

**Iron bioavailability and consumer acceptability of extruded
common bean (*Phaseolus vulgaris*) flour**

by

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Dedication

To Kwiki, my dear wife, who sacrificed a lot to see me through this journey.

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ABSTRACT

Iron deficiency anemia continues to be a major nutritional challenge worldwide and is mainly caused by dependence on staple foods which are low in iron bioavailability. In many parts of the world, the common bean (*Phaseolus vulgaris*) is an important source of iron. Bean iron has very low bioavailability and household/industrial processing technologies have been shown to improve iron bioavailability. In this study, iron bioavailability of 16 Ugandan bean varieties was determined using an in vitro digestion/Caco-2 cell culture model and modeled with respect to key influencing factors; phytate, polyphenol, ferritin and iron content. The effect of extrusion cooking on iron bioavailability was also established. Iron bioavailability of white seed coat bean varieties was significantly higher than in colored seed coat varieties. A reverse trend was observed in which colored varieties showed higher polyphenol content than white ones. These results indicated that iron bioavailability can be indirectly screened for by seed coat color. Regression modeling showed that only iron and polyphenol content significantly influence iron bioavailability in beans. The linear effects of polyphenol and iron decreased iron bioavailability while their interaction increased it.

Extrusion cooking process variables; raw material moisture content, extruder die temperature and feed flow rate were optimized with respect to bean iron bioavailability, paste viscosity and consumer acceptability of extruded flours. Extrusion cooking increased both iron content and iron bioavailability and gave consumer acceptable flours with reduced paste viscosity. The increase in iron content indicates possible contamination from extruder parts and may partly account for the increase in bioavailability. The optimal combination of extrusion variables was

15% moisture content, 120 °C die temperature and 3 kg/h feed flow rate. Model validation experiments revealed that most of the responses could be reliably predicted, save for iron bioavailability.

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ABBREVIATIONS

α -AI	α -amylases inhibitor
AACC	American Association of Cereal Chemists
BD	Bulk density
BP	Before Present
BBI	Bowman-Birk Inhibitor
CIAT	Centro Internacional de Agricultura Tropical
DFA	Desirability Function Approach
DRC	Democratic Republic of the Congo
Dwb	Dry Weight Basis
EDTA	Ethylenediaminetetraacetic acid
ER	Expansion ratio
FAO	Food and Agriculture Organization
Fe	Iron
GAE	Gallic acid equivalents
ID	Iron deficiency
IDA	Iron deficiency Anemia
IP6	Myo- inositol- 1, 2, 3, 4, 5, 6- hexakisphosphate
IP5	Myo- inositol penta phosphate
IP4	Myo- inositol tetra phosphate
IP3	Myo- inositol tri phosphate
IP2	Myo- inositol di phosphate
IP1	Myo- inositol mono phosphate
kDa	Kilo Dalton
MW	Molecular Weight
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20;
PHA	Phytohemagglutinins
PVDF	Polyvinylidene fluoride
RBA	Relative biological availability
RBV	Relative biological value or relative bioavailable value
rFerr	Recombinant ferritin
RSM	Response Surface Methodology
RTE	Ready To Eat
RVA	Rapid Visco Analyser
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-poly acrylamide gel electrophoresis
SRPs	Sulfur-rich proteins
TCA	Trichloacetic acid
UNICEF	United Nations International Children's Education Fund
WAI	Water absorption index
WSI	Water solubility index

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CHAPTER 1. GENERAL INTRODUCTION AND RESEARCH JUSTIFICATION

Common beans (*Phaseolus vulgaris L.*) are widely grown in Uganda and rank among the top three sources of proteins, calories and iron for the population (FAO, 2010). Beans contain 30-190 µg/g iron depending on variety (Graham *et al.*, 1999; CIAT, 2008; Beebe *et al.*, 2000) making them an important source of iron to populations dependent on staples for their nutrition. However, the bioavailability of iron in beans is very low (<5%) (CIAT, 2008; Petry *et al.*, 2010, Petry *et al.*, 2012) due to high content of polyphenols, phytic acid and fiber, factors known to inhibit iron absorption (Welch *et al.*, 2000; Hu *et al.*, 2006). These act by forming insoluble complexes with dietary iron in the gastro-intestinal tract thus inhibiting its absorption.

It is for this reason that iron deficiency is the most prevalent micronutrient deficiency in the world (WHO, 2010), especially in populations dependent on low iron bioavailability staples, such as beans. Strategies to alleviate iron deficiency include supplementation, fortification, biofortification and dietary diversification. However, in spite of these efforts, iron deficiency is still a major nutritional problem and new strategies need to be considered in addition to what is currently being implemented (Micronutrient Initiative, 2004). In the case of beans, strategies to improve their contribution to iron nutrition include: (1) increasing iron concentration but maintaining bioavailability, (2) maintaining the concentration and improving bioavailability, or (3) increasing both iron concentration and bioavailability. Bouis *et al.* (2011) projected that to achieve atleast 30% Estimated Average Requirements (EAR) of iron from beans for nonpregnant, non lactating women (1,460 µg/day) and children 4-6 yrs (500 µg/day), iron

content of beans ought to be increased from the current average of 50 $\mu\text{g/g}$ to 107 $\mu\text{g/g}$. This assumes a daily consumption of 200 g and 100 g a day for the women and children respectively, an iron bioavailability of 5% and processing losses of 15%. Biofortification, the use of conventional breeding techniques or genetic engineering to enhance the micronutrient content of staple food crops, is already being used to increase iron content of beans (Bouis, 2003). Using intra and inter specific breeding, high iron varieties (up to 190 $\mu\text{g/g}$) have been used as parental stock and progeny with iron content of up to 150 $\mu\text{g/g}$ realized (CIAT, 2008). Though some studies show an increase in total absorbed iron with increase in bean iron content (Tako et al., 2009; Tako et al., 2011) others have shown no such benefit (Donangelo et al., 2003; Petry *et al.*, 2012) highlighting the need to match biofortification efforts with strategies to increase iron bioavailability.

On the other hand, ferritin is the major iron storage protein in beans and the associated iron has been shown to be as bioavailable as ferrous sulfate (Davila-Hicks et al., 2004; Lonnerdal et al., 2006). Thus, ferritin is hypothesized to increase iron bioavailability in staple foods such as beans (Lukac et al., 2009). However, ferritin is susceptible to gastric digestion at physiological pH in the stomach (pH 2) and may release associated iron to interact with iron absorption enhancers and inhibitors (Hoppler et al., 2008) which may hamper its iron absorption promoting capacity. It is thus important to determine the relative contribution of known iron absorption inhibitors and other dietary modifiers in order to inform breeding programs.

Iron bioavailability can also be enhanced by food processing. Technologies that reduce polyphenol and phytic acid content as well as modifying the food matrix have been shown to

enhance iron bioavailability by 21-50%. These include milling, soaking, germination, dehulling and various heat treatments (Matella, 2005; Martín-Cabrejas, 2006; Nergiz and Gökgöz, 2007; Shimelis and Rakshit, 2007; Alonso et al., 2001). Of these, extrusion cooking has been shown to be the most effective methodology that enhances bioavailability without loss in iron content and effectively destroys anti-nutritional factors in beans (lectins, hemagglutinins, enzyme inhibitors) (Alonso et al., 2001). It also adds value to beans, significantly reducing cooking time and improving overall nutritional and sensory quality of beans.

To study the effect of influencing factors on iron bioavailability, the Caco-2 cell culture model is a cost effective technique, especially when large numbers of varieties and/or processing conditions need to be screened (Fairweather-Tait et al., 2005). Bioavailability is the relative measure of how well a nutrient is absorbed by the human body and becomes available for metabolic use. When determined in vitro using the Caco-2 cell culture model, iron bioavailability is often expressed as relative biological availability (RBA, also known as relative biological value, RBV) which is the bioavailability of the iron in a given compound/food matrix compared to the bioavailability of ferrous sulfate (a salt assumed to be 100% bioavailable) within the same experiment.

