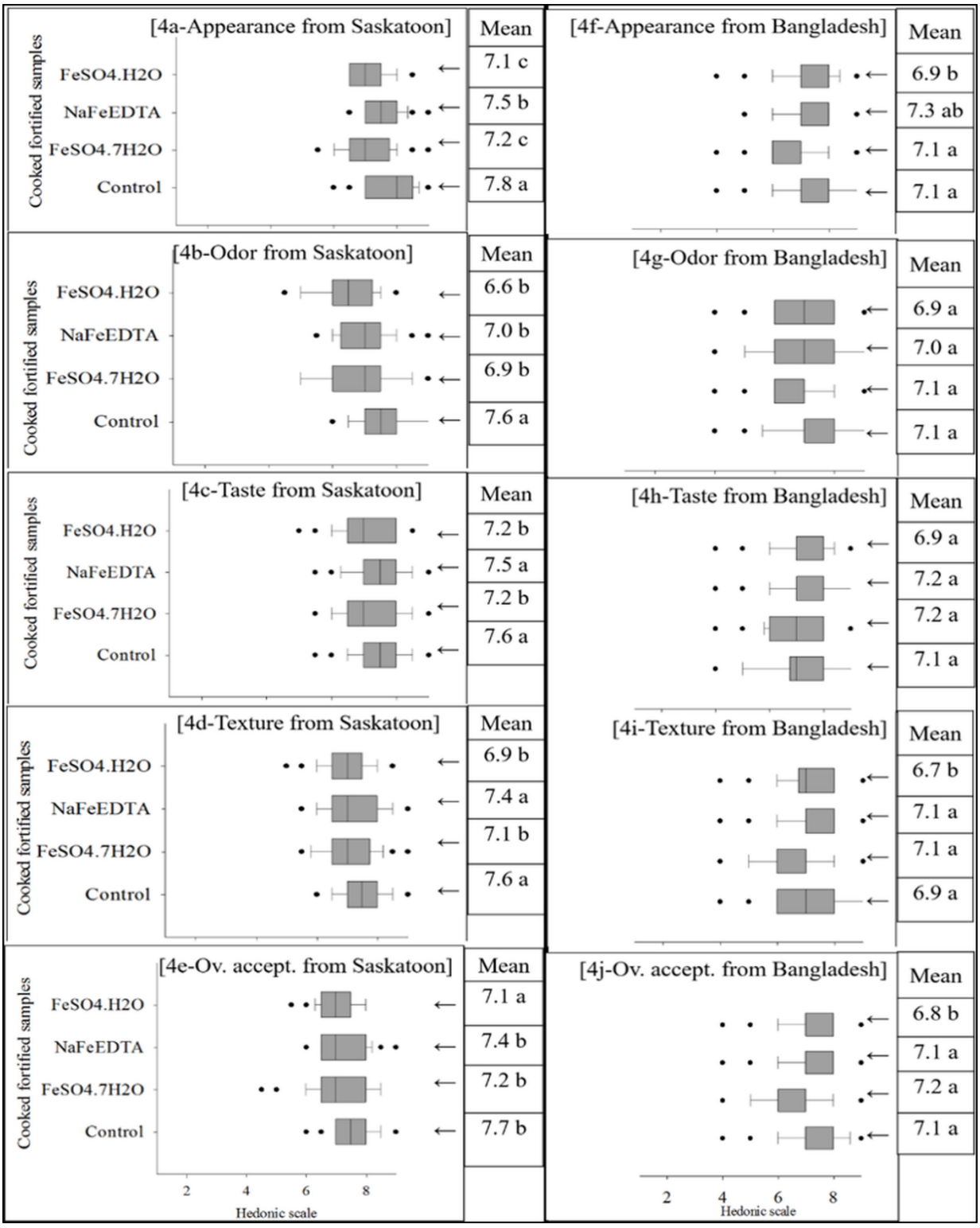
[4a-Appearance from Saskatoon]
Cooked fortified samples
FeSO4.H2O
NaFeEDTA
FeSO4.7H2O
Control
Mean
7.1 c
7.5 b
7.2 c
7.8 a
[4f-Appearance from Bangladesh]
Mean
6.9 b
7.3 ab
7.1 a
7.1 a
[4b-Odor from Saskatoon]
Mean
6.6 b
7.0 b
6.9 b
7.6 a
[4g-Odor from Bangladesh]
Mean
6.9 a
7.0 a
7.1 a
7.1 a
[4c-Taste from Saskatoon]
Mean
7.2 b
7.5 a
7.2 b
7.6 a
[4h-Taste from Bangladesh]
Mean
6.9 a
7.2 a
7.2 a
7.1 a
[4d-Texture from Saskatoon]
Mean
6.9 b
7.4 a
7.1 b
7.6 a
[4i-Texture from Bangladesh]
Mean
6.7 b
7.1 a
7.1 a
6.9 a
[4e-Ov. accept. from Saskatoon]
Mean
7.1 a
7.4 b
7.2 b
7.7 b
2
4
6
8
Hedonic scale
[4j-Ov. accept. from Bangladesh]
Mean
6.8 b
7.1 a
7.2 a
7.1 a
2
4
6
8
Hedonic scale

Figure 8.4. Box plot analysis of hedonic scores obtained for four cooked lentil samples (three fortificants at one Fe concentration (1600 ppm) plus one unfortified control) evaluated for appearance (a,f), odour (b,g), taste (c,h), texture (d,i) and overall acceptability (e, j) by 45 and 98 panelists in Saskatoon (a to e) and Bangladesh (f to j), respectively. Different letters after mean values indicate significant differences between treatments ($P < 0.05$). A nine-point hedonic scale (1 = dislike extremely to 9 = like extremely) was used.

8.3.5. Consistency assessment for sensory data based on Cronbach's alpha

Cronbach's alpha (CA) scores for all uncooked and cooked (both fortified and unfortified) samples are presented in Table 8.2. The CA scores for the ten uncooked samples were all greater than 0.75 with two exceptions, -$FeSO_4 \cdot 7H_2O$-fortified lentil (800 ppm) (0.66) and $FeSO_4 \cdot H_2O$-fortified lentil (800 ppm) (0.65). In Bangladesh, all samples had CA scores above 0.80. The overall mean CA scores for all variables for the uncooked samples were 0.93 and 0.94 for Saskatoon and Bangladesh, respectively.

Table 8.2. Internal Consistency Reliability of the sensory panellists' rating of uncooked red lentil and cooked dal samples in Saskatoon and Bangladesh

Treatment	Location	
Uncooked samples	Saskatoon	Bangladesh
Control	0.86	0.88
$FeSO_4 \cdot 7H_2O$-fortified lentil (800 ppm)	0.66	0.86
$FeSO_4 \cdot 7H_2O$-fortified lentil (1600 ppm)	0.88	0.91
$FeSO_4 \cdot 7H_2O$-fortified lentil (2800 ppm)	0.87	0.85
NaFeEDTA-fortified lentil (800 ppm)	0.81	0.80
NaFeEDTA-fortified lentil (1600 ppm)	0.89	0.80
NaFeEDTA-fortified lentil (2800 ppm)	0.86	0.85
$FeSO_4 \cdot H_2O$-fortified lentil (800 ppm)	0.65	0.92
$FeSO_4 \cdot H_2O$-fortified lentil (1600 ppm)	0.80	0.92
$FeSO_4 \cdot H_2O$-fortified lentil (2800 ppm)	0.85	0.93
All (ten) uncooked samples [a]	0.93	0.94
Cooked samples		
Control	0.90	0.93
NaFeEDTA-fortified lentil (1600 ppm)	0.85	0.92
$FeSO_4 \cdot H_2O$-fortified lentil (1600 ppm)	0.79	0.93
$FeSO_4 \cdot 7H_2O$-fortified lentil (1600 ppm)	0.89	0.91
All (four) cooked samples [a]	0.88	0.92

[a] Cronbach's alpha scores for all the ten uncooked and four cooked samples

8.4. Discussion

Sensory analysis originated in the mid-19th century and is considered a multidisciplinary science of various knowledge areas, including food science, psychology, sociology, statistics, human physiology and food preparation practices (Stone & Sidel, 2004). Sensory attributes are considered the most critical determinants of consumer acceptance of food (Guinard, 2004). In this study, sensory evaluation was considered a key means of understanding and evaluating the overall acceptance of iron-fortified lentil among panelists. The goal was to identify the best fortificant for lentil dal based on consumer preference.

Significant sensory differences were evident among the uncooked samples in both locations. Overall, scores for all sensory attributes and overall acceptability decreased with an increasing concentration of Fe in the fortificant, regardless of type. In Saskatoon, mean scores of the uncooked samples ranged widely, from 3.1 to 8.4, 4.1 to 7.5 and 3.3 to 8.2 for appearance, odour and overall acceptability, respectively. For all attributes, the control sample and the $FeSO_4{\cdot}7H_2O$-fortified sample (2800 ppm of Fe) had the highest and lowest mean scores, respectively. In Bangladesh, the corresponding scores fell into narrower ranges, from 4.6 to 8.0, 4.7 to 7.3 and 4.7 to 7.9 for appearance, odour and overall acceptability, respectively. For all attributes, the control sample and samples fortified with $FeSO_4{\cdot}H_2O$ (2800 ppm Fe) received the highest and lowest scores, respectively. These mean scores indicate that panelists evaluated the uncooked samples from "dislike moderately, score of 3" to "like very much, score of 8" in Saskatoon, and "neither like nor dislike, score of 5" to "like very much, score of 8" in Bangladesh. Moreover, in both locations, several panelists gave the highest hedonic score (like extremely, score of 9) for overall acceptability to the unfortified control and two NaFeEDTA-fortified samples (800 and 1600 ppm Fe). Overall, these results indicate that fortification with Fe did not have large

adverse effects on the acceptability of uncooked lentil to panelists. In particular, NaFeEDTA fortification did not change the visual organoleptic characteristics as much as did the other fortificants at any concentration.

Significant sensory differences were evident for the cooked lentil samples at the two study locations. The average scores for all attributes showed that panelists from Saskatoon assigned a wider range (6.6-7.8) of scores than did those from Bangladesh (6.7-7.3). This might be due to the fact that the geographical origin of the panelists in Saskatoon was much wider compared to those in Bangladesh. All panelists in Saskatoon were immigrants to Canada, having lived there for three to 25 years and having adopted more diverse food habits. Fifty percent of the Bangladeshi panelists in Saskatoon immigrated to Canada more than five years ago (data unpublished). Their food habits may have changed over time, which could affect their evaluations. To determine if this was the case, T-tests for unequal sample sizes were performed on data for panelists from Bangladesh (n = 98) and the Bangladeshi panelists who participated in Saskatoon (n=20). Scores were statistically different for five, three, and four of the ten uncooked samples for appearance, odour, and overall acceptability, respectively (Appendix 5). Bangladeshi panelists from Saskatoon scored all five attributes of the cooked samples higher (Appendix 6) than did panelists from Bangladesh, except for samples fortified with $FeSO_4 \cdot 7H_2O$ for odour, taste, texture and overall acceptability.

The other major group of panelists from Saskatoon was originally from India (n = 15). T-test results indicated no significant difference in scoring for most attributes for both uncooked and cooked samples compared to the Bangladeshi panelists, i.e. Bangladeshi and Indian panelists from Saskatoon scored samples similarly (Appendix 7 and 8). Although all panelists from Saskatoon in this study were familiar with lentil and the lentil soup prepared and served for the evaluation, some cultural factors may have influenced their scoring. Yao et al., (2003) reported that ranges in

hedonic scores differed for two groups of participants when evaluating the same food product, with a wider range obtained for Japanese compared to Korean panelists. The authors also have reported a cross-cultural effect on hedonic ratings when evaluating bulgogi (Korean traditional barbecued beef) with panelists from Korea and the USA (non-Korean). Verbeke, (2005) reviewed and stated that socio-cultural differences, education status, gender and annual income had an effect on choice of functional foods at the consumer level. Yao et al., (2003) also reported an effect due to translation of the evaluation form on scoring the same food by panelists from different countries. To mitigate this effect, the sensory evaluation forms, consent forms and questionnaires used in Bangladesh were translated into Bangla (and back-translated to English) to ensure the meaning was consistent with the English version of the forms used in Saskatoon. Despite this effort, the effect of translation might have been a factor in the narrower ranges of scores for the four cooked samples observed in Bangladesh compared to Saskatoon.

In this study, we selected four (three fortified and one control) lentil samples for the sensory acceptability study of cooked lentil dal. The three fortified lentil samples were fortified with an Fe concentration in the fortificant of 1600 ppm in each of the three fortificants. According to (FAO, 2017), the desirable intake of pulses is 50 g day^{-1} $person^{-1}$ and the World Health Organization and Food and Agriculture Organization of the United Nations recommended Estimated Average Requirements (EARs) for Fe at 10% bioavailability are 29.4 and 10.8 mg day^{-1} for 19-50 year-old females and males, respectively (WHO & FAO, 2006). Our previous study showed that 50 g of fortified lentil could provide more than 10 mg of Fe, which could meet a major part of the EARs (Podder et al., 2017). Also in the same study the lightness (L*), redness (a*) and yellowness (b*) of ten uncooked samples were measured using a HunterLab instrument (Hunter Associates Laboratory Inc., Reston, VA, USA). When the sensory data of three attributes (appearance, odour

and overall acceptability) of ten uncooked lentil samples obtained from both Saskatoon and Bangladesh were correlated with the L*, a* and b* scores using Pearson's correlation test, the results were significant at $P < 0.05$. Correlation coefficients between three attributes and L*, a* and b* scores were highly significant at $P < 0.001$ with a range from 0.88 to 0.97 (Appendix 9).

Another point of interest was whether consumer acceptance was the same for uncooked and cooked samples. A comparison of the scores for the four samples that were considered in both the cooked and uncooked panels showed that the relatively wide range in scores observed for the three uncooked fortified samples narrowed considerably after cooking. Beinner et al., (2010) observed no significant differences between cooked conventional and Fe-fortified rice after conducting sensory evaluation. Hof, (2006) conducted a consumer acceptance test with extruded samples of rice fortified with Vitamin A and C and two minerals, Fe and Zn and unfortified rice and two commercial samples of rice. The vitamin and mineral fortification did not affect sensory acceptability except for some appearance attributes. The reduced sensory variation in the cooked lentil samples in the present study might be due to the ingredients in the traditional recipe employed, which is typical for south Asian countries, including Bangladesh. The yellowness of turmeric (*Curcuma longa* L.) powder would reduce the darkness, and the pungent aroma of onion (*Allium cepa* L.) could affect the taste and odour profile of cooked dal prepared with fortified lentil.

Sensory measurements of any food product characteristics should be done carefully by following impartial presentation of the samples to the subjects, eliminating response bias, and using appropriate methods to improve the ability of panelists to evaluate (Delwiche, 2009). Panelists from Saskatoon had at least high school degree but in Bangladesh, approximately 7% of the panelist had < high school degree or did not attend school. This could be a limitation in this study in respect to representing participants from all levels. In this study, panelists from both

locations had no practical or theoretical knowledge of processing and fortifying lentil with Fe. They used their own perceptions to score the control and fortified samples without any bias. The sensory study in both locations showed that panelists could very easily discriminate fortified dal from the control when uncooked; however, panelist preferences were far more similar among the cooked samples. The addition of the recipe ingredients likely helped to maintain the traditional dal or soup colours and flavours within the range of acceptability.

The effect of fortification on sensory attributes of lentil dal should be minimized to achieve the greatest consumer acceptability. Taste, flavour, appearance, and texture are important factors for acceptability and consumption of any product. The effects of Fe fortification on sensory properties of food are highly variable, and depend on the specific Fe fortificant and food item (WHO & FAO, 2006). This includes potential changes in taste, colour and vitamin content (e.g., reduced vitamin C, which is an important factor for absorption and utilization of Fe) (Mehansho, 2006). Some natural food components such as anthocyanins, tannins and flavonoids can react with Fe and cause rancidity and other flavour changes (Bovell-Benjamin & Guinard, 2003). For instance, ferrous salts are more soluble and reactive than ferric salts with food components (Richardson, 1990). In this study, the sensory evaluation indicated that NaFeEDTA-fortification minimally affected consumer perception of colour, taste, texture, odour and overall acceptability of cooked lentil. This aligns with the results of our companion study in which appearance measurements using a Tristimular colorimetric scale (Wrolstad & Smith, 2010) resulted in the highest scores for the unfortified control samples followed by the NaFeEDTA-fortified lentil sample (1600 ppm Fe) (Podder et al., 2017).

Several studies illustrate the advantages of using NaFeEDTA as a Fe fortificant. For instance, NaFeEDTA has been approved as a safe fortificant by the FAO/WHO Expert Committee

on Food Additives to fortify foods (WHO, 2000). Moreover, the use of NaFeEDTA is preferred over ferrous sulphate, especially for pulse crops such as lentil that contain phytic acid, an antinutritional component (Hurrell, 2002b). NaFeEDTA is highly soluble in water and bioavailable, which allows more concentrated fortificant solutions to be used. Its colour also remains more stable after fortification because EDTA is stable to heat and humidity (Davidsson et al., 2002).

Cronbach's alpha (CA) was used to evaluate the reliability of the sensory data. Two reasons that favour the use of CA are the fact that it can be calculated easily with simple statistical analysis, and it considers both the variance and covariance relationships between panelists, creating a "proximity measure between evaluation profiles" (Pinto et al., 2014). The CA value for all treatments (both cooked and uncooked) showed that the fortified lentil dal and the control sample did not differ in context for all attributes, except for two samples in Saskatoon. This might be due to panelist inconsistency in scoring the samples. For instance, some panelists missed scoring some attributes for uncooked samples, which was considered as missing data. The missing values can affect the psychometric properties of the test (Huisman, 2000). Overall, however, the CA value indicated that panelists were highly consistent in evaluating all samples using the hedonic scales. The box plot for both uncooked and cooked lentil samples from both locations showed a few outliers which indicated that some panelists disliked the samples extremely. A few panelists commented that there was an oily smell associated with fortified lentil, and some noted a black spot in the region of the micropyle (where the whitish tip of the root of the embryonic seedling is visible when the lentil seed is dehulled. It is part of the embryonic seed axis which is activated early in the germination process when the seed initially absorbs water). In dehulled seed, the root embryo tissue in this region absorbs liquid in the crevice formed between the embryonic root and

the cotyledon, resulting in a slight discoloration caused by oxidation of the iron after the fortification process is completed.

The choice of Bangladesh as a study site is strategically important. In Bangladesh, different international and national organizations are actively collaborating with the national health sector by conducting studies with fortified foods. Salt and vegetable oil fortified with iodine and vitamin A, respectively, are becoming available in the Bangladeshi market (Ahmed et al., 2016). Moreover, efficacy studies are being conducted with staple foods like rice, wheat flour and sugar fortified with different micronutrients, including Fe. Lentil is considered a nutrient- dense, staple food, consumed daily as the cheapest source of protein, fibre, and micronutrients in South Asian countries, especially in Bangladesh. An acceptability trial carried out by the authors (Yunus et al., 2017, unpublished) in Bangladesh showed that adolescent girls of varying ages willingly consume lentils. A major part (~ 30%) of the adolescent girls in Bangladesh are anemic and Fe deficiency is considered the main cause (Ahmed et al., 2010). About 80% of the population in Bangladesh consume Canadian lentils and are familiar with their quality. “Dal vaat” (rice and lentil or other pulses) is a common meal in Bangladesh. The dish “hotchpotch” (made with rice and other pulses, mainly lentil) is a typical meal for 1-5-year-olds and school-aged children in South Asian countries. An advantage with fortified lentil lies in the likelihood that all lentils could be centrally processed and fortified, ensuring wide coverage with high quality. This benefit would also improve food quality, which is one of the biggest challenges. Rice is the primary staple food in Bangladesh. As with rice, there is no seasonal sporadic production of lentil which can lead to seasonal supply disruption. Also, there are many different varieties of rice, and household preferences are variable. Thousands of millers are involved in the rice supply system, and a significant proportion of the population consumes their own production. In Bangladesh rice is also fortified and marketed by

Nutrition International. To our knowledge, the World Food Programme does not enter commercial market channels and provides fortified products to vulnerable populations only.

The consumption rate of lentil in Bangladesh is 12 g/day/person (Sarker et al., 2004), far below the desirable intake rate of 50 g/day/person on the basis of previous results and current consumption patterns of the Bangladeshi population (FAO, 2017). A small amount of fortified lentil can provide a significant RDA of Fe for a human. Results from this study showed that the uncooked NaFeEDTA fortified lentil samples with 2800 ppm of Fe had significantly similar acceptance for all the attributes with the samples fortified with 800 and 1600 ppm of Fe. Thus, would help to reduce the amount of per capita lentil intake but can provide the similar amount of Fe from lentil fortified with 1600 ppm of Fe (Podder et al., 2017). In conclusion, Fe-fortified lentil can effectively and economically provide part of the solution to Fe micronutrient deficiency by providing a substantial amount of Fe from a minimum amount of lentil dal.

8.5. Conclusions

Lentil is consumed regularly as a staple food in all south Asian countries, where a significant percentage of the population suffers from Fe deficiency. Lentil contains a significant amount of non-heme Fe compared to other major cereal and legumes, all of which have low Fe concentration and low bioavailability. Our previous study illustrated that lentil is a potential vehicle for Fe fortification. In the current study, panelists' acceptability scores were higher for NaFeEDTA-fortified samples compared to $FeSO_4 \cdot 7H_2O$- and $FeSO_4 \cdot H_2O$-fortified samples. Although a significant difference in acceptability was observed between the control and NaFeEDTA-fortified lentil samples in the uncooked condition, the non-significant difference in the cooked condition indicates that fortification of lentil with NaFeEDTA is a promising approach.

Moreover, the non-significant difference between the samples fortified with 800 vs. 1600 NaFeEDTA in the uncooked condition, and the acceptance of the 1600 ppm samples in the cooked condition, indicate that the 1600 ppm concentration should be used in lentil fortification. At this level, 11-12 mg of dietary Fe can be obtained by consuming 50 g of fortified lentil, well within the normal range of daily consumption. This amount should meet the major part of the estimated average requirements for Fe of target populations.

Prologue to chapter 9

Iron bioavailability from Fe-fortified food depends on its absorption through the human gastrointestinal tract for systemic utilization. The bioavailability of Fe is affected by other factors including the presence of antinutritional factors, such as phytate. Fortified lentil with Fe fortificants can provide a significant amount of Fe and increase bioavailability. In the following chapter the Fe and phytic acid concentrations, the and relative bioavailability of Fe in different traditional Bangladeshi meal plan models featuring fortified and unfortified lentil dal will be described. The effect of addition of fortified or unfortified lentil dal on the Fe concentration and RFeB% of different meal models will also be described.

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CHAPTER 9

RELATIVE BIOAVAILABILITY OF IRON IN BANGLADESHI TRADITIONAL MEALS PREPARED WITH IRON-FORTIFIED LENTIL DAL

9.1. Introduction

Iron (Fe) deficiency is a public health problem and more than 30% (two billion) of the world population is anaemic, mainly due to Fe deficiency (WHO, 2018b). Fe deficiency is considered the major cause of anaemia, which mostly affects young children and pregnant and post-partum women (de Benoist et al., 2008). In Bangladesh, anaemia is a public health concern and 40% of adolescents are anaemic (Ahmed et al., 2010). In 2011, the national prevalence of anaemia in Bangladesh was 51% in children aged 6-59 months and 42% in non-pregnant women (Bangladesh Demographic and Health Survey, 2011). One of the major causes of Fe deficiency is low bioavailability of dietary Fe, especially in developing countries such as Bangladesh where diets are mostly cereal- and legume-based (Zimmermann et al., 2005).

Among legumes, lentil is one of the oldest and most important cultivated crops. Lentil is consumed in both developed and developing countries around the world, and is a potential whole food source that can provide micronutrients such as Fe, zinc (Zn), and selenium (Se) (Thavarajah et al., 2011). In some developing countries, lentil is considered a staple food due to its nutritive value, especially as an inexpensive protein source compared to animal protein. Studies investigating ways to increase Fe content and bioavailability have focused mainly on biofortification strategies using marker-assisted breeding, improved agronomic practices, and removal of the seed coat from lentil seed (DellaValle et al., 2013; Khazaei et al., 2017). However, Fe biofortification of food crops has several drawbacks, such as low bioavailability, limitations to increasing the total content in food crops, and insufficient consumption to show significant health benefits. The bioavailability of Fe

from lentil is often compromised due to the presence of antinutritional factors (e.g., phytate, polyphenols, cotyledon cell wall) in the seed (Glahn et al., 2016; Grusak, 2009). Fortification, on the other hand, often can overcome the inhibitors and provide significant bioavailable Fe (Hurrell, 2002) as long as the addition of Fe does not alter the appearance and taste of the target food product.

The main objective of any fortification program is to improve nutrient content and the nutritional quality of the added nutrients and thus help to eliminate or prevent deficiencies in the target population. Different strategies have been adopted to combat micronutrient deficiencies, such as biofortification, fortification, supplementation, dietary diversification, and nutrition education (Northrop-Clewes, 2013). All of these strategies have limitations depending on sociocultural and economic factors as well as the age and gender of the target population. These may be overcome by food fortification, which has proven to be a cost-effective way to add micronutrients to processed food and improve the dietary quality of a target population without changing their food habits (Allen et al., 2006). A systematic review of "micronutrient fortification of food and its impact on women and child health" revealed that fortification with micronutrients, including Fe, significantly increased serum Fe concentrations with no significant adverse effect on hemoglobin levels (Das et al., 2013).

Biofortification of lentil is not likely to have impact in much of the Bangladeshi population as the consumption rate of pulses for the population of Bangladesh is 12 g/day/person (Sarker et al., 2004), which is far below the desirable intake of 50 g/day/person that has been reported on the basis of previous studies and the current consumption pattern of the Bangladeshi population (FAO, 2017). To address this shortfall, improving the nutritional quality of lentil by Fe fortification could provide a significant amount of the required daily Fe from a minimum amount of lentil dal, without

having to increase the quantity of lentil in a given meal. To enable this approach, we previously developed a laboratory-scale protocol for fortifying de-hulled lentil seed (dal) using three Fe fortificants. NaFeEDTA was the most effective; at a fortificant Fe concentration of 1600 µg g^{-1}, NaFeEDTA provided 13-14 mg of additional Fe per 100 g of cooked lentil dal (Podder et al., 2017). The United States Food and Drug Administration (FDA) published a food fortification policy featuring six principles for food fortification (Dwyer et al., 2015; FDA, 2016). These are: "1) the nutrient intake without fortification is below the desirable content for a significant portion of the population; 2) the food being fortified is consumed in quantities that would make a significant contribution to the population's intake of the nutrient; 3) the additional nutrient intake resulting from fortification is unlikely to create an imbalance of essential nutrients; 4) the nutrient added is stable under proper conditions of storage and use; 5) the nutrient is physiologically available from the food to which it is being added; and 6) there is reasonable assurance that it will not result in potentially toxic intakes." All of these principles have been considered with respect to lentil fortification.

We also investigated the sensory acceptability of fortified lentil dal with respect to appearance, odor, taste, texture, and overall acceptability by lentil consumers (Podder et al., 2018). Fortification of lentil with NaFeEDTA minimally affected consumer perception of appearance, taste, texture, odour, and overall acceptability of cooked lentil compared to fortification with $FeSO_4 \cdot 7H_2O$ or $FeSO_4 \cdot H_2O$. Sensory acceptability was statistically similar to that of non-fortified lentil for almost all of the attributes.

The present study aimed to determine the concentration and relative bioavailability of Fe in different traditional Bangladeshi meal plan models featuring fortified and unfortified lentil dal. A Caco-2 cell bioassay was used to assess relative Fe bioavailability (RFeB%), expressed as a

percentage of that of an unfortified control red lentil sample that was included in each run of the bioassay. This lentil sample had a Fe concentration of 50 ug g^{-1}. Ferritin formation by Caco-2 cell monolayers is a sensitive and accurate measurement tool for *in vitro* assessment of Fe bioavailability in food (Glahn et al., 1998). The concentration of phytic acid (PA), a known inhibitor of Fe bioavailability, also was determined in the meal plan models.

9.2. Materials and Methods

9.2.1. Preparation of meal models

A total of 30 meal combinations were prepared and assessed with respect to Fe concentration, RFeB%, and PA concentration (appendix 10). Among these, models 1 to 11 and 15 to 25 featured either unfortified or fortified lentil dal, respectively, in different amounts (% by weight) along with other meal components. Three models (models 12 to 14) contained no lentil. The remaining five models (models 26 to 30) were prepared with only rice (model 26), vegetables (model 27), fish (model 28), unfortified cooked dal (model 29), or NaFeEDTA-fortified cooked dal (model 30). The fortified lentil had been treated with 2800 µg g^{-1} NaFeEDTA, which in previous work comparing various fortificants and concentrations thereof, was determined to have the least effect on appearance and consumer acceptability measures such as taste and texture (Podder et al., 2017; Podder et al., 2018). Lentil dal was prepared according to a traditional Bangladeshi recipe (Kohinoor et al., 2010) where lentil, deionized water, canola oil, salt, turmeric powder and onion were used as ingredients in a 15:70:4:3:2:6 ratio, by weight. Along with the dal, rice (white, boiled and unenriched), vegetables (mixture of carrot, cauliflower, brinjal, potato, sweet potato, onion, salt, turmeric, garlic, oil, and water at a 10:10:8:10:5:2:1:1:1:12:40 ratio, by weight) and fish (fish fillets, salt, turmeric, and oil at a 90:2:3:5 ratio, by weight) were used in

different ratios to prepare the meal models. All foods were cooked with 18 MΩ deionized water. Rice, fish, and vegetables were cooked in a traditional Bangladeshi fashion. Stainless steel cookware was used to prepare all meal components. Prepared dishes were cooled at room temperature for 2 h, frozen at -80°C for 24 h, freeze-dried using a FreeZone 12 L Console Freeze Dry System with Stoppering Tray Dryers (Labconco, model 7759040, Prospect Avenue, Kansas City, MO, USA) for 72 h, and stored at room temperature (DellaValle & Glahn, 2014). A 10-g sample from each freeze-dried cooked dish (models 1 to 30) was finely ground and sent to the USDA-ARS Robert Holley Center for Agriculture and Health (Ithaca, New York, USA) to determine Fe concentration, phytic acid concentration, and RFeB%. From the 10-g sample, 0.5 g of each of the three repetitions was used in the Caco-2 cell bioassay to estimate the RFeB% (DellaValle & Glahn, 2014; Glahn, 2009).