Therefore, the current study hypothesized that an optimal combination of extrusion cooking parameters significantly improves iron bioavailability and consumer acceptability of promising bean varieties through elimination of anti-nutritional factors and enhancement of desirable flavor. Two study objectives were formulated; (1) to screen for iron bioavailability in 16 Ugandan bean varieties and model the relationship with key influencing factors; iron,

polyphenol, phytic acid and ferritin content; (2) to optimize extrusion cooking variables with respect to iron bioavailability, sensory and physicochemical properties of the most promising bean variety.

Dissertation organization

The dissertation consists of a review of literature on the distribution of common beans and their nutritional importance, extrusion processing with emphasis on utility to beans; iron bioavailability and how it relates to bean nutrition; phyto ferritin and its relationship to iron bioavailability and finally the use of optimization techniques in product development. This is followed by two papers in preparation for submission to the *Journal of Agricultural and Food Chemistry*. The first paper is titled ‘White common beans (*Phaseolus vulgaris*) have higher in vitro iron bioavailability than colored seed coat varieties’. This study modeled the relationship between key bean composition factors and iron bioavailability using multiple regression techniques. The second paper is titled ‘Optimization of white common bean (*Phaseolus vulgaris*) extrusion cooking process.’ It involved utilization of Response Surface Methodology (RSM) techniques to optimize extrusion cooking conditions; raw material moisture content, extruder die temperature and raw material flow rate with respect to iron bioavailability, sensory, and physicochemical properties of extruded flour. It is then capped by a chapter on general conclusions from the study.

Authors’ roles

The authors of “White common beans (*Phaseolus vulgaris*) have higher in vitro iron bioavailability than colored seed coat varieties”, chapter 3, are Mutambuka M, Murphy PA, Hendrich S and Reddy MB. Mutambuka sourced the bean varieties, conducted Caco-2 cell

culture studies as well as chemical composition analysis of raw materials. The authors of “Optimization of white common bean (*Phaseolus vulgaris*) extrusion cooking process”, chapter 4, are Mutambuka M, Murphy PA, Hendrich S, Reddy MB and Lamsal PB. Mutambuka carried out the extrusion cooking experiments, sensory evaluation experiments, Caco-2 cell culture experiments and chemical composition analysis of extruded bean material. The author of the concluding chapter is Martin Mutambuka.

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CHAPTER 2. LITERATURE REVIEW

2.1 The common bean

2.1.1 Origin, distribution and classification

The common bean (*Phaseolus vulgaris* L.) is one of the most ancient crops of the New World and is currently the most important grain legume for direct human consumption in the world. It is a member of family Leguminosae, tribe *Phaseoleae* and subfamily Papilionoideae. The common bean is self-pollinating, diploid and characterized by growing habits as determinate or indeterminate bush beans and climbing beans. First domesticated in the upland regions of Latin America more than 7000 years ago, the common bean is now the most widely cultivated of all beans in temperate regions, and widely cultivated in semitropical regions. Current cultivars are believed to have evolved from a wild growing vine.

Two centers of origin for this crop have been identified as the highland regions of Mexico and Andean South America, each with distinct morphological, agronomic and allozyme patterns as well as seed protein variations (Gepts, 1988). An analysis of banding patterns of phaseolin, the major seed storage protein in beans, has revealed three predominant patterns (S= Sanilac, T= Tender green, C= Contender) and which have been used to provide reliable information about the corresponding gene pools (Gepts and Bliss 1986, Gepts et al 1986). Banding patterns were named after the variety in which they were discovered. The Mesoamerican gene pool is characterized by small seeds and type "S" while the Andean gene pool is characterized by larger seeds and two phaseolin types ("T" and "C"). The Mesoamerican gene pool is represented by

pinto, pink, black, white and some snap beans, whereas the Andean is represented by kidney, cranberry and many snap beans (Talukder et al 2010).

From the domestication centers, the beans spread following different routes (Gepts, 1988); the smaller seeded Mesoamerican lines spread through Mexico and Central America, via the Caribbean and northern South America to Brazil. On the other hand, the larger-seeded Andean type were probably introduced into Europe via the Iberian peninsula, from where they made it into Africa and northeastern USA through trade and immigrations. However, it is important to note that both Andean and Mesoamerican genotypes were disseminated to the same regions of the world. Currently, the Centro Internacional de Agricultura Tropical (CIAT) in Cali, Colombia, maintains a world collection of more than 40,000 accessions; including indigenous wild and weedy specimens, unimproved landraces, and pure lines of *Phaseolus vulgaris*, as well as numerous related species (CIAT, 2011).

In Uganda, close to 0.5 million tons of beans are grown by 53% of the farmers (UNHS, 2005); making them the fifth most important food crop next to plantains, cassava, maize and sweet potatoes. The per capita consumption averages 16 kg/person/year and they contribute 6% of daily calorie and 15% of protein intake (FAO, 2010). However, in the southwestern part of the country, production and consumption are highest with beans taking on an even greater importance. Indeed, the south western neighboring countries of Rwanda and Burundi have a per capita consumption of about 66 kg and 31 kg per year, respectively (Broughton *et al.*, 2003).

There is great phenotypic and genetic diversity in terms of the market classes grown by farmers in Uganda and the East African Highlands in general, ranging from red mottled, red, navy, cream, yellow, black, purple, and brown (Wortman *et al.*, 1998). Red-mottled bean varieties are the most important and account for about 22% of total bean production. Historically, the dominant gene pool in the region are Andean varieties. However, the Mesoamerican gene pool is on the rise (Blair *et al.*, 2010a) due to introduction of new improved climbing varieties (CIAT, 2010) which are from the Mesoamerican gene pool and the root rot disease to which Andean varieties are less resistant. Climbing bean varieties exhibit a higher yield in small space, large grains, good nitrogen fixation, reduced vulnerability to certain diseases and flexibility for various cropping systems. There is also a lot of inter-gene retrogression which may be due to the fact that beans are often cultivated in multi-cultures. This is a coping mechanism against biotic and abiotic stresses, environmental/climatic variability and with diversifying production in small plots, early and late maturing components are planted together providing harvestable products over a long period. However, there is growing demand by the urban consumers for pure lines instead of mixtures since these provide more uniformity and are easier to prepare. This, as well as introduction of new varieties, has threatened the existing diversity of genotypes in the region and reduced the genetic pool.

2.2 Nutritional importance of common beans to humans

Dry beans have been consumed since time immemorial with archeological evidence showing their consumption in Southeast Asia, the Middle East, Africa, the Americas, India and China 8000 to 10000 years BP. They offer a high nutrient-density and are a good source of starch,

protein, complex carbohydrates/dietary fiber as well as vitamins (Vitamin B6 and folate), phytochemicals and minerals (iron, zinc, and phosphorous) as shown in Table 2.1 (USDA, 2011). Important health benefits include reduced disease risk; diabetes (Villegas et al., 2008), coronary heart disease (Kabagambe et al., 2005), certain cancers (Deneo-Pellegrini et al., 2002; Key et al., 1997), enhanced longevity (Darmadi-Blackberry et al., 2004), lower total cholesterol, LDL-cholesterol, triglycerides, while increasing HDL-cholesterol (Shutler et al., 1989; Winham et al., 2007). Dietary Guidelines for Americans recommends 3 cups of beans per week (dry weight ~200 g).