9.2.2. Assessment of Fe concentration, RFeB%, and PA concentration

The concentrations of Fe for the 30 meal models were quantified with an inductively coupled argon-plasma emission spectrometer (iCAP 6500 series, Thermo Jarrell Ash Corp., Franklin, MA, USA) following the procedure of Glahn et al., (2017). Ferric chloride ($FeCl_3$) was used as the certified reference material in the iCAP analysis. Relative bioavailability of Fe for the 30 meal models was assessed using an established Caco-2 cell bioassay, where Caco-2 cell ferritin formation is used as the measure of cell Fe uptake and bioavailability (DellaValle et al., 2013; Glahn et al., 1998; Tako & Glahn, 2011). The bioavailability assessment was conducted on three replicates for each cooked lentil sample. Ferritin values from the fortified lentil samples were compared with the control lentil (CDC Robin; Fe concentration of 50 μg g^{-1}) to calculate the RFeB%, using the following equation: Relative Fe bioavailability (RFeB %) = [(ng ferritin of the lentil sample/mg protein of the lentil sample)/(ng ferritin/mg protein of the control lentil)] * 100

(DellaValle, et al., 2013). The resulting index of relative Fe bioavailability (RFeB%) is used hereafter. Phytic acid content was measured as phosphorous released by phytase and alkaline phosphatase via a colorimetric assay kit (K-PHYT 12/12, Megazyme International, Wicklow, Ireland) (Glahn et al., 2017).

9.2.3. Data analysis

Data were analyzed statistically using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) was used to verify differences in Fe concentration, RFeB%, and PA concentration among different meal models. The outcomes for the three variables (Fe concentration, RFeB%, and PA concentration) represented the three replicates of each sample. Fisher's least significant difference (LSD) was calculated with the level of significance set at $p < 0.05$. Paired *t*-test analysis was used to assess differences in the five variables in the meal models featuring fortified vs. unfortified lentil. The associations among Fe concentration, RFeB%, and PA concentration were assessed using Pearson correlations at a $p < 0.05$ significance level (DellaValle et al., 2013). Fe concentration, ferritin formation (ng ferritin/mg protein), RFeB%, PA, and PA:Fe molar ratio were compared to assess the effect of NaFeEDTA-fortified lentil (meal models 15 to 25) vs. unfortified lentil (meal models 1 to 11). A correlation analysis also was conducted for Fe concentration, PA concentration, and RFeB% to determine the relationships among these measures.

9.3. Results

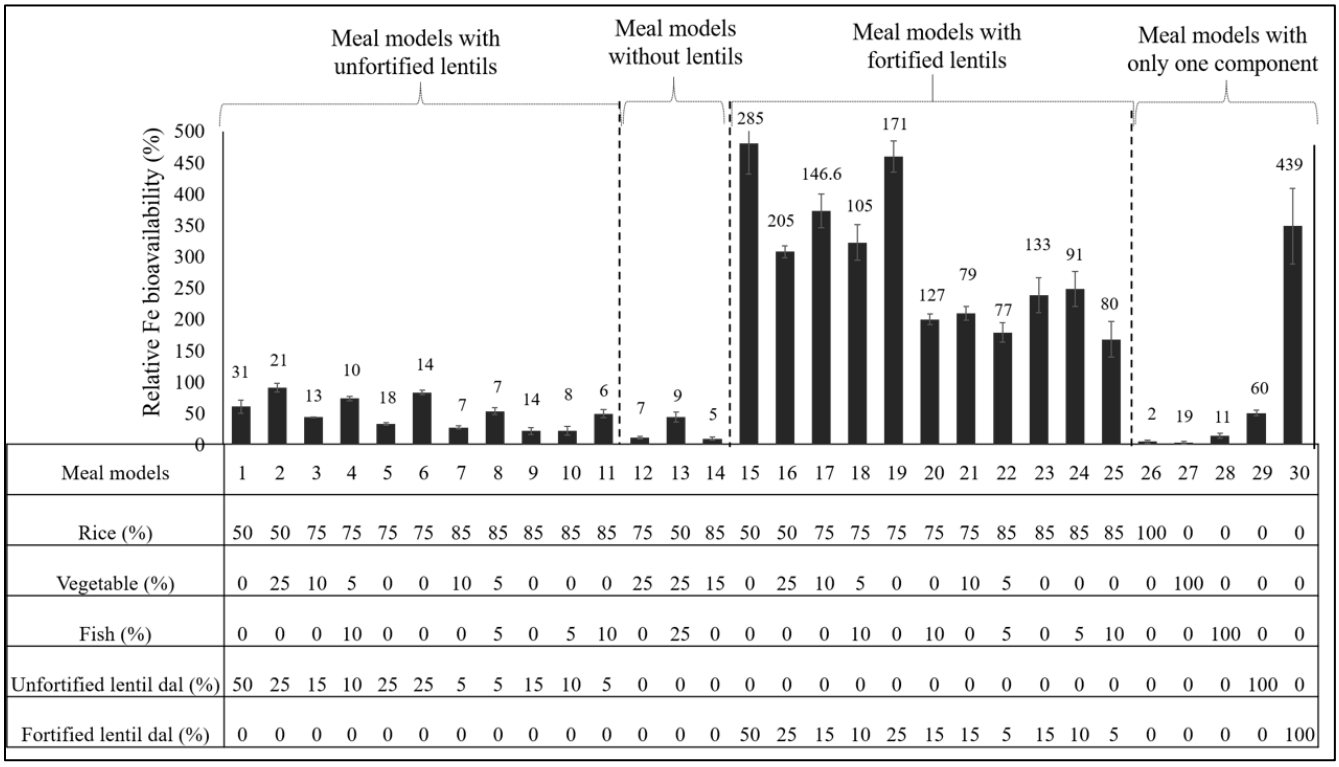
9.3.1. Fe concentration, RFeB%, and PA concentration

The average Fe concentration, RFeB%, and PA concentration of 30 meal model samples prepared with unfortified and fortified lentil are shown in Figure 1 and in appendix 11. Significant

differences were observed for Fe concentration, RFeB%, and PA concentration. The Fe concentration of the 30 meal plan models ranged from 2.1 µg g^{-1} (model 26; 100% rice) to 439.2 µg g^{-1} (model 30; 100% NaFeEDTA fortified lentil) and the PA concentration ranged from 1.2 mg g^{-1} (model 26; 100% rice) to 6.2 mg g^{-1} (model 29; 100% unfortified dal). RFeB% ranged from 3.7% (model 27; 100% vegetable) to 48.6% (model 15; 50% rice + 50% NaFeEDTA-fortified lentil); the control lentil had an RFeB% value of 30.9%. The highest Fe concentration, PA concentration, and RFeB% were found for meal models 30, 29, and 15, respectively. Among the 11 meal models (models 1 to 11) where unfortified lentil was used as a meal component (usage ranged from 5-50%, by weight), the highest Fe and PA concentrations were found in model 1, whereas the highest RFeB% was observed in model 2 (Figure 1). In meal models 15 to 25, where fortified lentil was used, the highest Fe and PA concentrations and RFeB% were observed in meal model 15 (Figure 1).

The iron concentrations for model 29 (100% unfortified lentil; Fe concentration 60 µg g^{-1}) and model 30 (100% NaFeEDTA-fortified lentil; Fe concentration 439.2 µg g^{-1}) indicate that lentil was the main component providing Fe across all of the meal plans (Figure 1). This also is reflected in the six models (12, 13, 14, 26, 27, 28) that contained no lentil and had low Fe concentrations (Figure 1) compared to models containing either fortified or unfortified lentil. Fish, vegetables, and rice did not notably affect Fe concentration as these components contain low amounts of Fe. The vegetable curry contained a higher amount of Fe (19.4 µg g^{-1}) than did fish (11.4 µg g^{-1}) or rice (2.1 µg g^{-1}). The main component of meal models 2 to 14 and 16 to 25 was rice, ranging from 75 to 85%, by weight. Although the largest amounts of PA were found in unfortified lentil (6.2 mg g^{-1}) followed by fortified lentil dal (4.6 mg g^{-1}), the contribution of PA would have been mainly from rice, which comprised the major part of most meal models. For instance, meal models 9 and

23 had similar amounts of rice (85%) and lentil dal (15%), but the former contained unfortified dal and the latter, fortified dal. PA concentrations in meal models 9 and 23 were 2.4 and 1.7 mg g^{-1}, respectively, of which 1.02 mg g^{-1} was contributed by rice.



Meal models	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Rice (%)	50	50	75	75	75	75	85	85	85	85	85	75	50	85	50	50	75	75	75	75	75	85	85	85	85	100	0	0	0	0
Vegetable (%)	0	25	10	5	0	0	10	5	0	0	0	25	25	15	0	25	10	5	0	0	10	5	0	0	0	0	100	0	0	0
Fish (%)	0	0	0	10	0	0	0	5	0	5	10	0	25	0	0	0	0	10	0	10	0	5	0	5	10	0	0	100	0	0
Unfortified lentil dal (%)	50	25	15	10	25	25	5	5	15	10	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0
Fortified lentil dal (%)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	50	25	15	10	25	15	15	5	15	10	5	0	0	0	0	100

Figure 9.1. Relative iron bioavailability (RFeB%) and Fe concentration (µg g^{-1}, above each bar) of 30 traditional Bangladeshi meal plan models containing unfortified lentil (meal models 1-11), no lentil (meal models 12-14), fortified lentil (meal models 15-25) and single components (meal models 26-30), assessed using a Caco-2 cell bioassay.

Among the six meal models (1, 5, 9, 15, 19, 23) in which rice and lentil were the only ingredients, increasing the amount of rice generally decreased the Fe concentration, PA concentration, and RFeB%. The meal model that included rice (50%), fish (25%), vegetables (25%), and no lentil (model 13) contained a very low amount of Fe (8.7 µg g^{-1}) but it was of higher relative bioavailability, which could be due to the low amount of PA in the meal. Models 4, 8, 18,

and 22 contained similar amounts of vegetable (5%), but model 8 and 22 contained 10% more rice and 5% less fish and dal compared to models 4 and 18. This resulted in decreased Fe concentration, PA concentration, and RFeB%.

9.3.2. Comparison between meal models containing unfortified vs. fortified lentil

A comparison of Fe concentration, ferritin formation (ng ferritin/mg protein), relative Fe bioavailability (% of control lentil), PA concentration, and PA:Fe molar ratio between meal model groups featuring unfortified lentil (models 1 to 11) vs. fortified lentil (meal models 15 to 25) revealed significant differences for all parameters considered. Specifically, the average Fe concentration was significantly ($p \leq 0.001$) higher in meal models with fortified lentil (136.2 µg g^{-1}) compared to those with unfortified lentil (13.5 µg g^{-1}). Ferritin formation (52.5 vs. 15.8 ng ferritin/mg protein) and RFeB% (290.0 vs. 51.2%) also were significantly ($p < 0.001$) higher in meal models with fortified lentil. PA concentration (2.1 vs. 2.4 mg g^{-1}, $p = 0.03$) and PA:Fe molar ratio (1.5 vs. 16.9) were significantly ($p \leq 0.001$) lower in meal models with fortified lentil.

9.3.3. Correlations between measured variables

Correlation coefficients between measured variables are presented in Table 9.1. Significant correlations were observed between Fe concentration and RFeB%, RFeB% and PA:Fe molar ratio, and Fe concentration and PA:Fe molar ratio when all meal models were considered. Significant correlations between Fe concentration and RFeB% as well as between RFeB% and PA:Fe molar ratio were observed for meal models with fortified lentil (models 15 to 25) but not unfortified lentil (models 1 to 11). Fe concentration and PA:Fe molar ratio had an inverse relationship for all meal models containing either unfortified or fortified lentil.

Table 9.1. Pearson correlation coefficients for iron (Fe) concentration vs. relative Fe bioavailability (RFeB%), bioavailability vs. phytic acid (PA):Fe molar ratio, and Fe concentration vs. PA:Fe molar ratio.

Meal model	[Fe] vs. RFeB%	RFeB% vs. PA:Fe molar ratio]	[Fe] vs. PA:Fe molar ratio
All (models 1 to 30)	0.832**	-0.722**	-0.627**
(n = 30)	(< 0.001)	(< 0.001)	(< 0.001)
Unfortified lentil (models 1 to 11)	-0.142	0.351	-0.628*
(n = 11)	(0.685)	(0.299)	(0.0364)
Fortified lentil (model 15 to 25)	0.801**	-0.763**	-0.628*
(n = 11)	(0.001)	(0.004)	(0.036)

**. Correlation is significant at the 0.01 level (2-tailed); *. Correlation is significant at the 0.05 level (2-tailed).

9.4. Discussion

Lentil fortification programs have been initiated with the aim of improving the Fe content in lentil because lentil serves as a major side dish in many countries, including Bangladesh. Due to poor absorption of intrinsic Fe from lentil, improvement in the Fe concentration in lentil dal and the increased absorption of Fe through fortification is a potential strategy to combat micronutrient malnutrition. In this study we assessed the bioavailability of Fe, using a Caco-2 cell bioassay, from a variety of traditional Bangladeshi meal models that contained either Fe-fortified or unfortified lentil.

In Bangladesh, the prevalence of anaemia in adolescent girls is ~30%, with iron deficiency considered the main cause (Ahmed et al., 2010). Socioeconomic conditions also are reported to be a factor that, along with nutritional deficiency, influence dietary problems in rural Bangladeshi women, who consume lentil three (60%) or four (12%) times per week (Sheema et al., 2016). Lentil consumption also is increasing with the increasing price and reduced availability of animal protein. One study of the dietary habits of 384 rural women from northern Bangladesh revealed that 92% of respondents eat hotchpotch, a typical and traditional Bangladeshi dish with a pulse (usually lentil) and rice (Sheema et al., 2016). Thus, lentil fortification could be a potential approach to supplying a major part of the required amount of Fe to vulnerable people with Fe deficiency in Bangladesh.

Micronutrient bioavailability from fortified food depends on its absorption through the gastrointestinal tract for systemic utilization (Moretti et al., 2014). Bioavailability is the result of three major steps: digestibility (solubility of Fe in digesta), absorbability in the circulation system, and final processing and incorporation into a functional compartment of the body (Armah, 2014; Wienk et al., 1999). Different approaches, such as the chemical balance method, solubility or dialyzability, Caco-2 cell bioassay, hemoglobin repletion method, isotopic methods, and area under the curve for serum iron have been used to estimate non-heme iron absorption (Armah et al., 2013). Other algorithms or combinations thereof have been used to assess Fe uptake based on Fe absorption from a single or complete meal (Armah et al., 2013). In this study, a Caco-2 cell bioassay was used to measure Fe absorption. This model mimics conditions in the small intestine, and ferritin formation in the Caco-2 cell monolayers is considered as iron uptake (DellaValle & Glahn, 2014). Some limitations have been reported for the *in vitro* Caco-2 cell bioassay, for example, the *in vitro* model cannot fully mirror the human gut system that involves the effect of

body Fe status and gut microflora on Fe uptake (DellaValle & Glahn, 2014). Considering these limitations, although this *in vitro* model is not a substitute for an *in vivo* model, it is a highly sensitive, cost-effective, and quick tool to measure Fe availability in foods (DellaValle & Glahn, 2014; Glahn et al., 1998). Moreover, this model was found to be strongly correlated ($R = 0.968$, $p < 0.001$) with human Fe absorption studies (Yun et al., 2004), and with human and animal efficacy studies of Fe absorption from biofortified crops (Tako et al., 2016). This model, therefore, can be considered to be thoroughly validated as a predictor of Fe absorption by humans. PA content was measured using a colorimetric assay kit, which is widely used as it gives accurate and reliable data, and saves cost and time (Reason et al., 2015). Sometimes this kit gives more accurate result than HPLC and quality controlling is easier than using HPLC if the person running the system is less experienced. However, a limitation to the use of this kit is that it cannot measure *myo*-inositol in either its free or phytase/alkaline phosphatase released forms (Reason et al., 2015).

Iron absorption is influenced by both endogenous and exogenous factors (Hunt, 2005). The recipe used to prepare the various meal models used herein included different spices (turmeric, onion, garlic) and fat (canola oil). Bio-accessibility of Fe increased by 26.3% and 17.2% when 3.0 g of onion and 0.5 g of garlic, respectively, were cooked with 10 g of chickpea (Gautam et al., 2010; Greger & Mulvaney, 1985). This could be due to the presence of sulfur-containing amino acids in *Allium* species that are reported to influence mineral status in animals. Moreover, spices also may contain phytic acid (inositol hexakisphosphate) and polyphenolic compounds (e.g., tannic acid and chlorogenic acid) (Hunt, 2003). The fortified and unfortified lentil used in the meal preparations are non-heme iron sources. Most polyphenols are located in the lentil seed coat, and the dehulled lentil used in this study would contain a low level of polyphenols, which would contribute to increased non-heme iron absorption in populations with limited Fe storage (Mennen et al., 2005).

Turmeric is used extensively in countries of the Indian sub-continent, including Bangladesh. The most active constituent of turmeric is curcumin, a polyphenolic diketone. Curcumin forms a complex with solubilized Fe in aqueous solution with either Fe (II) or Fe (III) ion (Bernabé-Pineda et al., 2004; Borsari et al., 2002; Tuntipopipat et al., 2006) and does not inhibit Fe absorption in young women (DellaValle & Glahn, 2014). Vegetables also contain significant amounts of vitamin A, carotenoids, and indigestible carbohydrates and the effect of these components on Fe absorption is unresolved (Hurrell & Egli, 2010). Some vegetables used in this study to prepare vegetable curry, such as potato and sweet potato, contain a higher amount of Fe compared to the fish and the other vegetables used. This may explain the higher amount of Fe in vegetable (19.4 μg g^{-1}; meal model 27) than in fish (11.4 μg g^{-1}; meal model 28). A similar result also was found in another study conducted with traditional Bangladeshi meals (DellaValle & Glahn, 2014).

Lentil consumption varies with age, gender, food habit, price, and availability of lentil in the market. The amount of vegetables in the meal models ranged from 5 to 25%, similar to traditional Bangladeshi meals. Fish comprised only 5 or 10% of the meals because the fish price in local markets is high and the consumption rate much lower than for other food items in the regular meal. Two meal models (models 3 and 17) are unique and represent hotchpotch, a ubiquitous meal for 1- to 5-year-olds and school-aged children in Bangladesh. In suburban areas of Bangladesh, “dal vaat” (rice and lentil or other pulses) is a common meal. Dried fish also is prevalent, and small amounts of dried fish with rice and lentil (models 6, 11, 20, and 25) also is a popular and widespread meal for local people in Bangladesh. The 30 meal models considered herein were designed with either unfortified or fortified lentil in varying amounts (5, 10, 15, 25, or 50%). Preliminary data (not shown) indicated that consumers prefer a thicker soup, which

requires more lentil. This is favourable, as a higher amount of lentil dal in a meal will help to provide more of the required supply of Fe, and will increase the relative bioavailability.

The choice of NaFeEDTA-fortified lentil was based on the results of our two previous studies with respect to consumer acceptability (Podder et al., 2017, Podder et al., 2018). Moreover, in the context of bioavailability, NaFeEDTA has proven to be more suitable than $FeSO_4$ as a fortificant in legume-based flours (Abizari et al., 2012; Brouwer, 2012). In cowpea flour, higher PA:Fe molar ratios (3.0:1 to 3.3:1) are related to low iron absorption (Abizari et al., 2012). PA chelates with positively charged multivalent cations such as Fe, Zn, Mg, and Ca, forming insoluble complexes that precipitate in the neutral pH condition of the intestine, thus decreasing Fe absorption (Schlemmer, 2009). In models 29 (100% unfortified lentil) and 30 (100% NaFeEDTA-fortified lentil), the PA content was 6.2 and 4.6 mg g^{-1} and the RFeB% was 50.6 and 349.2%, respectively (appendix 11). These differences could be attributed to: (i) the higher Fe concentration in the NaFeEDTA-fortified lentil, (ii) the lower PA content in the NaFeEDTA-fortified lentil, or (iii) the fortification process, as dephytinization can inactivate phytates to a large extent (Schlemmer et al., 2009).

In this study, PA concentration was assessed using a PA (total P) test kit (Megazyme International, Ireland). However, the concentration of polyphenolic components also could differ between fortified and unfortified lentil dal due to the effect of the fortification process. The PA concentration in the unfortified lentil meal (model 29) was significantly higher than in the fortified lentil dal meal (model 30). Thus, the PA:Fe molar ratio also was reduced from 8.8 in meal model 29 to 0.9 in meal model 30 (appendix 11). This could be due to dephytinization during the fortification process. A previous study reported that for Fe-fortified fonio porridge, dephytinization and fortification reduced the PA:Fe molar ratio from 24:1 to 0.3:1 (Koréissi-Dembélé et al., 2013).

Again, a significant inverse correlation was found between RFeB% and the PA:Fe molar ratio. A similar result with respect to RFeB% and PA:Fe molar ratio was observed for meal models prepared with dehulled lentil and whole lentil (DellaValle & Glahn, 2014).

Although no recommendations are in place for lentil fortification, the World Health Organization (WHO) has recommended some Fe fortificants and appropriate doses for fortification of wheat flour in 13 countries (Pachón et al., 2015). The FAO/WHO recommended nutrient intakes (RNIs) of Fe (mg) for females and males 19-50 years of age are 29.4 and 13.7 mg, respectively, based on 10% bioavailability (WHO & FAO, 2006). In this study, the amount of fortified lentil ranged from 5 - 50% in meal models 15 to 25. These meal models feature the fortified lentil as part of the meal, and not as a supplement. The meal model with fortified lentil only (model 30; 100% NaFeEDTA-fortified lentil) can provide ~43.9 mg of Fe from 100 g of cooked dal (dry basis). This means that 100 g (dry basis) of meal model 19, which contains 25% fortified lentil, would contain ~11 mg of Fe. This could provide a major portion of the recommended nutrient intakes (RNIs) of Fe for adult males and females aged 19-50 mentioned in (WHO & FAO, 2006). Because the tolerable upper intake level of Fe for adults is 45 mg/day (National Institutes of Health, 2016), the meal model with fortified lentil only (50 g person^{-1}) also is safe for human consumption.

The study results showed that lentil was the major contributor of Fe and that the relative bioavailability of Fe increased when NaFeEDTA-fortified lentil was used in different meal models. Since different amounts of either fortified or unfortified lentil were used in different meal models, and the RNIs are advised on the basis of age, gender, pregnancy, and lactation period, recommendations for use of appropriate amounts of Fe-fortified lentil can be given for target populations. In this study, PA content was measured and considered to be the key inhibitor of Fe absorption. Since the PA concentration was significantly lower in the fortified lentil, it may be

possible that levels of inhibitory polyphenolic also were reduced in the fortified lentil, thereby increasing Fe absorption. However, it has been shown that not all polyphenolic compounds inhibit Fe absorption, and some have been identified as potential promoters of Fe uptake (Hart et al., 2017; Hart et al., 2015).

9.5. Conclusion

Per capita global consumption of lentil is increasing rapidly. In some regions, however, the per capita consumption rate is actually decreasing due to higher demand. Fe-fortified lentil can provide a higher amount of Fe from a smaller amount of fortified lentil compared to unfortified lentil. This study demonstrated that lentil fortification is a promising and simple approach to help alleviate Fe deficiency, especially for countries in the developing world like Bangladesh, where most of the population consumes lentil in their daily meals.

CHAPTER 10

GENERAL DISCUSSION

Iron (Fe) is the fourth most abundant element on earth and is an essential micronutrient for plant and animal health. Globally, 1.62 billion people suffer from Fe deficiency anemia (Quintaes et al., 2017). To cope with this global health problem, various strategies were developed over the last few decades, including micronutrient supplementation, food fortification, biofortification, dietary diversification, nutrition education, public health intervention and food safety implementation measures. These strategies may be stand alone or may be implemented in combination. Fortification is well known and has been practiced for several decades. Biofortification and fortification to improve Fe concentration in different food crops and food products, respectively, is gaining momentum as a strategy for coping with Fe deficiency. In the body of research in this thesis, both approaches were investigated with the common goal of enhancing Fe status in lentil dal. Baseline research required to develop a biofortification strategy for lentil was initiated a decade ago at the Crop Development Centre, while the food fortification strategy for lentil dal is a more recent approach.

The significant outputs from investigations involving the biofortification strategy were (i) development of a standard protocol to determine the minimum amout of seed required for accurate and precise F-AAS analysis of whole lentil seed samples of wild and cultivated lentil, (ii) estimation of the accumulated quantity of Fe in three harvest periods in the field, and its environmental interaction with Fe concentration in developing lentil seeds, (iii) a genetic comparison of a set of RILs in which higher (compared to parents) Fe concentration, and genotype by environment interaction for SFeC was observed, and (iv) identification of potential SNP markers associated with Fe concentration in lentil seeds. Using the Fe fortification approach we

were able (i) to develop a standard laboratory scale protocol for fortification of lentil dal that can be easily developed into large scale production of fortified lentil, (ii) to determine the sensory properties of both uncooked and cooked, Fe-fortified, dehulled red lentil dal and thereby determine the most appropriate Fe fortificant from the consumer point of view, and finally, (iii) to determine the concentration and relative bioavailability of Fe in a series of traditional Bangladeshi meal plan models that included both fortified and unfortified lentil dal.

Lentil is one of the oldest cultivated crops, and its global per capita consumption is increasing. In some developing countries lentil is considered a partially staple food due to its nutritive value and is a relatively inexpensive protein source compared to animal sources. Lentil is widely consumed on a global scale, and a wide range of variability exists in the context of methods of processing and cooking (Global Crop Diversity Trust, 2014). Globally, the harvested area of pulse crops, including lentil, is about 10% -tenth of the harvested area under all cereal crops, but pulses have significant effects on crop diversification, on soil health improvement, on enhancing ecosystem resilience, and on the health and nutrition of humans and animals (Akibode & Maredia, 2011). The global annual harvested area, and yield of lentil have increased from 2010 to 2016 by 27 and 33%, respectively, (FAOSTAT, 2017). In 2016, the lentil area harvested in Canada was 2.17 M ha followed by India with 1.54 M ha (FAOSTAT, 2017).

Biofortification is mainly dependent on plant breeding activities to address micronutrient deficiency. More than 20 million people now consume biofortified food (Bouis & Saltzman, 2017). Biofortification has two major advantages over other micronutrient intervention systems, such as long-term cost-effectiveness and availability by the underserved or rural populations (Bouis & Saltzman, 2017). Since 1972, the Crop Development Centre of the University of Saskatchewan developed and released a series of lentil varieties with disease resistance, herbicide and lodging

tolerance, and with improved seed characteristics and higher yield for many market classes (Morrall, 1997; Slinkard & Vandenberg, 1995). The CDC also has been conducting the research required for a long-term breeding strategy to develop micronutrient enriched lentil varieties with increased concentrations of Fe, Zn, Se, folates, and carotenoids. A core collection of wild species accessions of the genus *Lens* were screened for micronutrients concentration and it was observed the few available accessions of *Lens lamottei* accumulated higher amounts Fe and Zn in their seeds (unpublished data). This result led us to conduct the studies reported in Chapters 3 to 6. The goal was to use the broad genotypic variation that is present in the landraces and wild accessions to improve the future biofortification program.

In our first study, we reported an accurate determination of Fe in lentil seeds by F-AAS from whole lentil seed samples of both wild and cultivated lentil species. F-AAS is widely used for estimating seed mineral concentrations due to its relatively low cost in comparison to newer technologies (e.g. inductively coupled plasma mass spectrometry (ICP-MS), and inductively coupled plasma atomic emission spectrometry (ICP-AES)) that provide quicker estimates but are relatively expensive compared to F-AAS methods. In our new method, the sample preparation does not require grinding, the procedure is rapid and simple, and therefore useful for routine analysis. For analysis of concentrations of minerals such as Fe, Zn, Se, Mn for large numbers of samples, F-AAS can be an appropriate method. Seed number per sample is an important consideration, because the production of seeds from wild accessions of lentil, especially under field conditions, is relatively difficult and unpredictable compared to seed production of the cultivated species. Some wild species accessions do not produce sufficient seed quantities to allow analysis, and seeds of the wild species of *Lens* are much smaller than those of the cultivated species. In our study, for instance, the 100-seed weight of *Lens ervoides* (IG 72815) and *Lens*

nigricans (IG 116024) accessions were about 0.5 g, whereas, 100 seed weight of *Lens culinaris* accession (CDC Greenland) was 6.9 g. This difference between wild and cultivated species may have influenced the estimation of Fe using F-AAS because seed sample sizes < 0.3 g of wild and < 0.5 g of cultivated species showed inconsistency for estimation of seed Fe concentration. Another reason could be the over-digestion of smaller seed sample sizes when using the existing higher digestion matrix. Overall, however, the results can be used to minimize the amount of valuable and rare seeds used for micronutrient analyses of seed samples of wild lentil species and their interspecific hybrid with cultivated lentil.

Although previous literature described the influence of environment on Fe accumulation, transport, and storage in the seed of different crops, the variation for seed Fe concentration over the maturity stages of indeterminate lentil plants was unknown. Results of Chapter 4 revealed that Fe concentration of seeds of different *Lens* species or genotypes were significantly different based on genotype, but not for inter-harvest or harvest × genotype interaction, except at the Crop Science Field Lab location in 2014. The 2014 cropping year at Saskatoon experienced higher than average rainfall compared to 2015. This set of environmental conditions might have influenced Fe accumulation, resulting in the observed significant differences among Fe uptake for the three sequential harvests in 2014. Apart from 2014, the non-significant difference could be due to a Fe metabolic homeostasis for the entire Fe accumulation period at maturity stage. Garcia and Grusak (2015) observed no significant differences in Fe concentration in leaves, pod walls, and seeds of the model legume *Medicago truncatula* during the reproductive stage. Since we did not analyze any other plant part for Fe accumulation, it is not possible to predict the relative amount of Fe accumulated from soil and transported to the other parts of lentil plant prior to storage in the seeds. Vasconcelos et al., (2014) suggested that apart from the reductase activity in leaf and pod wall,

other factors may limit seed Fe concentration. In transgenic soybeans with a constitutively expressed AtFRO2 iron reductase gene, Fe concentration in pod walls and leaves was greatly increased (500%), but the seed Fe concentration remained relatively low (only 10% increased). Further studies could be conducted to determine if Fe accumulation varies in different parts of lentil plants as a way to identify possible causes of differential variation of Fe concentration in seeds. Among the 7 seven lentil species, only *Lens lamottei* accessions had consistently higher Fe concentration than other species studied here. Kundu, (2016) reported similar results for seed Zn concentration. It may be the case that similar genes confer higher Fe and Zn accumulation in lentil seeds. However, further research could determine if the concentration of other minerals in *Lens lamottei* is higher than in the other species. Acceleration of a biofortification program, by hybridization followed by backcrossing, can be developed to introgress genes from *Lens lamotteii* that might lead to increased Fe accumulation.