Table 2.1: Nutrient composition of dry beans

Nutrient*	Composition (per 100g, dwb)
Energy, Kcal	308
Protein (g)	20.8
Total lipid (g)	1.3
Ash (g)	3
Carbohydrate (g)	56.9
Total dietary fiber (g)	17.2
Total sugars (g)	0.8
Calcium (mg)	105.72
Iron (mg)	6.21
Magnesium (mg)	145.44
Phosphorus (mg)	332.12
Potassium (mg)	1076.8
Sodium (mg)	43.67
Zinc (mg)	2.23
Copper (mg)	0.6
Manganese (mg)	1.11
Selenium (µg)	3.96
Vitamin C, total ascorbic acid (mg)	8.93
Thiamin (mg)	0.56
Riboflavin (mg)	0.24
Niacin (mg)	1.76
Pantothenic acid (mg)	0.86
Vitamin B-6 (mg)	0.34
Folate, total (µg)	243.81
Choline, total (mg)	58.74
Vitamin B-12 (µg)	0
Vitamin A (IU)	0.21
Vitamin E (α-tocopherol) (mg)	0.8
Vitamin K (phylloquinone) (µg)	16.71

*Average of black, navy, pinto, kidney, great northern and red beans. Source:
www.nal.usda.gov/fnic/foodcomp/search

The following sections look at the biochemistry and physicochemical properties of cooking beans.

2.2.1 Proteins

Typically beans contain 15-30% protein on a dry weight basis. Water-soluble albumins and salt-soluble globulins, respectively, account for up to 10 - 30% and 45 - 70% of the total proteins (dwb) (Sathe et al., 1984). Glutelins (12 - 30%) and protease inhibitors (0.3%), non-extractable proteins and non-protein nitrogen make up the rest. The albumin fraction is typically composed of several different proteins, with phytohemagglutinin accounting for 10% of total proteins while a single globulin, phaseolin, dominates the globulins and may account for up to 50 to 55% of the total proteins (dwb). Osborne (1894) first extracted and characterized bean protein and found two major globulins that required a certain amount of ionic strength for solubilization in aqueous media. Later, Danielsson (1949) showed that these globulins (vicilin-like and legumin-like) had sedimentation coefficients of 7S and 11S, molecular weights of 186,000 and 331,000 and isoelectric pH of 4.8 and 5.5 respectively.

The 7S vicilin type protein in beans is the major storage protein (accounting for over 50% of the total seed proteins) and is known as phaseolin (also variously referred to as globulin G1 fraction, euphaseolin, α globulin and Glycoprotein II) (Derbyshire et al., 1976). It is regarded as an oligomeric protein consisting of three polypeptide subunits, α , β and γ , with a molecular weight distribution ranging from 43 to 53 kDa (Lawrence et al., 1994). The group is typically composed of pre-proproteins of MWs in the range 50 – 75 kDa and may be glycosylated. Both Raman spectroscopy (Yin et al., 2011) and circular dichroism spectroscopy (Deshpande and Damodaran,

1989) show that the secondary structure of native phaseolin is 50% β -sheets, 31% random coils, 10.5% α -helixes and 8.5% β -turns. The three-dimensional structure of bean 7S globulins has been determined using x-ray crystallography and shows disk shaped trimeric proteins with diameters of ~ 90 Å and thicknesses of 30 - 40 Å (Lawrence et al., 1994). There is great genetic variability in expression of phaseolin and its banding patterns have been utilized to trace origin of bean varieties (Mesoamerican vs. Andean) (Gepts and Bliss 1986, Gepts et al 1986). This variation may be attributed to the large number of genes encoding for phaseolin, the extent of glycosylation and posttranslational proteolytic processing of the pre-proproteins. Mannose, xylose and glucosamine are the carbohydrate constituents that have been identified to be associated with phaseolin, and may be linked at two major sites, Asn²⁵² and Asn³⁴¹ (Sturm et al., 1987).

Several authors have investigated the functional properties of the 7S protein fraction in beans and have shown its unique potential in food systems (Sathe and Salunkhe, 1981a, b; Plietz et al., 1987, Deshpande and Damodaran, 1989; Kimura et al., 2008; Yin et al., 2011). Uniqueness of functional properties of phaseolin may be related to the positions and level of glycosylation associated with it. Water absorption capacity of albumins and globulins is 3.18 and 2.77 g/g respectively (Sathe and Salunkhe, 1981a, b) and is a function of presence of carbohydrate moieties and pH, increasing with increase in glycosylation and pH. Oil holding capacity of albumins and globulins are 3.29 and 3.23 g/g, respectively; similar to soybean glycinin but much lower than β -conglycinin. The isoelectric pH of bean proteins are in the range of pH 4-5 and they are more soluble at alkaline pH than at acidic pH.

Kimura et al., (2008) compared functional properties of 7S and 11S globulins of beans, soybean, pea, black cowpea, red cowpea and fava bean. Solubility of bean 7S protein was very high and similar to that of soybean 7S globulin and higher than 7S globulins of the other legumes, which may be attributed to glycosylation of soybean and bean 7S globulins. Salt type, pH and ionic strength are the most important parameters affecting bean protein solubility, with dilute alkalis being most effective (Sathe and Salunkhe, 1981b). Sodium carbonate and potassium sulfate were the most effective salts. The foaming and emulsifying properties of bean proteins are fair, with some bean varieties showing excellent surface activity (Sathe and Salunkhe, 1981a, Kimura et al., 2008). 7S globulins from soybean and bean gave smaller average particle sizes and thus better emulsifying abilities than those from pea, fava bean, and red and black cowpea, especially at $\mu=0.08$ (Kimura et al., 2008). 11S globulins did not show any difference in emulsifying ability across the varieties and were poor emulsifiers. Bean 7S globulin showed an excellent ability to form stable emulsions compared to the 11S globulins and 7S globulin from other legumes. The excellent emulsion stability shown by bean 7S globulin over soybean 7S globulin cannot be explained by glycosylation alone, as both types show similar levels of glycosylation. However, the difference in the positions of the carbohydrate moieties may account for this anomaly.

Bean 7S proteins are thermally stable compared to similar fractions from other legumes (Kimura et al., 2008) showing suitability for production of foods requiring high thermal stabilities. This suggests that forces, which are sensitive to ionic strength, for example, hydrophobic interactions,

play a more important role in the maintenance of the structures of cowpea, pea, and soybean 7S globulins than those of bean 7S globulins. However, hydrophobic interactions are important for the maintenance of the structure of bean 11S globulin. Surface hydrophobicity of bean 7S protein was lowest among the legumes, showing suitability for foods requiring low surface hydrophobicity. The foaming capacity and foam stability of bean globulin proteins are poor when compared with many animal proteins partly because they typically have compact and rigid structure. The smallest gelation concentration of albumins and globulins are 18 and 20% (Sathe and Salunkhe, 1981a).

In beans, the 11S legumin type globulins form a minor component to the protein content (Derbyshire et al., 1976). The native legumins typically have sedimentation coefficients of 11 to 13S and are not glycosylated. One polypeptide from each of the α and β subunits are linked via disulfide bond(s) to form a polypeptide of MW ~ 60,000 (Lawrence et al., 1994). Six such polypeptides (each of MW ~ 60,000) are thought to constitute the native molecule (hexamer) with MWs of 320,000 to 400,000. Typically, legumin-like proteins are not glycosylated but show molecular heterogeneity which may be due to the flexible region of the 11S type proteins that is susceptible to proteolysis (Plietz et al., 1987). Both legumin and vicilin proteins contain (N-terminal) leader sequences that are removed co-translationally prior to packaging the mature proteins in membrane bound protein bodies.

In addition to phaseolin 7S and 11S globulins, beans also contain 17 - 18S and 2~3S proteins which may contain aggregated and dissociated forms of the main storage proteins, respectively,

as well as other smaller MW proteins (Sun et al., 1974). The 2 - 3S fraction of bean proteins contains many small proteins, including trypsin-chymotrypsin inhibitors and some enzymes. Trypsin inhibitors occur in appreciable amounts in beans and contain significant amounts of sulfur amino acids compared with other proteins (Grant et al., 1995). The double headed Bowman-Birk type trypsin and chymotrypsin inhibitors are the most important protease inhibitors in beans (Lajolo and Genovese, 2002). They are small molecular weight (MW 8-10kDa) cysteine-rich polypeptides containing a large number of disulfide bonds which makes them heat stable. They consist of two binding sites located at opposite sides of the molecule and are able to form a 1:1:1 stoichiometric enzyme-inhibitor complex with trypsin and chymotrypsin (Bergeron and Nielsen 1993). Several isoforms of Bowman-Birk inhibitor (BBI) have been isolated from common beans (Wu et al., 1990; Bergeron and Nielsen 1993). Their heat stability is an important factor in protein nutritional quality since they require higher temperatures and/or prolonged time for inactivation. Earlier interest in BBI was based on poor raw bean protein utilization by rodents and associated pancreatic hypertrophy in animals with pancreas size > ~0.3% (as % body weight) (Grant et al., 1995). However, pancreatic hypertrophy is not observed in larger animals. Instead, an anticarcinogenic effect has been reported in humans with BBI implicated in the treatment of different types of cancer (Armstrong et al., 2000).

Beans also contain amylase inhibitors, notably of which are the α -amylases inhibitors (α -AI). Three isoforms of α -AI (α -AI1, α -AI2, α -AIL) have been isolated and characterized in beans, with α -AI1, the isoform with anti-amylase activity in humans, being most widely distributed (Iguti and Lajolo, 1991). The α -amylase inhibitors α -AI1 and α -AI2 exist in their native form as

typical lectin tetramer structures ($\alpha_2\beta_2$) with a molecular weight of approximately 56.7 kDa. α -AI have an amino acid sequence homology of about 50–90% with bean phytohemagglutinins (PHA) (Moreno and Chrispeels, 1989). However, there are major differences in the number of surface loops in the three dimensional structures of these bean proteins (Rougi et al., 1993). PHA has three loops, α -AIL has one shortened loop, while the loops are completely absent in α -AI1 and α -AI2. Therefore, α -AI has no carbohydrate-binding activity due to lack of carbohydrate-binding loops that are present in PHA (Pueyo et al., 1993). Moist heat typically inactivates most of the carbohydrase inhibitors and they are therefore not nutritionally important.

Beans also contain lectins, also referred to as phytohemagglutinins (PHA), glycoprotein I or protein II (Lis and Sharon, 1980). Native lectins are typically tetrameric proteins (MW range 85,000 to 150,000) and are glycosylated with sedimentation patterns similar to the vicilin-type proteins. They are composed of two different types of subunits; a leucoagglutinating sub unit (34 kDa) and erythroagglutinating sub unit (36 kDa) (Leavitt et al., 1977). These subunits are synthesized in the endoplasmatic reticulum and then randomly combined to produce five isolectins that are assigned the structures L4, L3E1, L2E2, L1E3, and E4. Both bean lectin subunits contain the characteristic N-glycosylation sequence; subunit E at Asn12, Asn60 and Asn80, subunit L only at Asn12 and Asn60. In the mature proteins, only the first two sites are actually glycosylated. The glycan at Asn12 belongs to the high mannose type, while glycan at Asn60 belongs to the complex type containing xylose and fucose (Thomas et al., 1996). All legume lectins possess two bound metal ions (one calcium ion and one transition metal ion, mainly Mn^{2+}) per monomer, in the vicinity of the sugar binding site. The presence of these two

bound metal ions is vital for the sugar binding capabilities of the legume lectins (Bardocz et al., 1995). Lectins have a unique property of being able to bind disaccharides in a highly specific fashion (Pusztai et al., 1989). Most animal cell membranes contain these sugar molecules. The toxic activity of lectins derives from their resistance to proteolysis and ability to bind tightly to sugars present on cells in the small intestines causing food poisoning or long term inhibition of nutrient absorption (Pusztai et al., 1989). However, heat treatment (e.g. boiling for 12 minutes) has been shown to completely eliminate the hemagglutinating activity, though slow heating below boiling point may not. Also, fermentation for 72 h at 42 °C completely removed lectins from lentil flour (Cuadrado et al., 2002). Other biochemical properties of lectins are agglutination of erythrocytes, mitogenic activity (induction of mitosis of lymphocytes), agglutination of malignant cells, and specificity for human blood groups (Lis and Sharon, 1986). They have also been shown to potently and selectively inhibit HIV-1 and HIV-2 in MT4-cells (Balzarini et al., 1992).

Another class of bean proteins is the sulfur-rich proteins (SRPs) which are nutritionally important since bean protein is generally deficient in sulfur containing amino acids (Burow et al., 1993). They are high in methionine and account for $\leq 5\%$ of total seed proteins. The bean SRPs are thought to be similar to the soybean ones and are composed of several polypeptides (MW range 17,000 to 45,000). The final class of bean proteins are the enzymes (amylases, lipases, proteases, peptidases) bioactive peptides and small polypeptides.

Nutritionally, the contribution of cooked bean protein is curtailed by relatively low digestibility (65 - 85%) and a low supply of sulfur amino acids, methionine and cysteine (Sathe et al., 1984;

Montoya et al., 2008a). The low digestibility of bean protein may be due to the food matrix, protease inhibitors and resistant protein fractions. Bean proteins are contained within cell walls that remain primarily intact during cooking and may enter the small intestine encased within fibrous cell walls, limiting access of proteases. Bean protease inhibitors and lectins have been discussed in an earlier part of this section. Digestibility of the different protein fractions also differs. Montoya et al. (2008a) found that the degree of hydrolysis of 43 different types of phaseolin ranges from 57% to 96%. They attributed the variations to differences in subunit composition, subunit precursor origin (α or β) and trypsin susceptibility between phaseolin subunits (Montoya et al., 2008b; Montoya et al., 2009). For the legumin (11S) fraction, only α -polypeptides may be partially degraded, while β polypeptides remain intact, even after heat treatment (Momma, 2006). Degree of hydrolysis of cooked albumins and glutelins is very low (13–18%). The low degree of hydrolysis of 2S proteins may be due to a high number of disulphide bridges and the presence of carbohydrates (Moreno et al., 2005).

2.2.2 Carbohydrates

Dry beans contain about 70% carbohydrate. Starch (43 - 45%), non-starch polysaccharides or fiber (18 - 20%), α -galactosides (stachyose, verbascose, and raffinose; 3 - 5%), and sucrose (3 - 5%) are the major types of carbohydrate. Typically, dry bean starches contain less amylose (10-44 %) than amylopectin (Reddy et al., 1984; Wani et al., 2010), but the percentage of amylose is higher than most cereal or tuber starches. The amylose fraction of bean starch has a high degree of polymerization (1000 - 1200). Bean starch predominantly exhibits a type C X-ray diffraction

pattern and has low rates of gelatinization but high rate of retrogradation. The latter two properties predispose it to a low starch bioavailability/ glycemic index. Further, retrograded starches escape digestion and provide substrate for colonic micro biota thus enhancing colonic health. Also, bean starch is embedded within cell walls limiting enzyme accessibility and further contributing to reduced glycemic index.

Bean α -galactosides are not digested in the upper part of the small intestine due to a lack of the enzyme, α -galactosidase and thus contribute to colonic micro biota fermentable material. Soaking is the most common treatment for partial elimination of these oligosaccharides and addition of bicarbonate enhances their removal due to the greater permeability obtained by partial solubilization of the cell wall (Ibrahim, 2002). Germination for 48 h at 20 °C removes 40 - 60% of bean oligosaccharides. Bean fiber contains equal amounts of water soluble and insoluble fractions. The most important fiber components are cellulose, hemicelluloses and lignin, but pectins are also present (Reddy et al., 1984). These also contribute to fermentable substrate to colonic micro biota, alongside a number of other health effects such as increase fecal volume and thus reduce transit time, lower blood cholesterol and reduced risk of some cancers, cardiovascular diseases and diabetes (Flight and Clifton, 2006).