Study 5 determined the seed Fe concentration of *Lens culinaris* × *Lens ervoides* interspecific hybrid RILs and their parents across three environments. Significant effects of genotype, location, and genotype × location were observed for seed Fe concentration. More than 80% and 35% of the interspecific RILs had significantly higher seed Fe concentration compared to the *L. culinaris* parent 'Eston' in RIL populations LR-26 and LR-59, respectively. The continuous distribution of seed Fe concentration for RILs in both populations indicated that the Fe concentration is quantitatively inherited, and that it is significantly influenced by environment. Gregorio, (2002) reported that environment had a significant influence on bean seed Fe concentration, but that high-Fe bean genotypes accumulated more Fe compared to low-Fe genotypes when grown at the same location in the same growing season. In our study, there were some RILs had higher Fe concentration but lower yield. An inverse relationship of Fe

concentration with both seed size and hundred seed weight of lentil landraces was reported by Karaköy et al., (2012). Moreover, incompatibility of various physiological traits due to the chromosomal rearrangements of interspecific RILs might have influenced the yield potential. Iron concentration in soil is heterogeneous and may also influence the accumulation of Fe (Li et al., 2016).

Association mapping is now considered a promising approach for "mining" the elite genes within available germplasm population, compared to the traditional QTL mapping approach (Zhang et al., 2014). Association mapping uses a diverse set of individual lines, such as breeding populations, landraces, and random mating population of wild species (Singh & Singh, 2015). In our study, significant variation was observed for seed Fe concentration in 138 cultivated lentil genotypes across four environments. The mean seed Fe concentration in Canadian accessions was higher than in the international accessions, which could be due to poor adaptation of the latter group in the Canadian environment. However, the association mapping analysis showed that 9 SNPs were associated with lentil seed Fe concentration. A number of candidate genes with SNP markers associated with Fe concentration were also detected. These markers can be validated in the interspecific mapping populations (LR-26 and LR-59) that were studied for seed Fe concentration (in chapter 5). Overall, these SNPs can be used for marker-assisted selection to improve Fe concentration in lentil seeds.

Developing Fe-rich lentil through biofortification is a long-term approach, inherently more difficult than producing fortified lentil products. Fe concentration in the seed is quantitatively inherited and involves several genes that are highly influenced by environment. Maintaining both yield and the desired increase in Fe concentration may be very difficult due to the possibility of

negative correlation for seed Fe concentration and seed yield. (Liu et al., 2014) reported a significant negative correlation between grain Fe and Zn concentration with grain yield.

Fortification of lentil on a large scale is relatively easy using the appropriate concentration of Fe. Fortification strategies have been used to combat Fe deficiency, especially in countries where people are vulnerable to Fe deficiency. Among the various fortificants approved by the WHO for food fortification, we initially chose three to conduct the study, selected on the basis of solubility, potential interaction with composition of the food vehicle (lentil) relative Fe bioavailability, and the cost of fortificant. To our knowledge, no fortificants have yet been recommended to fortify any legumes or pulses. After evaluation of the selected Fe-fortificants, NaFeEDTA was considered to be the most appropriate fortificant in consideration of ease of fortification, consumer acceptability, and relative Fe bioavailability.

Fe fortification may affect other qualitative attributes, such as interaction with proteins, polyphenols and other minerals. The sensory study results showed that consumers helped to determine the effect of fortification on lentil dal by evaluating appearance, odour, taste, texture and overall acceptability of fortified lentils. In this study, consumer acceptability was evaluated with panelists who regularly consume lentils. A significant difference was observed between nine uncooked and four cooked samples. Overall, the control and the NaFeEDTA-fortified uncooked samples (at fortificant Fe concentration 800 and 1600 ppm) had the highest rank score for all attributes. However, the score difference among four cooked samples (fortificant Fe concentration 1600 ppm) was negligible at both locations. This could be because the ingredients (turmeric, onion, etc.) used to cook the lentil suppressed the darkness and metallic taste (due to Fe fortificants) of cooked fortified lentil. Among the four cooked samples, NaFeEDTA-fortified (fortificants Fe

concentration 1600 ppm) sample had relatively similar acceptance by panelists in comparison to the control.

In this study, we conducted colorimetric analysis of fortified lentils and correlated the results with the sensory attributes. The uncooked and cooked samples were evaluated on the basis of five and three attributes, respectively. Results showed highly significant positive correlation for all attributes. Other attributes that can also be used to evaluate the samples. This could help with principal component analysis to assess the contribution of each attribute, and to select the main components that influence the overall acceptability of evaluated samples. T-tests results of scores obtained from Bangladesh and the Bangladeshi panelists who participated in Saskatoon were significantly different for some uncooked and cooked samples. It may well be that food habits of Bangladeshi immigrants change over time after immigration to Canada. The internal consistency reliability (CA) value indicated that panelists were highly consistent in evaluating all samples using the hedonic scales.

Both the intrinsic and the fortificant Fe are non-heme Fe that has relatively low bioavailability compared to heme-Fe. Moreover, lentil seed has antinutritional factors (e.g., phytate, polyphenols, cotyledon cell walls) that inhibit Fe absorption. Breeding for lower concentration of antinutritional factors, for example phytates, can be an option to increase bioavailability. Low phytate pea showed higher bioavailability of Fe in a previous study (Liu, 2014). Results from the relative bioavailability study showed that the Fe fortification process reduced the phytic acid concentration. This could be due to dephytinization (Koréissi-Dembélé et al., 2013) of PA during fortification. Dephytinization can remove or reduce the PA concentration and it occurs by several processes such as milling, soaking, fermenting, boiling, roasting (Gupta et al., 2013). A study of ferritin Fe bioavailability suggested that the mineral Fe core inside the

ferritin protein shell can be absorbed from legume crops, even in the presence of the known inhibitors, phytate, and tannic acid (Kalgaonkar & Lönnerdal, 2008).

Fe and PA concentration, and relative Fe bioavailability (%) from Fe-fortified and unfortified lentil dal were significantly different when used in various meal compositions in thirty meal models. The Fe concentration in lentil was increased from 60 to 439 µg g-1 after fortifying it with 2800 ppm NaFeEDTA, and thereby increased the RFeB% by 79%, as estimated by Caco-2 cell ferritin formation. A small amount of fortified lentil can provide a significant amount of bioavailable Fe that can meet a substantial part of the daily requirement of Fe for lentil consumers who consume less than the average daily recommended amount of lentil. Fe bioavailability can be influenced by other components in meals. In this study, the meal models had different food components, such as vegetables, rice, and fish in various proportions. There may be some components within foods that can either increase or decrease Fe bioavailability. For example, ascorbic acid and polyphenolic compounds have a significant effect on increasing and decreasing Fe absorption, respectively (Yun et al., 2004).

The fortification protocol developed in the laboratory was used in a commercial lentil processing plant on a trial basis. The objective was to determine the feasibility and efficacy of the protocol in terms of cost and time. The time and cost are directly related to the total production cost that would influence fortified lentil price. In the commercial trial, two additional steps were required at the end of the process. The fortificant was sprayed onto dehulled lentil seed, and this was followed by coating the dal with 0.5 to 1.0 % vegetable oil. The price of the fortificant is $ 14 US kg^{-1} and is a sufficient quantity to fortify ~780 kg of lentil. The cost of bulk fortificant would be less. Some extra cost of production may be required for initial modification of the current process. The total cost difference between fortified and unfortified lentil would be small, however,

a formal detailed economic analysis would be required to fully assess the production cost of fortified lentil at the commercial level.

Another concern may arise regarding the removal of fortified Fe from the fortified dal during rinsing prior to cooking. In this fortification protocol, we washed the fortified lentil one time with tap water before cooking. The Fe concentration was reduced by 25% (data unpublished) compared to the unwashed sample. It would be possible to distribute fortified lentils in consumer-ready form through the packaging information which could aware consumers that there is no washing required prior to cooking. A positive factor in regard to the traditions of cooking lentil is that, unlike rice, lentil is prepared in the style of stew or soup, and the cooking water is retained, resulting in little chance of loss of Fe from the soup after cooking. In another small study, Fe-fortified lentil samples were kept inside an artificially prepared chamber where the environment was controlled at high humidity (80-90%) and temperature (25-29˚C) as a simulation of the south Asian retail market environment. Colorimetric attributes of the lentil dal samples were recorded after one month. After a treatment consisting of one-month exposure to high humidity and temperature, a significant difference was found for L*, a* and b* scores between unfortified and fortified lentil samples, but no difference was found between fortified treated and fortified untreated samples (Figure 10.1).

Results from these studies helped with the design of a pilot study that was designed to identify feasible field implementation strategies using fortified lentil dal to improve bioavilable Fe uptake of adolescent girls in Bangladesh (Yunus, 2018). Another study will be conducted in the near future to determine the efficacy of using an Fe-fortified lentil dietary intervention to improve the Fe status of non-pregnant adolescent Bangladeshi rural girls (Fakir Yunus, University of Saskatchewan, personal communication).

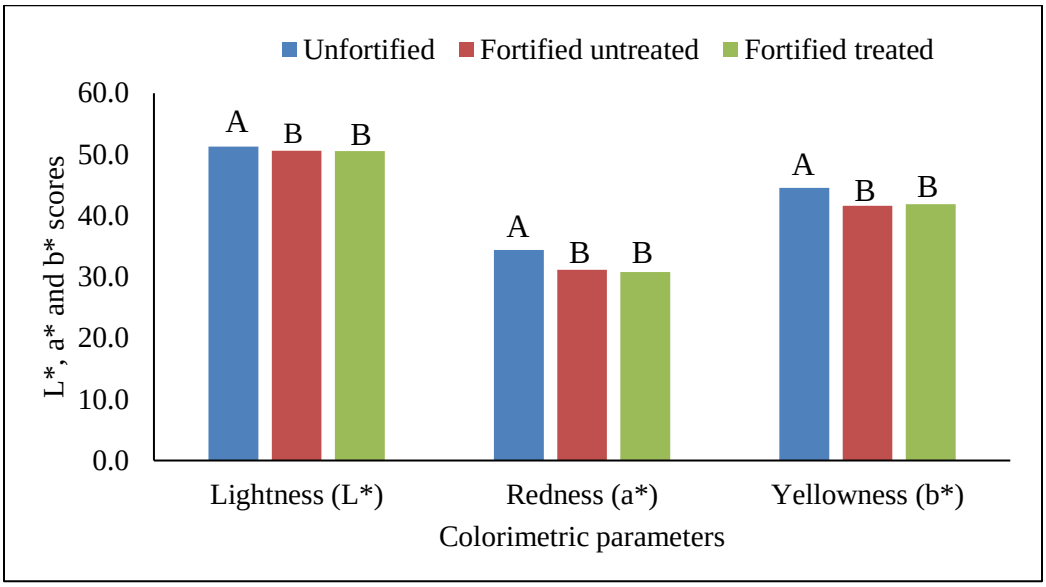


Figure 10.1. Lightness (L*), redness (a*) and yellowness (b*) scores of unfortified, fortified, and fortified red lentil dal samples exposed to high heat and humidity. Samples were prepared using NaFeEDTA at Fe concentrations of 1600 ppm and analysed after one month of storage.

In conclusion, the overall outcome of this research can help to significantly and cost-effectively increase the amount of bioavailable Fe in lentil. An interdisciplinary approach involving biofortification and fortification may provide an effective and practical approach to mitigate Fe deficiency. As the biofortification approach is a long term stretegy and the bioavailability of Fe is relatively low compared to the Fe from animal sources, the short term approach of fortification can help to provide a rapid supply of adequate amounts of the daily requirement of Fe.

CHAPTER 11

CONCLUSIONS

This series of experiments revealed that both biofortification (long term) and fortification (short term) can contribute to increasing Fe concentration in lentil. The main conclusions from the research are summarized below.

1) The protocol developed for precise and reliable estimation of Fe using F-AAS for minimum seed sample sizes will help in future assessments of seed concentrations of other minerals, saving time and cost.
2) Environmental interactions affecting lentil seed Fe accumulation were significant and need to be considered carefully before selecting genotypes or RILs for future lentil breeding with the objective of increasing seed Fe concentration. Transgressive segregants with higher seed Fe concentration were observed for two populations of interspecific lentil RILs. Selected genotypes with both higher and lower seed Fe concentration can be used to design future genetic investigations.
3) SNP markers identified from the association mapping study may provide an opportunity to validate them in bi-parental populations for deeper investigation of the genetics of increasing seed Fe concentration in lentil.
4) Considering the complete set of results from experiments involving the development of Fe fortification protocols for dehulled lentil, sensory evaluation, and bioavailability, NaFeEDTA was the most suitable Fe fortificant for lentil dal.

Overall, both biofortified and fortified (with NaFeEDTA) lentil dal can offer simple and low-cost solutions for alleviation of human health problems associated with Fe micronutrient malnutrition.

CHAPTER 12

FUTURE RESEARCH

Among the crops that are important in the human diet, most research work is based on improving yield and quality of staple carbohydrate-rich crops such as rice, wheat, potato, etc. Ensuring future food and nutritional security for healthy living and environmental balance in food production systems will require that more research effort be allocated to improvement of nutrient-rich crops and foods. The overall aim of this study was to investigate strategies for increasing the Fe concentration in lentil and lentil products using both biofortification and fortification strategies.

Initially, an optimized F-AAS procedure was developed to analyze seed Fe concentration with a minimum amount of seed from wild lentil species as a means of minimizing cost and the seed quantity required for analysis. Other micronutrients are present in lentil seeds that are both economically and nutritionally important for plants and animals. It may be worthwhile to investigate whether the developed analytical technique is suitable for the study of other micronutrients. It could be compared inductively coupled plasma mass spectrometry (ICP-MS) techniques that are now available in the CDC at the University of Saskatchewan. The use of ICP-MS would allow simultaneous analysis of the concentration of more than 20 micronutrients from the same sample.

The Fe concentration in the seven *Lens* species and the market classes of cultivated lentil was found to be stable across sampling times during the harvest period of both indeterminate and determinate types. Those genotypes that consistently maintained higher seed Fe concentration can be further evaluated for their other agronomic characteristics and yield. The outcome may provide deeper understanding of the relationship between Fe concentration and seed yield. The two *Lens*

lamottei accessions had higher seed Fe concentrations compared to genotypes of the other six lentil species. More *Lens lamottei* accessions can be collected and evaluated to observe if variability exists for even higher seed Fe concentration. It will be necessary to determine how the higher seed Fe concentration of this species can be used in hybridization programs to determine if the high seed Fe concentration is genetically transferable to cultivated lentil.

A wide range of variability for seed Fe concentration was observed in two interspecific lentil RIL populations, both involving *Lens ervoides*. A few of the RILs with higher or lower seed Fe concentration can be used for further evaluation on a small scale for other agronomic characteristics and yield potential. The outcomes of this research may help in the selection of specific RILs with high and stable Fe concentration. Quantitative trait loci also could be identified for Fe concentration in interspecific genotype because genotypic data for the LR-26 population will become available in the near future. An inheritance study for seed Fe bioavailability also could be developed for accessions from those interspecific RILs with consistently higher seed Fe concentration. The SNP markers identified from the association mapping study can be validated in different biparental population to identify QTLs involve in seed Fe accumulation. It also may be of interest to use recently derived advanced backcross populations for further study of seed Fe concentration.

In the Fe fortification study, only red football lentil dal was used. In the future, studies could identify the fortification potential of more product types, including yellow and green cotyledon colors of dehulled lentil. Moreover, split lentil is widely consumed, and theoretically has a higher total surface area per unit mass of dal. This should be lead to more Fe fortificant per unit surface area, thereby increasing the fortification efficiency.

The sensory analysis was conducted mainly using consumers from Bangladesh where lentil is consumed as football style dal on a regular basis as a relatively cheap source of protein and minerals. Fortified lentil products could be evaluated for acceptance by consumers from other countries where lentil is frequently consumed, and where large segments of the population are Fe-deficient.

The bioavailability of Fe was estimated using the in-vitro Caco-2 cell culture bioassay. Since the in vivo technique is more expensive and time consuming compared to the in vitro method, a small-scale study could be conducted to validate the results of the in vitro procedure.