2.2.3 Lipids

Beans contain between 1- 3% lipid depending on variety and growth environment. Lipids in beans are stored in oil bodies or sphaerosomes in the cotyledon, which differ in size and relative abundance (Sathe et al., 1984, Holland et al., 1991, Onwuliri and Obu, 2002). Neutral lipids are the predominant class of lipids and are primarily made up of triglycerides, accompanied by

smaller proportions of free fatty acids, sterols and sterol esters (Onwuliri and Obu, 2002).

Phospholipids and glycolipids are also present in appreciable amounts. The majority of the fatty acids are unsaturated. Oleic (7-10 %), linoleic (21-28%) and linolenic (37-54%) are the major unsaturated fatty acids in beans and make up 64-87% of total lipids.

2.2.4 Minerals

Beans are a significant dietary source of several essential minerals and are particularly rich in potassium, iron, zinc, magnesium, phosphorus, copper and manganese (Table 2.1). Others include calcium and selenium. Potassium makes up 25-30% of the mineral content of beans, making beans an important food with respect to heart health. Phosphorous in beans is mainly associated with phytic acid and may not be bioavailable. However, phosphorous deficiency is not critical in human health and thus phytates are not of concern for this particular mineral. Zinc and iron are the minerals of most nutritional interest in beans since their deficiency is highly prevalent and beans are critical sources of these minerals. However, the bioavailability of iron and zinc from beans is rather low due to presence of polyphenols and phytic acid (see section 2.3). Mineral content is genetically determined with Andean beans having higher iron concentrations and Mesoamerican higher in zinc (Islam et al., 2002). Recent studies have identified quantitative trait loci (QTL) controlling iron and zinc accumulation (Blair *et al.*, 2009, Blair *et al.*, 2010b). In regard to varieties commonly grown in East and Central Africa, inter-gene pool introgressed genotypes tend to have higher seed iron concentration, followed by Andean and finally the Mesoamerican (Blair et al., 2010a). Seed zinc content is higher in the Mesoamerican and lowest in Andean, with introgressed varieties in-between.

The CIAT germplasm bank contains beans with iron content between 30 - 190 µg/g depending on variety (Graham *et al.*, 1999; CIAT, 2008; Beebe *et al.*, 2000). This genetic variability and the high iron content of related species (*P. polyanthus* and *P. coccineus* which contain up to 127 µg/g of iron) has spurred interest in breeding for high iron varieties, a process known as biofortification (Bouis, 2003). Using high iron parents, progeny with iron content of up to 150 µg/g (almost twice the iron content of low iron content market classes) have been bred (CIAT, 2008). The concentration of seed micronutrients is also affected by environmental interactions (CIAT, 2008). Recent work by CIAT (2008) has indicated it is possible to increase seed levels of Fe and Zn through addition of inorganic nitrogen, phosphorus and potassium.

2.2.5 Vitamins

Dry beans are an excellent source of the water-soluble vitamins thiamin, riboflavin, niacin and folate (Table 2.1). The vitamin content of beans varies widely among varieties and reduces on cooking by 70-75% (Augustin, 1981). The bioavailability of vitamin B6 in beans is also an area of interest with Gregory and Kirk (1981) suggesting the cell wall matrix and non-digestible polysaccharides and lignins may inhibit vitamin B6 absorption. Rockland *et al.* (1977) showed that both soaking and cooking led to significant reduction in bean water soluble vitamins. Cooking however, led to the greatest loss in vitamin but depended on variety and not on cooking time. Up to 50% losses in vitamin were reported after soaking and cooking.

2.2.6 Antinutritional factors

2.2.6.1 Phytic acid

Phytic acid is the primary storage form of both phosphorous and inositol in plant seeds. It is a hexahydric cyclic alcohol and exists as the hexaphosphoric ester of myo-inositol (Figure 1).

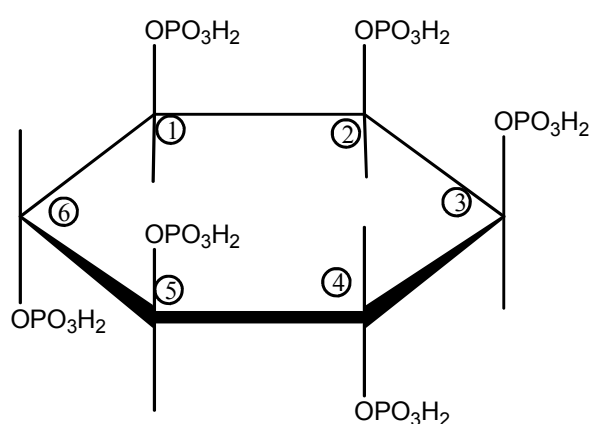


Figure 2.1: Chemical structure of phytic acid (Source, Reddy and Sathe, 2001).

Phytic acid, also known as myo-inositol 1,2,3,4,5,6 hexakisphosphate (IP6) or phytate when in salt form, is the principal inositol phosphate in plants (65- 80%) though other inositol mono and polyphosphates (inositol penta-(IP5), tetra- (IP4), triphosphate (IP3), diphosphate (IP2) and monophosphate (IP1) exist in plants (Dorsch et al 2003). The lower ester forms may exist naturally but may also be products of IP6 enzymatic or non-enzymatic/thermal hydrolysis during food processing. Phytate accumulates in the seeds during the ripening period and the associated phosphorus and inositol are not utilized by monogastric animals because they lack the intestinal digestive enzyme phytase.

Phytic acid comprises about 1-5% of legumes, cereals, oil seeds, pollens and nuts (Cheryan 1980). In legumes, phytate can be found in the protein bodies of the cotyledon (Schlemmer et al., 2009). The anti-nutritional effect of phytate is due to its ability to form strong, mainly insoluble complexes with divalent and monovalent minerals such as iron, zinc, magnesium, copper, calcium and potassium. This is mainly due to its chemical structure. Phytic acid contains 12 replaceable protons; 6 are strongly dissociated with pK of about 1.8, 2 are weak acid functions with a pK of 6.3 and 4 are very feebly dissociated (pK of 9.7) (Cheryan, 1980). This suggests that at all pH values normally encountered in foods, phytic acid will be strongly negatively charged indicating tremendous potential for complexing or binding positively charged molecules, such as cations or proteins. Due to its multiplicity of reactive phosphate groups, phytic acid can complex a cation within a phosphate group itself, between two phosphate groups of a molecule, or between phosphate groups of different molecules. Thus, lower ester forms (IP1-IP5) bind less minerals and the complexes are relatively more soluble. Thus, phytic acid strongly chelates with cations such as calcium, magnesium, zinc, copper, iron and potassium to form insoluble salts, adversely affecting their absorption. Phytates also form complexes with proteins resulting in decreased protein solubility, enzymatic activity and proteolytic digestibility. However, consumption of phytates has been shown to have some favorable effects. These include anti-carcinogenic (Shamsuddin, 2002; Vucenik and Shamsuddin, 2003), anti-oxidant (Minihane and Rimbach, 2002) and reduced blood glucose response (Thompson, 1993). Others include reducing cholesterol and triglycerides, prevention of renal stone development, removal of traces of heavy metal ions and even inhibition of HIV-1 replication (Kumar et al., 2010).

Phytic acid is a particularly strong inhibitor of iron absorption. The inhibitory effect of phytic acid on iron absorption was first studied by Widdowson and McCance (1942) who found decreased iron levels in subjects who consumed brown bread instead of white bread, though the former provided a 50% higher iron intake. This group and other workers went ahead to show inhibitory effect of phytic acid in both monogastric animals and humans and later Hallberg et al., (1989a) showed that this relationship was dose dependent. Hallberg et al., (1989b) showed that 7, 89 and 887 mg of phytic acid served to healthy male and female subjects as part of a meal containing 4.1 mg of iron reduced iron absorption by 18, 62 and 82% respectively. This inhibitory effect could however be overcome by ascorbic acid (Hallberg et al., 1989b, Siegenberg et al., 1991) and EDTA (Troesch et al., 2009). Several authors have studied phytic acid in common beans and show that it comprises 0.2-2.8% of seed weight (Reddy and Sathe, 2001). Contribution of phytic acid to inhibition of iron absorption in beans has been studied using both human studies (Lynch et al., 1984; Donangelo et al., 2003; Pertry et al., 2010; Pertry et al., 2012) and in vitro digestion/Caco-2 cell culture (Hu et al., 2006; Beiseigel et al., 2007). However, the relative contribution of phytic acid to iron absorption in beans is still a complex question since polyphenols and fiber contribute extensively to inhibition of iron bioavailability.

2.2.6.2 Polyphenols

Phenolic compounds are substances possessing an aromatic ring bearing one or more hydroxyl groups, including their functional derivatives. They are one of the most numerous and ubiquitous groups of plant metabolites arising from two main synthetic pathways; the shikimate pathway and the acetate pathway (Shahidi, 2000). This is an extremely wide and complex group

of plant substances and in their simplest classification can be grouped phenolic acids and derivatives, flavonoids and tannins (Bravo, 1998). Phenolic acids include low molecular weight phenolic compounds, such as phenol itself but also include hydroquinone and derivatives, and phloroglucinol (Robbins, 2003). Phenolics with a C₆-C₁ structure such as phenolic acids (e.g., gallic, vanillic, syringic, p-hydroxybenzoic) and aldehydes (e.g., vanillin, syringaldehyde, p-hydroxybenzaldehyde) are common. Phenylpropanoid derivatives (C₆-C₃) are also common and include chromones, coumarins and hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, sinapic) and derivatives. The flavonoids are sub classed into anthocyanins (e.g., pelargonidin, malvidin, cyanidin), flavones (e.g., epigenin, luteolin, diosmetin), isoflavones (e.g., daidzein, genistein, glycitein), flavonols (e.g., quercetin, myricetin, kaempfeol), flavanones and flavanols (Merken and Beecher, 2000). Tannins are high molecular weight compounds and can be subdivided into two major groups; hydrolysable and condensed tannins (Martin-Tanguy et al., 1977). Hydrolysable tannins are easily hydrolyzed with acid, alkali or hot water and by enzymatic action (Porter, 1989). They consist of gallic acid derivatives and can be subdivided into gallotannins and ellagitannins. Condensed tannins or proanthocyanidins consist of falvan-3, 4-ol (catechin, epicatechin, etc) monomeric units with a flavan-3, 4-diol or leucoanthocyanidin molecule as its precursor.

The polyphenol content of beans is in the range of 0.19-8 mg/g seed and is a function of bean seed coat color (Elias et al., 1979; Bressani and Elias, 1980; Hu et al., 2006, Luthria and Pastor-Corales, 2006). Beans contain a wide range of polyphenols including phenolic acids, proanthocyanidins, anthocyanidins as well as flavonols. Flavonol glycosides (astragalin,

quercetin 3-O- β -D-glucopyranoside-(2 \rightarrow 1)-O- β -D-xylopyranoside, and quercetin 3-O- β -D-glucopyranoside), tannins and anthocyanins (cyanidin 3-diglucoside, 3,5-diglucoside, pelargonidin 3-glucoside, 3,5-diglucoside, malvidin glucosides, delphinidin 3-O-glucoside, malvidin 3-O-glucoside, and petunidin 3-O-glucoside) are present in the seed coat (Beninger et al., 1998, 1999; Takeoka et al., 1997). In dehulled beans, presence of caffeic, p-coumaric, sinapic and ferulic acids have been reported (Garcia et al. 1998). Isoflavones (daidzein, genistein, glycitein) and their glycosylated forms have been identified in various bean varieties (Romani et al., 2004; Antonelli et al., 2005) and their content shown to increase with germination (Díaz-Batalla et al., 2006).

Tannins in beans are linear polymers of flavan-3-ol (catechin and gallocatechin) and flavan-3 to 4-diol (leucocyanidin and leucodelphinidin) units (Martin-Tanguy et al., 1977). There are differences in the content of condensed tannins of beans depending on the color of seed coats. The white varieties of beans usually contain lower concentrations of tannins than those with red, black or bronze seed coats (Bressani and Elias, 1980; Elias et al., 1979). However, Marquardt et al. (1978) reported that white varieties of faba beans have higher concentrations of tannins than those with dark testa.

Polyphenols have diverse biological functions; both in the plant and in human health. In plants, they act as phytoalexins, anti-feedants, attractants for pollinators, contributors to plant pigmentation/sensory characteristics of fruits and vegetables, antioxidants and protective agents against UV light, amongst others (Naczek and Shahidi, 2006). In food, polyphenols have been

reported to have preservation properties and are used as natural colorants. In human health, the high redox potential is an important property for their anti-oxidant functions allowing them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (Shahidi, 2000). Further, isoflavones possess estrogenic, antiestrogenic, anticarcinogenic and antioxidant activities (Zhang et al., 2009) that have been linked to a reduction in osteoporosis, cardiovascular disease, prevention of cancer and treatment of menopause symptoms.

2.2.6.2.1 Interactions with minerals

Polyphenols may form a stable complex with a wide range of minerals (Conrad, 1970; McDonald et al., 1996). Polyphenols containing o-dihydroxyphenyl (catechol) and o-trihydroxyphenyl (galloyl) such as proanthocyanidins (catechol groups and galloyl groups) and hydrolysable tannins (galloyl groups) are the most potent iron chelators (Brune et al 1989, Hurrell et al 1999). In plants, this reaction has been suggested to play a significant role in defense against microorganisms (Scalbert, 1991; Mila and Scalbert, 1994), but is of vital nutritional significance in humans as it lowers mineral bioavailability. Iron chelating phenolics have been identified in legumes, tea, vegetables, wines, sorghum and coffee (Brune et al., 1989; Hallberg and Rossander, 1982; Hurrell et al., 1998). This inhibition may be due to formation of insoluble iron (III)–phenol complexes, thus making the iron unavailable for absorption in the gastrointestinal tract (Slabbert, 1992). On the other hand, addition of ascorbic acid increases the absorption of nonheme iron in the presence of polyphenols due to the reduction of Fe (III) to Fe (II) (Hallberg et al., 1989a; Siengenberget al., 1991).

2.2.6.2.2 Remedies for anti-nutritional effects of food phenolics

Several methods for lowering the potential antinutritional effect of phenolics in the diet have been evaluated (Salunkhe et al., 1989). These include supplementing the diet with phenol-binding materials, dehulling, cooking/steaming/extrusion cooking, soaking in water and chemical solutions, and promoting metabolic detoxification (by fermentation or addition of external polyphenol oxidase). Soaking and cooking kidney beans decreased polyphenol contents by about 70% with losses from both soaking and cooking being of similar magnitude and mainly due to leaching into water (Shimelis and Rakshit 2007). Heat processing may lower levels of assayable tannins because of the formation of insoluble complexes between tannins and seed proteins or other hydrophobic compounds (Butler, 1989; Shimelis and Rakshit 2007). Ammoniation, treatment with alkaline solutions, acids and formaldehyde (Deshpande and Cheryan, 1983), have been shown to significantly decrease the content of assayable tannins. Breeding for beans with low polyphenol content with no adverse effects on pathogen resistance and seed coat color is also another approach (Beebe et al., 2000).