Development of suitable packaging for fortified lentil products also is fundamental for efficient distribution, storage and sale. Packaging can differ on the basis of consumer preference in different regions of the world. An effective global consumer survey could be conducted in lentil consuming areas to protect market advantage, to maintain product quality, and to reduce the risk of food adulteration.

Like Fe deficiency, Zn deficiency is a major world health problem equal in scale to that of Fe deficiency. In some regions of the world, Fe and Zn deficiency occur together. Lentil products that are fortified simultaneously with Fe and Zn can provide significant health benefits to primary consumers. A preliminary experiment was conducted on a trial basis to simultaneously fortify lentil with both Fe and Zn, and this showed that double fortification is feasible. More research will be required, and in the future, research can be conducted to develop lentil products prepared with multiple fortificants to simultaneously mitigate multiple nutrient deficiencies in humans.

CHAPTER 13

SUMMARY

- Results from the seed optimization study can be used to minimize the amount of valuable and rare seeds used for micronutrient analyses of samples of wild lentil species and their interspecific hybrids.
- Genotypes with contrasting Fe concentration could be used to conduct experiments for better understanding of Fe accumulation and homeostasis in lentil.
- The trend of seed Fe accumulation was similar over the entire seed maturity stage, but that substantial variability was observed among genotypes. This variability can be exploited in future breeding programs.
- A wide range of variation for seed Fe concentration was observed among the LR-26 and LR-59 RILs based on an evaluation in three environments.
- In both populations, broad sense heritability was higher than 0.50, even though the effects of G, E, and G × E were significantly different.
- Selection of RILs with higher (> 80 ppm) and lower (< 60 ppm) Fe concentration could be used to develop new RILs that could provide more diverse populations for use in inheritance studies to identify QTL for seed Fe concentration in lentil.
- Among the genotypes in an association mapping panel, a wide range of variability was observed for seed Fe concentration.

- Two SNPs had a strong association ($-\log10\ P \geq 4.36$) with seed Fe concentration and a number of candidate genes contained SNP markers associated with seed Fe concentration.

- NaFeEDTA fortified lentils had the best appearance amongst all fortified samples tested, and was close to the appearance of the control.

- Consumers reliably preferred NaFeEDTA as the most suitable Fe fortificant for dehulled lentils based on ratings of five attributes.

- Fortification of lentils with NaFeEDTA increased the Fe concentration from 60 to 439 µg g-1 and relative Fe bioavailability from 50% to 350%.

- Phytic acid levels were reduced from 6.2 to 4.6 mg g-1 and PA:Fe molar ratios were reduced from 8.8 to 0.9 when fortified lentil was added to traditional Bangladeshi meals.

- Fortified lentil can contribute significant amounts of bioavailable Fe to populations at risk of Fe deficiency.

CHAPTER 14

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CHAPTER 15

APPENDICES

Appendix 1. Seed Fe concentration of LR-26 interspecific RILs and their parents grown at Sutherland farm (2014 and 2015) and Crop Science Field Laboratory, University of Saskatchewan in 2015.

Genotype	Fe conc. (ppm)	SE	P Value	Difference to Eston	Genotype	Fe conc. (ppm)	SE	P Value	Difference to Eston
Eston	65.1	3.3	--	--	LR-26-171	78.9	1.9	**	H
IG 72815	57.7	2.4	**	L	LR-26-173	64.8	3.2	NS	S
LR-26-4	84.5	5.8	**	H	LR-26-175	61.7	3.3	NS	S
LR-26-7	87.3	1.9	**	H	LR-26-180	69.9	3.4	*	H
LR-26-12	59.9	1.9	*	L	LR-26-181	80.4	2.5	**	H
LR-26-13	63.8	3.2	NS	S	LR-26-182	73.2	1.5	**	H
LR-26-18	66.3	1.9	NS	S	LR-26-183	67.3	2.4	NS	S
LR-26-19	70.4	3.2	*	H	LR-26-184	58.6	1.5	*	L
LR-26-20	61.6	1.7	NS	S	LR-26-186	66.5	1.9	NS	S
LR-26-22	67.1	1.5	NS	S	LR-26-187	70.1	2.0	*	H
LR-26-23	51.7	1.0	**	L	LR-26-193	73.3	2.6	**	H
LR-26-29	57.4	0.4	**	L	LR-26-194	79.0	2.5	**	H
LR-26-30	65.2	0.1	NS	S	LR-26-196	70.1	1.7	*	H
LR-26-32	61.1	3.3	NS	S	LR-26-198	67.4	1.5	NS	S
LR-26-36	71.6	0.7	*	H	LR-26-200	79.3	4.8	**	H
LR-26-41	69.3	0.8	NS	S	LR-26-202	79.4	1.8	**	H
LR-26-43	65.8	1.6	NS	S	LR-26-203	76.3	1.3	**	H
LR-26-45	71.6	2.7	*	H	LR-26-206	83.5	1.0	**	H
LR-26-47	81.9	3.8	**	H	LR-26-210	65.5	2.6	**	H

LR-26-49	81.3	5.3	**	H	LR-26-215	62.9	1.0	NS	S
LR-26-54	73.4	2.4	**	H	LR-26-216	56.2	2.4	*	L
LR-26-55	79.8	3.2	**	H	LR-26-219	61.7	2.5	NS	S
LR-26-56	68.8	3.6	NS	S	LR-26-220	73.2	3.5	**	H
LR-26-57	66.9	3.8	NS	S	LR-26-227	73.9	0.8	**	H
LR-26-62	54.2	1.8	*	L	LR-26-228	100.0	4.1	**	H
LR-26-63	54.9	.	**	L	LR-26-233	70.9	2.8	**	H
LR-26-64	62.6	3.9	NS	S	LR-26-228	77.0	3.2	**	H
LR-26-67	78.4	3.7	**	H	LR-26-238	75.7	1.8	**	H
LR-26-77	52.2	1.6	**	L	LR-26-239	78.8	3.0	**	H
LR-26-78	73.3	2.0	**	H	LR-26-240	64.5	0.7	NS	S
LR-26-79	65.8	1.8	NS	S	LR-26-241	64.0	3.4	NS	S
LR-26-83	56.8	5.1	***	L	LR-26-233	93.8	3.0	**	H
LR-26-84	71.7	2.2	**	H	LR-26-244	78.0	1.3	**	H
LR-26-85	68.7	3.8	NS	S	LR-26-246	67.8	3.0	***	H
LR-26-90	70.9	2.6	*	L	LR-26-251	59.5	1.9	NS	S
LR-26-91	74.1	5.7	**	L	LR-26-252	63.0	3.1	NS	S
LR-26-95	71.4	3.9	**	L	LR-26-253	79.5	3.4	***	H
LR-26-98	73.0	1.0	*	L	LR-26-254	81.4	3.9	***	H
LR-26-99	81.5	4.4	***	L	LR-26-256	66.7	1.7	NS	S
LR-26-105	80.5	2.9	***	L	LR-26-257	76.4	1.4	**	H
LR-26-107	75.3	4.2	**	L	LR-26-261	67.1	2.0	NS	S
LR-26-108	68.6	3.4	NS	S	LR-26-262	70.4	1.8	**	H
LR-26-111	81.1	3.6	***	H	LR-26-266	76.4	2.8	***	H
LR-26-112	67.9	1.7	NS	S	LR-26-267	81.8	3.6	***	H

LR-26-113	64.0	1.6	NS	S	LR-26-269	75.1	1.6	**	H
LR-26-115	71.3	3.9	*	H	LR-26-273	70.3	1.3	**	H
LR-26-116	68.6	7.1	NS	S	LR-26-274	65.1	2.3	NS	S
LR-26-117	71.2	3.2	**	H	LR-26-275	76.0	2.7	***	H
LR-26-118	72.8	2.5	***	H	LR-26-276	63.9	2.7	NS	S
LR-26-121	75.2	1.8	***	H	LR-26-280	82.4	4.1	**	H
LR-26-122	69.8	3.9	*	H	LR-26-281	62.9	1.3	NS	S
LR-26-123	81.0	1.2	**	H	LR-26-282	81.5	2.5	***	H
LR-26-125	86.9	7.3	***	H	LR-26-283	78.6	5.3	***	H
LR-26-127	74.4	3.5	**	H	LR-26-288	80.1	2.4	***	H
LR-26-132	82.9	3.8	***	H	LR-26-290	60.0	1.9	**	L
LR-26-134	51.0	1.8	**	L	LR-26-292	65.3	2.2	NS	S
LR-26-135	74.2	4.1	***	H	LR-26-293	70.7	4.5	*	H
LR-26-136	81.2	3.7	***	H	LR-26-294	77.6	4.1	**	H
LR-26-138	73.4	5.5	**	H	LR-26-296	76.0	3.5	**	H
LR-26-139	60.0	3.1	*	L	LR-26-297	69.6	1.8	**	H
LR-26-140	63.0	2.8	NS	S	LR-26-298	91.2	4.7	**	H
LR-26-145	72.9	3.3	***	H	LR-26-300	74.7	3.2	***	H
LR-26-151	63.5	4.1	NS	S	LR-26-301	80.3	3.0	***	H
LR-26-156	74.3	1.9	***	H	LR-26-303	76.4	3.8	**	H
LR-26-157	73.6	2.1	***	H	LR-26-307	61.2	2.3	NS	S
LR-26-161	78.0	1.2	**	H	LR-26-311	76.5	3.7	***	H
LR-26-165	84.7	3.0	***	H	LR-26-312	65.8	1.9	NS	S

NS – non-significant Fe conc. with *L. culinaris* parent 'Eston'; H – significantly higher Fe conc. than 'Eston'; S – Significantly similar to Eston; L – significantly lower Fe concentration than 'Eston'; *,**,*** - significantly different seed Fe concentration than *L. culinaris* parent Eston at $p \leq 0.05$, 0.01, and 0.001 level, respectively.

Appendix 2: Seed Fe concentration of LR-26 interspecific RILs and their parents grown at Sutherland farm (2014 and 2015) and Crop Science Field Laboratory, University of Saskatchewan in 2015.

Genotype	Fe conc. (ppm)	SE	P Value	Difference to Eston	Genotype	Fe conc. (ppm)	SE	P Value	Difference to Eston
Eston	68.7	1.7			LR-59-59	69.5	1.4	NS	S
L01827A	62.1	2.0	*	L	LR-59-60	75.2	4.7	*	H
LR-59-1	80.4	2.6	***	H	LR-59-62	68.8	4.5	NS	S
LR-59-5	95.9	2.8	***	H	LR-59-70	79.6	5.4	***	H
LR-59-6	65.2	1.3	NS	S	LR-59-74	67.9	2.6	NS	S
LR-59-7	78.1	4.1	**	H	LR-59-76	63.5	3.8	*	L
LR-59-10	73.5	2.6	*	H	LR-59-78	63.4	4.2	*	L
LR-59-11	78.3	3.2	***	H	LR-59-80	68.9	3.8	NS	S
LR-59-14	62.1	2.9	*	L	LR-59-81	82.1	1.7	**	H
LR-59-15	90.9	2.7	**	H	LR-59-86	63.3	2.4	**	L
LR-59-23	70.5	2.7	NS	S	LR-59-87	67.3	2.9	NS	S
LR-59-25	78.6	3.2	***	H	LR-59-89	73.0	2.5	NS	S
LR-59-27	73.4	3.9	**	H	LR-59-90	61.8	3.7	*	L
LR-59-29	63.3	2.6	*	L	LR-59-91	61.7	2.2	**	L
LR-59-30	61.6	2.2	**	L	LR-59-103	63.9	3.3	NS	S
LR-59-34	73.5	4.6	**	H	LR-59-104	71.7	6.4	NS	S
LR-59-35	66.4	2.1	NS	S	LR-59-105	75.9	3.1	*	H
LR-59-36	62.4	2.9	**	L	LR-59-106	81.1	10.1	**	H
LR-59-42	64.5	2.8	NS	S	LR-59-112	75.2	0.8	**	H
LR-59-43	69.4	2.3	NS	S	LR-59-122	83.6	3.3	**	H

LR-59-44	76.7	5.8	***	H	LR-59-126	67.6	2.8	NS	S
LR-59-47	71.8	1.8	NS	S	LR-59-127	53.8	2.7	***	L
LR-59-53	70.5	0.9	NS	S	LR-59-128	73.9	2.0	**	H
LR-59-55	78.4	1.6	***	H	LR-59-132	64.7	3.1	NS	S
LR-59-56	72.9	4.0	NS	S	LR-59-133	64.7	4.5	NS	S

NS – non-significant Fe conc. with *L. culinaris* parent 'Eston'; H – significantly higher Fe conc. than 'Eston'; L – significantly lower Fe concentration than 'Eston'; *, **, *** - significantly different seed Fe concentration than *L. culinaris* parent Eston at $p \leq 0.05$, 0.01, and 0.001 level, respectively.

Appendix 3. Average values of Iron (Fe, ppm) concentrations in studied lentil accessions (across locations and years). SPG, Saskatchewan Pulse Growers farm; STH, Sutherland.

Accessions	Fe 2013 SPG	Fe 2013 STH	Fe 2013	Fe 2014 SPG	Fe 2014 STH	Fe 2014	Fe TOTAL
3156-11	86.3	71.9	79.1	80.5	76.7	78.6	78.8
CDC Asterix	89.0	72.5	80.8	83.8	78.4	81.1	80.9
CDC Blaze	92.4	81.7	87.1	91.2	74.0	82.6	84.8
CDC Cherie	76.7	64.4	70.5	73.1	70.5	71.8	71.2
CDC Dazil	81.5	74.0	77.7	89.4	79.5	84.4	81.1
CDC Glamis	79.5	81.7	80.6	70.1	72.3	71.2	75.9
CDC Grandora	88.8	83.8	86.3	69.0	76.2	72.6	79.5
CDC Greenland	73.7	72.6	73.2	63.9	71.2	67.6	70.4
CDC Greenstar	85.0	80.7	82.8	82.5	78.7	80.6	81.7
CDC Imax	92.7	84.2	88.4	88.2	85.8	87.0	87.7
CDC Imigreen	89.7	73.5	81.6	80.5	77.3	78.9	80.2
CDC Impact	89.4	82.0	85.7	82.3	78.0	80.2	83.0
CDC Impala	86.0	85.2	85.6	88.1	79.5	83.8	84.7
CDC Imperial	88.6	81.3	84.9	91.6	85.5	88.5	86.7
CDC Impower	78.9	74.6	76.7	76.7	78.3	77.5	77.1
CDC Impress	72.5	76.4	74.4	60.3	69.2	64.8	69.6
CDC Improve	96.0	87.6	91.8	76.4	85.5	81.0	86.4
CDC Imvincible	85.3	83.5	84.4	85.5	84.9	85.2	84.8
CDC KR-1	77.0	73.2	75.1	72.9	70.4	71.6	73.4
CDC LeMay	78.7	67.9	73.3	76.2	73.1	74.7	74.0
CDC Matador	96.3	79.9	88.1	98.4	84.0	91.2	89.6
CDC Maxim	84.5	78.4	81.4	78.9	76.2	77.5	79.5
CDC Meteor	82.1	75.5	78.8	70.0	70.8	70.4	74.6
CDC Milestone	79.3	73.0	76.2	74.8	73.9	74.3	75.3
CDC Peridot	79.9	71.1	75.5	69.3	72.3	70.8	73.2

CDC Plato	81.0	78.0	79.5	78.4	74.8	76.6	78.1
CDC QG-1	72.9	68.1	70.5	82.1	70.3	76.2	73.4
CDC Redberry	85.7	79.1	82.4	83.7	78.6	81.1	81.8
CDC Redbow	85.2	73.9	79.6	74.3	75.1	74.7	77.1
CDC Redcap	78.4	76.4	77.4	78.4	69.5	74.0	75.7
CDC Redcliff	70.4	63.9	67.2	74.2	67.5	70.9	69.0
CDC Redcoat	90.6	80.3	85.4	87.7	75.8	81.8	83.6
CDC RedRider	80.7	74.0	77.4	75.6	73.6	74.6	76.0
CDC Redwing	95.3	92.2	93.8	92.9	84.5	88.7	91.3
CDC Richlea	81.9	81.6	81.8	84.8	79.4	82.1	81.9
CDC Robin	87.8	84.2	86.0	89.3	75.8	82.5	84.3
CDC Rosebud	87.7	74.7	81.2	81.7	75.6	78.6	79.9
CDC Rosetown	89.7	86.1	87.9	95.6	88.8	92.2	90.1
CDC Rosie	95.7	81.0	88.4	90.9	85.7	88.3	88.3
CDC Rouleau	86.2	70.6	78.4	84.9	79.7	82.3	80.3
CDC Royale	79.3	64.4	71.8	72.1	65.6	68.9	70.4
CDC Ruby	94.7	81.9	88.3	91.2	81.9	86.6	87.4
CDC SB-1	69.9	63.5	66.7	64.9	63.1	64.0	65.3
CDC Sedley	94.4	86.1	90.2	91.8	83.4	87.6	88.9
CDC Sovereign	85.4	77.5	81.4	79.9	77.2	78.5	80.0
CDC Vantage	92.7	78.1	85.4	77.5	82.0	79.8	82.6
CDC Viceroy	80.6	74.8	77.7	80.0	79.6	79.8	78.7
Crimson	76.7	72.8	74.8	68.2	70.6	69.4	72.1
Eston	82.3	70.6	76.4	74.4	72.0	73.2	74.8
ILL 1139	74.0	66.8	70.4	62.8	64.6	63.7	67.1
ILL 1220	88.3	83.1	85.7	86.3	78.7	82.5	84.1
ILL 1337	101.5	82.2	91.8	93.4	88.1	90.8	91.3
ILL 1553	83.4	81.0	82.2	83.2	78.8	81.0	81.6
ILL 1762	80.2	70.9	75.6	71.3	69.9	70.6	73.1
ILL 1861	75.5	67.5	71.5	67.9	69.4	68.6	70.1
ILL 1983	59.0	56.1	57.6	50.5	61.4	56.0	56.8

ILL 2194	63.7	55.0	59.3	69.5	58.2	63.9	61.6
ILL 2217	94.0	79.3	86.6	75.3	85.2	80.3	83.4
ILL 2290	93.1	81.7	87.4	88.3	86.2	87.2	87.3
ILL 242	97.1	84.3	90.7	84.9	89.6	87.2	89.0
ILL 2433	71.1	68.1	69.6	66.6	67.7	67.2	68.4
ILL 2501	56.2	61.7	58.9	56.7	62.5	59.6	59.3
ILL 2526	59.1	54.9	57.0	52.9	61.7	57.3	57.2
ILL 2607	53.6	56.2	54.9	49.5	53.0	51.3	53.1
ILL 2684	68.2	63.0	65.6	68.1	67.2	67.6	66.6
ILL 2789	51.1	64.2	57.7	52.0	62.1	57.0	57.4
ILL 28	77.8	70.0	73.9	57.7	64.6	61.1	67.5
ILL 293	70.7	68.4	69.6	61.1	64.3	62.7	66.1
ILL 3025	59.2	59.9	59.6	41.5	51.7	46.6	53.1
ILL 313	81.7	76.2	79.0	64.9	77.2	71.0	75.0
ILL 3347	49.5	58.8	54.2	60.8	56.0	58.4	56.3
ILL 3502	68.7	62.3	65.5	75.7	68.3	72.0	68.8
ILL 3597	54.4	55.4	54.9	50.7	53.8	52.2	53.6
ILL 4164	65.5	63.1	64.3	55.9	64.4	60.2	62.2
ILL 4359	58.8	62.3	60.5	57.2	68.2	62.7	61.6
ILL 4400	88.2	77.3	82.8	70.7	81.6	76.2	79.5
ILL 4605	68.9	73.2	71.1	67.5	79.6	73.5	72.3
ILL 4609	66.5	66.7	66.6	63.7	72.7	68.2	67.4
ILL 4665	76.5	72.1	74.3	70.4	71.4	70.9	72.6
ILL 4671	92.4	89.1	90.8	89.7	91.3	90.5	90.6
ILL 4740	72.6	66.9	69.7	64.1	67.2	65.6	67.7
ILL 4768	65.5	61.1	63.3	62.5	64.0	63.2	63.3
ILL 4783	85.1	79.4	82.2	79.9	84.3	82.1	82.1
ILL 4804	71.1	62.3	66.7	69.9	70.1	70.0	68.4
ILL 4875	88.5	76.6	82.6	73.1	80.9	77.0	79.8
ILL 4956	83.4	79.4	81.4	83.8	74.4	79.1	80.2
ILL 5058	94.9	85.0	90.0	81.7	82.4	82.1	86.0

ILL 5151	71.0	69.8	70.4	61.9	74.1	68.0	69.2
ILL 5209	64.8	62.8	63.8	69.1	72.0	70.6	67.2
ILL 5490	83.9	86.0	84.9	71.5	84.4	77.9	81.4
ILL 5511	77.1	80.1	78.6	77.3	79.3	78.3	78.4
ILL 5883	64.7	61.8	63.2	69.5	68.1	68.8	66.0
ILL 5945	87.3	64.0	75.6	72.5	67.3	69.9	72.8
ILL 618	99.6	82.7	91.1	84.4	83.3	83.8	87.5
ILL 6182	74.0	84.8	79.4	66.3	69.2	67.7	73.6
ILL 624	84.0	76.7	80.4	77.9	76.9	77.4	78.9
ILL 6853	86.3	75.2	80.7	71.4	69.1	70.2	75.5
ILL 6967	80.6	81.2	80.9	81.9	73.9	77.9	79.4
ILL 7089	79.5	73.7	76.6	82.4	67.1	74.8	75.7
ILL 7585	80.9	76.8	78.9	72.7	73.8	73.2	76.0
ILL 7747	69.2	65.9	67.5	63.2	63.9	63.5	65.5
ILL 80	86.0	72.6	79.3	73.0	79.5	76.3	77.8
ILL 9	65.0	66.2	65.6	59.7	63.5	61.6	63.6
ILL 927	94.8	79.0	86.9	83.8	81.3	82.6	84.7
Indian Head	87.7	79.3	83.5	96.3	80.3	88.3	85.9
Laird	87.6	83.3	85.5	74.8	77.0	75.9	80.7
PI 178939	72.4	77.8	75.1	75.2	74.3	74.7	74.9
PI 178971	85.7	84.3	85.0	86.2	92.1	89.1	87.1
PI 217949	52.3	58.7	55.5	65.9	61.7	63.8	59.6
PI 251032	91.1	76.3	83.7	82.0	77.8	79.9	81.8
PI 273664	76.5	68.0	72.2	58.3	66.4	62.3	67.3
PI 297284	97.0	87.9	92.4	89.2	90.0	89.6	91.0
PI 298631	89.9	80.6	85.2	86.6	85.5	86.0	85.6
PI 298922	86.3	75.9	81.1	81.5	76.9	79.2	80.1
PI 299121	63.7	62.6	63.1	62.8	71.7	67.3	65.2
PI 299126	71.5	69.5	70.5	68.0	66.4	67.2	68.9
PI 299215	101.7	84.1	92.9	94.0	91.8	92.9	92.9
PI 300250	66.5	71.5	69.0	70.8	73.3	72.0	70.5

PI 308614	73.7	72.8	73.2	67.9	77.6	72.8	73.0
PI 320954	81.2	75.3	78.2	69.1	81.5	75.3	76.8
PI 329169	92.1	80.7	86.4	74.8	93.9	84.4	85.4
PI 339283	77.1	70.6	73.8	69.2	75.1	72.2	73.0
PI 339285	81.7	75.7	78.7	73.0	78.1	75.6	77.1
PI 339292	96.7	86.7	91.7	90.4	87.7	89.0	90.3
PI 343026	83.4	75.4	79.4	84.9	88.6	86.8	83.1
PI 357225	76.1	76.4	76.2	85.2	81.9	83.6	79.9
PI 368647	89.2	80.4	84.8	77.0	81.5	79.3	82.0
PI 420929	84.8	78.1	81.4	76.3	88.4	82.4	81.9
PI 426803	59.9	58.4	59.1	46.6	60.1	53.4	56.2
PI 431662	86.1	71.2	78.6	81.9	79.9	80.9	79.8
PI 431679	94.8	76.4	85.6	82.0	81.8	81.9	83.8
PI 431705	96.3	84.9	90.6	100.3	83.6	92.0	91.3
PI 431710	90.2	77.4	83.8	86.7	87.8	87.3	85.5
PI 431714	87.5	83.7	85.6	81.6	76.9	79.2	82.4
PI 431717	96.7	86.1	91.4	81.2	85.6	83.4	87.4
PI 431756	79.1	71.0	75.0	78.3	78.4	78.3	76.7
W6 27764	53.1	59.6	56.4	51.9	73.9	62.9	59.7
W6 27766	64.3	69.8	67.1	62.9	67.2	65.1	66.1

Appendix 4. List of candidate genes for significant SNP markers. The sequence of markers were used for blast search in the DNA database of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome#) and Phytozome against MT 4.0v1 (https://phytozome.jgi.doe.gov/pz/portal.html#!search?show=BLAT&method=Org_Mtruncatula)

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Marker	Candidate
LcC25737p350	*Medicago truncatula* clone JCVI-FLMt-7K9 unknown mRNA
LcC01329p253	PREDICTED: *Cicer arietinum* photosynthetic NDH subunit of lumenal location 4, chloroplastic (LOC101497210), transcript variant X2, mRNA
LcC24316p626	*Medicago truncatula* transmembrane protein, putative mRNA
LcC11104p161	*Medicago truncatula* fatty acid/sphingolipid desaturase mRNA
LcC01714p78	*Medicago truncatula* clone JCVI-FLMt-3K9 unknown mRNA
LcC01908p896	*Medicago truncatula* flavonoid O-methyltransferase-like protein mRNA
LcC06625p437	*Medicago truncatula* cytosolic Fe-S cluster assembly factor NUBP1-like protein mRNA
LcC07856p82	*Medicago truncatula* thiazole biosynthetic enzyme mRNA
LcC21183p306	*Medicago truncatula* hypothetical protein mRNA
LcC06739p564	*Cicer arietinum* nuclear transport factor 2-like (LOC101504278), mRNA
LcC04105p1090	No significant similarity found
LcC18132p1029	*Medicago truncatula* 1-aminocyclopropane-1-carboxylate oxidase mRNA
LcC01084p238	*Medicago truncatula* RPM1-interacting protein 4 (RIN4) family protein mRNA
LcC05435p444	*Medicago truncatula* kinesin-like protein for actin-based movement protein mRNA
LcC09698p304	*Medicago truncatula* S3 self-incompatibility locus-linked pollen 3.15 protein mRNA
LcC11556p306	*Medicago truncatula* stem-loop-binding protein of 41 kDa protein B mRNA
LcC05176p392	*Medicago truncatula* tplate-like protein partial mRNA
LcC02533p226	*Medicago truncatula* alanine-glyoxylate aminotransferase-like protein partial mRNA

Appendix 5. T-tests results for sensory evaluation data for ten uncooked and four cooked lentil samples evaluated by panelists from Bangladesh (n=98) and Bangladeshi panelists who participated in Saskatoon (n=20)

	Appearance			Odour			Overall acceptability		
Uncooked samples	Df[1]	Cal t[2]	Tab t[3]	Df	Cal t	Tab t	Df	Cal t	Tab t
Control	29	1.86	2.05	37	0.76	2.02	33	1.24	2.04
$FeSO_4 \cdot 7H_2O$ 800ppm	21	3.07*	2.08	22	1.12	2.07	22	2.79*	2.07
$FeSO_4 \cdot 7H_2O$ 1600ppm	31	0.5	2.05	34	1.78	2.04	31	0.64	2.04
$FeSO_4 \cdot 7H_2O$ 2800ppm	27	6.65*	2.05	25	2.73*	2.06	29	4.41*	2.05
NaFeEDTA 800ppm	21	11.22*	2.08	33	2.32*	2.04	32	2.25*	2.04
NaFeEDTA 1600ppm	30	0.09	2.05	37	3.2*	2.02	24	0.25	2.06
NaFeEDTA 2800ppm	24	2.56*	2.06	24	1.08	2.07	22	1.61	2.07
$FeSO_4 \cdot H_2O$ 800ppm	23	0.93	2.07	40	0.95	2.02	28	0.72	2.05
$FeSO_4 \cdot H_2O$ 1600ppm	27	1.52	2.05	27	0.22	2.05	28	1.02	2.05
$FeSO_4 \cdot H_2O$ 2800ppm	28	3.72*	2.05	23	1.14	2.07	28	3.33*	2.05

	Appearance			Odour			Taste			Texture			Overall acceptability		
Cooked Samples	Df	Cal t	Tab t	Df	Cal t	Tab t	Df	Cal t	Tab t	Df	Cal t	Tab t	Df	Cal t	Tab t
Control	33	4.85*	2.04	34	3.49*	2.04	36	2.93*	2.04	30	3.37*	2.03	30	2.85*	2.05
NaFeEDTA 1600ppm	27	2.54*	2.05	34	0.87	2.04	24	0.19	2.07	25	0.68	2.06	29	0.86	2.05
$FeSO_4 \cdot H_2O$ 1600ppm	29	3.17*	2.05	22	1.54	2.07	27	2.14*	2.05	26	2.59*	2.06	25	2.35*	2.07
$FeSO_4 \cdot 7H_2O$ 1600ppm	38	0.32	2.02	34	2.41*	2.04	28	0.87	2.05	37	1.58	2.04	36	1.33	2.02

[1] - Degrees of freedom; [2]- Calculated t value; [3] - Tabulated t value; *- Statistically significant difference of sensory evaluation data evaluated by panelists from Bangladesh and Bangladeshi panelists who participated in Saskatoon.