2.3 Iron bioavailability

In the literature, the term ‘bioavailability’ is defined differently depending on the background of the author, the field and specific interest (Wienk et al., 1999). Fairweather-Tait and Hurrell, (1996) defined mineral bioavailability as the fraction of the ingested nutrient that is absorbed and subsequently utilized for normal physiological functions. The bioavailability of iron in mixed human diets is low and variable depending on host and dietary factors. Host factors include, among others, body iron stores, erythropoietic activity and enterocyte exposure to iron; a reduction in iron stores will result in increased efficiency of absorption. Dietary factors include

form of iron (heme vs. nonheme) and presence of other dietary components ingested with the meal (Dunn et al., 2007). Heme iron comes from the breakdown of hemoglobin and myoglobin in meat, fish, and poultry whereas nonheme iron exists as ferric or ferrous salts in both plant and animal foods. Dietary heme iron is directly absorbed into the enterocytes with virtually no influence by dietary factors, so its bioavailability may reach up to 30% (Fairbanks, 1994). Dietary nonheme iron, on the other hand, is commonly affected by dietary factors that chelate the iron and either enhance or inhibit absorption (Hallberg and Hulthen, 2002). The result is a bioavailability that may range from < 1-10% (Hallberg and Rossander, 1982). Factors that have been shown to inhibit nonheme iron absorption include phytic acid, fiber (cellulose, hemicelluloses, lignin, cutin, etc.), polyphenols, oxalic acid, Calcium, certain proteins/amino acids, haemagglutinins (lectins) and heavy metals (Cd, Hg, Pb, etc.). Promoters of nonheme iron absorption include organic acids (ascorbic acid, fumarate, malate, citrate), hemoglobin, 'the meat factor', certain amino acids (met, cys, his, lys) and β -carotene (Hallberg and Hulthen, 2002). Effect of phytic acid and polyphenols on iron bioavailability has been discussed under section 2.2.6 of this manuscript.

Iron bioavailability can be assessed at digestibility (availability), transport into intestinal enterocytes (uptake), efflux across basolateral membrane of enterocytes (absorption), retention or endogenous excretion in urine and feces (retention), transport to tissues (utilization) and transport to storage sites (body stores) (Fairweather-Tait et al., 2005). In vitro, animal and human methodologies have been developed to determine iron bioavailability at each of these steps. At the digestion step, iron bioavailability can be accessed by solubility (Shackleton and

McCance, 1936; Narasinga Rao and Prabhavathi, 1978), dialyzability (Miller et al., 1981) and use of human gastric juice (Bezudova et al., 1978) methods. Iron solubility measures the fraction of iron that is soluble in dilute aqueous or dilute acid extracts in vitro, in vivo (by collecting digesta samples from humans) or ex vivo in animals. Narasinga Rao and Prabhavathi (1978) showed that percentage ionizable iron correlated very well with percent iron absorption in a human absorption study. They also demonstrated inhibitory effect of phytate and tannic acid as well as promoting effects of ascorbic acid and meat. However, subsequent studies have shown that this method has poor correlation with either Caco-2 cell culture or human field trials and thus not a useful predictor of iron bioavailability (Platt and Clydesdale, 1984; Forbes et al., 1989; Pynaert, 2006). The iron dialyzability method is an improvement to the solubility procedure and introduce in vitro digestion supported by gradual and reproducible pH adjustment from gastric to intestinal levels as well as use of a dialysis membrane with a specified molecular weight cut off to allow only low molecular weight, soluble iron (Miller et al., 1981). Determination of iron bioavailability using this method correlated highly with iron absorption studies in humans (Schriker et al., 1981; Forbes et al., 1989) though not all dialyzable iron is bioavailable. Although it can be used to screen for iron bioavailability and for effect of enhancers and inhibitors, the magnitude of the effect and sometimes the direction may differ from human studies, calling for careful interpretation of results (Fairweather-Tait et al., 2005).

At the uptake and absorption steps, in vitro (Caco-2 cell culture model), in man, and animals methods (using native iron or isotope labeled iron) have been developed. These methods recognize the fact that not all soluble or dialyzable iron is absorbed and thus a need to measure

iron absorption across the basolateral membrane of enterocytes. The Caco-2 cell culture model, a cell line developed from a human adenocarcinoma, has been the most commonly used in vitro iron absorption method (Alvarez-Hernandez et al., 1991; Halleux and Schneider, 1991). The cell line spontaneously differentiates in cell culture to form a polarized epithelial monolayer with many of the characteristics of enterocytes (e.g., production of enzymes, well developed microvilli and tight junctions, etc). Caco-2 cells are grown on porous membranes in bicameral chambers for 12-18 days to ensure maximal trans epithelial electrical resistance. In vitro digested food samples are then added to the apical or upper chamber and uptake allowed to proceed for 1-24 hrs. A dialysis membrane may be added to prevent contact with digestive enzymes or the digest is heat treated to inactivate enzymes. The food sample may be radio labeled and the radioactivity in the cells, apical and basal chambers determined and used to calculate percentage uptake. Otherwise, Caco-2 cell ferritin formation is determined and used as surrogate for iron uptake (Glahn et al., 1998). Iron deficient cells are more effective in iron uptake than cells grown on normal iron levels. A further modification of the method is use of cells grown on solid plastic plates which are then harvested after iron uptake and ferritin formation determined (Proulx and Reddy, 2006). This model has been used to show that Fe (II) is better up taken than Fe (III) and that ascorbate enhances bioavailability (Alvarez-Hernandez et al., 1991). Further, enhancing effects of meats and inhibiting effects of bran, tea and phytates have been shown (Au and Reddy, 2000). The model has been shown to better approximate human iron absorption (Au and Reddy, 2000; Yun et al., 2004). Subsequently, the Caco-2 cell model has been used to determine effects of numerous inhibitors and enhancers as well as effect of processing and fortificants on iron bioavailability from numerous food matrices. However,

the in vitro digestion/Caco-2 cell culture model has numerous shortcomings; the fact that they are derived from colonic adenocarcinoma cells means that they may behave differently from normal, small intestine enterocytes. It has also been reported that transepithelial resistance in Caco-2 cells is much higher than in human small intestine and resembles that of human colon (Amine and Hegsted, 1975). The absence of a mucin layer, which may play a significant role in intestinal iron absorption may also be problematic. Caco-2 cell monolayer shows a low carrier expression, resulting in very low transport rates, so that a scaling factor may be required (Lennernäs et al., 1996). Further, there is question of standardization of the procedure with various passage numbers and incubation periods reported in literature.

Animal models can be used to measure iron bioavailability at uptake, absorption, retention and utilization stages. Rats (AOAC, 2012), chicks (Tako and Glahn, 2010; Tako et al., 2011) and pigs (Howard et al., 1993) are the most commonly used animal models. Usually, the animals are depleted of iron by feeding an iron-deficient diet or by periodical bleeding. However, the need for depleting iron stores may be avoided by using weanling animals with a similar iron status. The use of the rat model is complicated by the fact that the physiology of iron absorption is different from humans, especially by the fact that they are able to synthesize ascorbic acid, and they possess intestinal phytase activity. Thus, animal models have been shown to deviate significantly from human studies (Reddy and Cook, 1991). Though less frequently used, the pig model may more resemble the human iron absorption model. At retention step of iron bioavailability, human chemical balance studies have been used (Moore et al., 1943; Rosado et al., 1992). Human chemical balance studies measure the amount of iron that is absorbed or

retained by the body after accounting for fecal iron losses (McCance and Widdowson, 1937; Rosado et al., 1992) or measure post-absorption plasma iron (Moore et al., 1943). They were the method of choice for determining iron absorption before the use of radioisotopes was introduced and are still useful in situations where radioisotope facilities are not available or exposure to ionizing radiation is not advisable. Radioisotope balance techniques were introduced in the late 1940s (Dubach et al., 1948) and have an advantages of obtaining estimates of endogenous excretion. However, problems arise if the radioisotope does not exchange completely with the native iron. Stable isotopes, on the other hand, have increasingly replaced radio-isotopes (Weaver, 1988) and eliminate health risks from ionizing radiation, do not decay, and can be used freely in analytical or other sample or food processing equipment without fear of radioactive contamination. Other methods have made use of tissue concentrations of iron (plasma/serum iron, serum ferritin, serum transferrin receptors, hemoglobin regeneration and hemoglobin incorporation) as surrogates of iron bioavailability (Olszon et al., 1978).