Appendix 6. Average score for sensory evaluation data for ten uncooked and four cooked lentil samples evaluated by panelists from Bangladesh (n = 98) and Bangladeshi panelists (n = 20) who participated in Saskatoon

	Appearance		Odour		Overall acceptability	
Uncooked samples	BD1[a]	BD2[b]	BD1[a]	BD2[b]	BD1[a]	BD2[b]
Control	8.0	8.4	7.3	7.5	7.9	8.1
$FeSO_4 \cdot 7H_2O$ 800ppm	6.7	5.5	6.5	6.1	6.4	5.4
$FeSO_4 \cdot 7H_2O$ 1600ppm	5.5	5.6	5.6	6.1	5.5	5.7
$FeSO_4 \cdot 7H_2O$ 2800ppm	5.6	3.1	5.2	4.0	5.2	3.4
NaFeEDTA 800ppm	7.1	5.1	6.5	7.1	6.9	7.4
NaFeEDTA 1600ppm	7.0	7.0	6.3	7.1	6.9	6.8
NaFeEDTA 2800ppm	7.0	6.3	6.6	6.3	6.8	6.4
$FeSO_4 \cdot H_2O$ 800ppm	6.6	6.3	6.3	6.6	6.5	6.3
$FeSO_4 \cdot H_2O$ 1600ppm	5.5	5.0	5.7	5.6	5.5	5.2
$FeSO_4 \cdot H_2O$ 2800ppm	4.7	3.2	4.7	4.1	4.6	3.3

	Appearance		Odour		Taste		Texture		Overall acceptability	
Cooked Samples	BD1[a]	BD2[b]	BD1[a]	BD2[b]	BD1[a]	BD2[b]	BD1[a]	BD2[b]	BD1[a]	BD2[b]
Control	7.1	8.1	7.1	7.9	7.0	7.7	6.9	7.8	7.1	7.7
NaFeEDTA 1600ppm	7.3	7.0	7.1	7.3	7.2	7.3	7.3	7.3	7.1	7.3
$FeSO_4 \cdot H_2O$ 1600ppm	6.9	7.5	6.9	7.3	6.9	7.5	6.7	7.4	6.8	7.4
$FeSO_4 \cdot 7H_2O$ 1600ppm	7.1	7.2	7.1	6.6	7.2	7.0	7.1	6.8	7.2	6.9

[a] – Bangladeshi panelists; [b]- Bangladeshi panelists who participated in Saskatoon

Appendix 7. T-tests results for sensory evaluation data for ten uncooked and four cooked lentil samples evaluated by panelists from Bangladesh (n = 20) and India (n = 15) who participated in Saskatoon

	Appearance			Odour			Overall acceptability		
Uncooked samples	Df[1]	Cal t[2]	Tab t[3]	Df	Cal t	Tab t	Df	Cal t	Tab t
Control	28	0.10	2.05	29	0.674	2.05	31	0.74	2.04
$FeSO_4 \cdot 7H_2O$ 800ppm	30	2.83*	2.04	30	2.83*	2.04	31	0.97	2.04
$FeSO_4 \cdot 7H_2O$ 1600ppm	24	9.24*	2.06	27	0.08	2.05	28	0.58	2.05
$FeSO_4 \cdot 7H_2O$ 2800ppm	21	14.66*	2.08	31	0.23	2.04	31	5.35*	2.04
NaFeEDTA 800ppm	29	0.372	2.05	25	0.84	2.06	21	4.08*	2.08
NaFeEDTA 1600ppm	31	2.88*	2.04	28	0.43	2.05	30	1.22	2.04
NaFeEDTA 2800ppm	31	1.34	2.04	29	0.11	2.05	31	1.33	2.04
$FeSO_4 \cdot H_2O$ 800ppm	28	1.24	2.05	26	1.78	2.06	31	0.2	2.04
$FeSO_4 \cdot H_2O$ 1600ppm	27	4.61*	2.05	27	0.34	2.05	30	1.06	2.04
$FeSO_4 \cdot H_2O$ 2800ppm	26	8.71*	2.06	30	0.37	2.04	31	0.75	2.04

	Appearance			Odour			Taste			Texture			Overall acceptability		
Cooked samples	Df	Cal t	Tab t	Df	Cal t	Tab t	Df	Cal t	Tab t	Df	Cal t	Tab t	Df	Cal t	Tab t
Control	29	1.77	2.05	25	1.63	2.06	31	0.38	2.04	31	0.76	2.04	30	0.19	2.04
NaFeEDTA 1600ppm	31	1.7	2.04	31	1.62	2.04	30	1.06	2.04	31	0.37	2.04	30	0.34	2.04
$FeSO_4 \cdot H_2O$ 1600ppm	28	0.91	2.05	25	0.36	2.06	31	0.44	2.04	25	0.9	2.06	28	0.75	2.05
$FeSO_4 \cdot 7H_2O$ 1600ppm	29	2.49*	2.05	30	1.37	2.04	31	1.36	2.04	29	1.33	2.05	30	1.02	2.04

[1] - Degrees of freedom; [2]- Calculated t value; [3] - Tabulated t value; *- Statistically significant difference of sensory evaluation data evaluated by panelists from Bangladesh and India who participated in Saskatoon.

Appendix 8. Average scores for sensory evaluation data for ten uncooked and four cooked lentil samples evaluated by Bangladeshi (n = 20) and Indian panelists (n = 15) who participated in Saskatoon

Uncooked samples	Appearance		Odour		Overall acceptability	
	BD[a]	India	BD[a]	India	BD[a]	India
Control	8.4	8.4	7.5	7.3	8.1	8.3
$FeSO_4 \cdot 7H_2O$ 800ppm	5.4	5.8	6.1	7.3	5.5	6.0
$FeSO_4 \cdot 7H_2O$ 1600ppm	5.6	6.3	6.1	6.2	5.7	6.1
$FeSO_4 \cdot 7H_2O$ 2800ppm	3.6	5.3	3.9	4.1	3.4	4.9
NaFeEDTA 800ppm	7.7	7.8	7.1	6.8	7.4	6.1
NaFeEDTA 1600ppm	7.0	7.8	6.7	6.5	6.8	7.2
NaFeEDTA 2800ppm	6.3	6.7	6.3	6.3	6.3	6.7
$FeSO_4 \cdot H_2O$ 800ppm	6.3	6.0	6.6	6.0	6.3	6.4
$FeSO_4 \cdot H_2O$ 1600ppm	4.9	6.7	5.6	5.7	5.2	5.5
$FeSO_4 \cdot H_2O$ 2800ppm	3.2	4.7	4.1	4.3	3.3	3.6

Cooked samples	Appearance		Odour		Taste		Texture		Overall acceptability	
	BD[a]	India	BD[a]	India	BD[a]	India	BD[a]	India	BD[a]	India
Control	8.0	7.6	7.8	7.3	7.7	7.6	7.7	7.5	7.7	7.7
NaFeEDTA 1600ppm	7.6	7.3	7.2	6.8	7.2	7.4	7.3	7.2	7.3	7.2
$FeSO_4 \cdot H_2O$ 1600ppm	7.3	7.1	6.5	6.4	7.0	7.2	6.7	7.0	6.9	7.1
$FeSO_4 \cdot 7H_2O$ 1600ppm	7.5	6.9	7.3	6.7	7.4	7.1	7.4	7.0	7.4	7.1

[a] – Bangladesh;

Appendix 9. Correlation coefficients between sensory acceptability score from both Saskatoon and Bangladesh for three attributes of uncooked lentil samples (appearance, odour and overall acceptability) and colorimetric data (lightness (L*), redness (a*) and yellowness (b*) score) obtained from HunterLab.

	Saskatoon			Bangladesh		
	Lightness	Redness	Yellowness	Lightness	Redness	Yellowness
Appearance (n = 10)	0.93	0.97	0.96	0.88	0.91	0.92
Odor (n = 10)	0.96	0.95	0.94	0.95	0.92	0.92
Overall acceptability (n = 10)	0.94	0.96	0.96	0.92	0.95	0.95

L*, Lightness; a*, redness; b*, yellowness; all the correlation coefficients were found significant at $p < 0.001$

Appendix 10: Description of the 30 meal models

Meal models	Rice (%)	Vegetable (%)	Fish (%)	Unfortified dal (%)	NaFeEDTA-fortified dal (%)
Meal models with fortified lentil					
Model 1	50	0	0	50	0
Model 2	50	25	0	25	0
Model 3	75	10	0	15	0
Model 4	75	5	10	10	0
Model 5	75	0	0	25	0
Model 6	75	0	10	15	0
Model 7	85	10	0	5	0
Model 8	85	5	5	5	0
Model 9	85	0	0	15	0
Model 10	85	0	5	10	0
Model 11	85	0	10	5	0
Meal models without lentil					
Model 12	75	25	0	0	0
Model 13	50	25	25	0	0
Model 14	85	15	0	0	0
Meal models with fortified lentil					
Model 15	50	0	0	0	50
Model 16	50	25	0	0	25
Model 17	75	10	0	0	15
Model 18	75	5	10	0	10
Model 19	75	0	0	0	25
Model 20	75	0	10	0	15
Model 21	75	10	0	0	15
Model 22	85	5	5	0	5
Model 23	85	0	0	0	15
Model 24	85	0	5	0	10
Model 25	85	0	10	0	5
Meal models, each contain one component at 100%					
Model 26	100	0	0	0	0
Model 27	0	100	0	0	0
Model 28	0	0	100	0	0
Model 29	0	0	0	100	0
Model 30	0	0	0	0	100

Appendix 11: Iron (Fe) concentration, relative Fe bioavailability (RFeB%), and phytic acid (PA) concentration (mean ± SD) and PA:Fe molar ratio of 30 meal plan models composed of varying percentages by volume of the amounts of rice, vegetable curry, fish and dal (lentil dish prepared with either fortified or unfortified lentil)

	Rice (%)	Veg (%)	Fish (%)	Unfortified lentil dal (%)	Fortified lentil dal (%)	Fe ($\mu g\ g^{-1}$)	RFeB%	PA ($mg\ g^{-1}$)	PA:Fe molar ratio
Meal models with unfortified lentil									
Model 1	50	0	0	50	0	30.8±0.6	61.0±10.8	3.9±0.0	10.6
Model 2	50	25	0	25	0	20.9±0.6	91.1±6.9	3.1±0.2	12.5
Model 3	75	10	0	15	0	12.7±0.9	44.7±0.1	2.2±0.1	14.4
Model 4	75	5	10	10	0	9.8±0.7	73.8±3.1	2.2±0.1	18.9
Model 5	75	0	0	25	0	18.1±0.1	33.5±1.9	2.7±0.2	12.8
Model 6	75	0	10	15	0	14.4±1.6	83.6±3.8	2.6±0.1	15.4
Model 7	85	10	0	5	0	7.1±0.2	27.4±2.5	1.6±0.1	19.7
Model 8	85	5	5	5	0	7.5±0.5	53.6±5.3	1.9±0.1	21.0
Model 9	85	0	0	15	0	13.7±1.3	22.0±5.2	2.4±0.2	14.6
Model 10	85	0	5	10	0	8±0.4	22.5±6.94	1.6±0.18	17.4
Model 11	85	0	10	5	0	5.7±0.4	49.8±6.69	1.9±0.03	28.3

Meal models without lentil									
Model 12	75	25	0	0	0	6.7±0.3	11.4±2.4	1.7±0.1	21.1
Model 13	50	25	25	0	0	8.7±0.6	44.2±8.3	2.2±0.2	21.1
Model 14	85	15	0	0	0	5.2±0.1	9.6±2.6	1.5±0.0	24.8
Meal models with fortified lentil									
Model 15	50	0	0	0	50	285.2±28.3	480.6±48.8	2.8±0.2	0.8
Model 16	50	25	0	0	25	204.8±34.4	308.0±9.7	2.5±0.1	1.0
Model 17	75	10	0	0	15	146.6±52.6	373.0±26.8	1.9±0.1	1.1
Model 18	75	5	10	0	10	105±25.1	322.7±28.3	2.0±0.0	1.6
Model 19	75	0	0	0	25	170.9±23.1	460.1±25.1	2.2±0.1	1.1
Model 20	75	0	10	0	15	126.8±28.3	200.4±8.4	2.0±0.0	1.3
Model 21	75	10	0	0	15	78.8±13.9	209.5±10.8	1.8±0.1	2.0
Model 22	85	5	5	0	5	76.6±10.8	179.0±15.4	1.9±0.1	2.1
Model 23	85	0	0	0	15	132.8±15.3	239.0±27.8	1.7±0.0	1.1
Model 24	85	0	5	0	15	91.2±7.1	248.8±28.2	1.9±0.1	1.7
Model 25	85	0	10	0	5	79.8±2.8	168.4±28.1	2.0±0.1	2.1
Meal models, each contain one component at 100%									
Model 26	100	0	0	0	0	2.1±0.8	5.3±2.5	1.2±0.0	46.6

Model 27	0	100	0	0	0	19.4±0.5	3.7±1.9	2.5±0.1	10.8
Model 28	0	0	100	0	0	11.4±0.2	14.0±4.0	1.4±0.1	10.4
Model 29	0	0	0	100	0	60±0.6	50.6±4.3	6.2±0.1	8.8
Model 30	0	0	0	0	100	439.2±30.8	349.2±60.4	4.6±0.1	0.9

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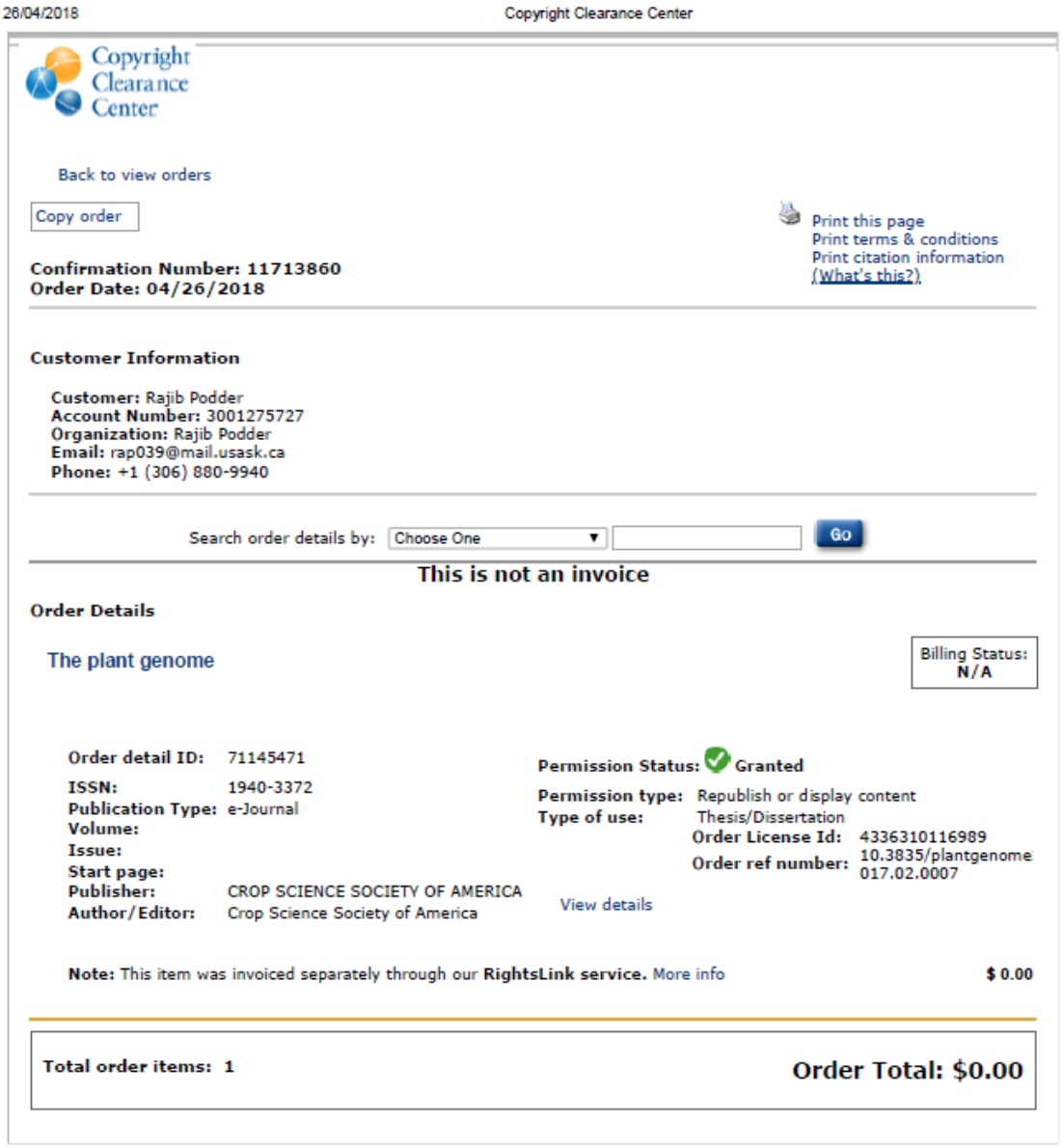

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