2.3.1 Iron bioavailability from beans

Though beans show a high iron content (30-190 $\mu\text{g/g}$ seed) bioavailability of this iron is very low and variable, depending on variety. Polyphenols, phytic acid and some fractions of bean proteins have been implicated in lowering bean iron bioavailability. Solubility/dialyzability methods typically over estimate iron bioavailability from beans while Caco-2 cell culture/animal studies and human absorption studies are more or less in agreement on the fraction of bean iron that is bioavailable. However, what is in agreement is that white beans provide more bioavailable iron than colored beans due to the lower polyphenol content.

2.3.1.1 Iron solubility and dialyzability studies

Initial solubility studies by Shackleton and McCance (1936) indicated high percentage of ionizable iron in raw, cooked and tinned beans (>70%) when extracted in dilute acid solutions. However, on implementing in vitro peptic digestion (pepsin and HCl at pH 2 for 2 hrs at 37 °C) and pancreatic digestion (pancreatin and bile salts at pH 7 using NaHCO₃ for 4 hrs at 37 °C) proposed by Miller et al., (1981) to the iron solubility method, but without a dialysis membrane, Lombardi-Boccia et al., (1994) showed an iron solubility of 55% in whole white bean flour which reduced with peptic digestion (to 4%) but increased with pancreatic digestion (to 18%). This showed inhibitory effects of protein digestion intermediate peptides but over estimated iron bioavailability as compared to iron dialyzability studies of the same samples. Solubility of iron associated with globulin fractions was 40-48% while that associated with albumins was very low (<10%). Solubility of iron associated with the albumin and globulin fractions increased with peptic and reduced with pancreatic digestion, more so in the albumin fraction. Using a similar method, Pynaert et al., (2006) showed that an infant complementary food consisting of germinated, autoclaved and dried finger millet (65.2%) and kidney beans (19.1%), roasted peanuts (8%) and mango puree (7.7%) had 18.8% soluble iron which was significantly higher than an unprocessed combination of the ingredients (4.4%). However, a Caco-2 cell culture model did not show a significant difference in the absolute iron uptake between the processed and unprocessed samples (1.3 and 3.4 nmol/mg cell protein), a result that had earlier been seen in human field trials (Mamiro et al., 2004). Taken together, these two studies clearly demonstrate inability of the iron solubility method to predict iron bioavailability in these particular food matrices.

However, with inclusion of a dialysis membrane, Lombardi-Boccia et al., (1994) were able to show iron dialyzability results more in agreement with in vitro digestion/Caco-2 cell culture and human absorption studies. Percentage dialyzable iron from mottled and white bean was 2.5 and 3.6% respectively; a result in agreement with many Caco-2 (Hu et al., 2006, Ariza-Nieto, 2007; Beiseigel et al., 2007) and human absorption studies (Beiseigel et al., 2007; Petry et al., 2010; Petry et al., 2012). This method was able to predict higher bioavailability of white beans over colored seed coated ones in agreement with human studies (Petry et al., 2012) but not the enhancing effects of dehulling and cooking of whole bean seeds (Lombardi-Boccia et al., 1995). Iron dialyzability was used by the same group to determine bioavailability of iron associated with various bean protein fractions and to study effect of various protein digestion residues (Lombardi-Boccia et al., 1994). Iron dialyzability from whole white bean flour, albumin, G1 globulin and G2 globulin fractions was 2.28, 0.4, 2.99 and 5% respectively. A similar trend was obtained for protein dialyzability i.e. albumin < GI < G2. The low bioavailability of iron associated with the albumin fraction was blamed on its resistance to digestion, the low percentage of dialyzable amino acids and the phytic acid associated with it. A high amount of cysteine associated with globulin digestion as well as its higher susceptibility to digestion may have enhanced bioavailability of associated iron. Iron dialyzability procedures have also been used to study effect of extrusion cooking (Lombardi-Boccia et al., 1995b; Ummadi et al., 1995; Drago et al., 2007), and showed no significant effect of the heat process.

2.3.1.2 Caco-2 cell and animal studies

A combination of in vitro digestion and Caco-2 cell culture in studying iron bioavailability offers the best in vitro method whose results correlate well with human absorption studies and

predictive equations developed to relate the two (Yun et al., 2004). These methods have mainly been used to screen for iron bioavailability in a large number of varieties and also to compare effect of different treatments and dietary factors. When the cells are grown on porous membranes in bicameral chambers, it is also possible to study effect of host factors on iron bioavailability through application of treatments to the basal side. Glahn et al., (1998) used an in vitro digestion/Caco-2 cell culture model to study iron bioavailability from various foods and eliminated use of radioisotopes, pioneering the use of ferritin formation by the Caco-2 cells as surrogate for iron uptake. This model was able to predict enhancing effect of ascorbic acid and superior bioavailability of heme (beef and fish) over nonheme iron (corn and beans). Tako et al., (2009a) used the same method for determining iron bioavailability from red and white beans; comparing the in vitro digestion/Caco-2 cell culture method with a pig model. They showed that though the cell culture method showed a significantly higher bioavailability from white beans over the red variety, the same could not be said of the pig model, which showed no significant difference. From the pig model, hemoglobin repletion efficiency, a measure of percentage of ingested iron that is incorporated into hemoglobin was 20.8 and 23.5% for the white and red bean diets, respectively. The lack of polyphenol effect (red beans had a higher polyphenol content) on iron bioavailability in the pig model may have been due to the adaptation of the animals to chronic exposure to polyphenols. Beiseigel et al. (2007) compared the Caco-2 cell culture model with human uptake studies and showed some discrepancy between the two methods. Subjects of varying levels of iron status absorbed iron equally from both the red and white bean samples (2.1 and 3.0% respectively). These results were in contrast to the in vitro results where the white beans gave significantly higher iron uptake relative to the red beans. The

discrepancy could be explained by the fact that the samples were extrinsically labeled and may not have equilibrated well with the intrinsic iron of the red bean sample. However, the Caco-2 model was in agreement with the human subjects for maize samples in the same study, showing reliability of the method. Other Caco-2 cell culture studies have confirmed enhanced iron bioavailability from white beans as compared to colored seed coat varieties (Hu et al., 2006, Ariza-Nieto, 2007; Laparra et al., 2008; Laparra et al., 2009). Rat models (Welch et al., 2000), poultry models (Tako and Glahn 2010) and human studies (Petry et al, 2012) have confirmed similar seed coat color effects, i.e., a higher bioavailability of iron from white as compared to colored seed coat beans. The pig model was used to show that biofortified black (Tako et al., 2009b) and red mottled (Tako et al., 2011) beans provided more iron than standard varieties.

2.3.1.3 Human absorption studies

Though in vitro methods are useful for screening for iron bioavailability in large numbers of samples, ultimately human studies will be needed to verify results. Lynch et al., (1984) determined iron bioavailability from different legumes (soy beans, black beans, lentils, mung beans and split peas) in humans using radioisotopes. Male subjects with normal iron status were used in the study and the legumes administered in a soup meal labeled by the extrinsic tag method. Mean percent absorption was low (0.8-1.9%) and was not significantly different among the legumes. Donangelo et al., (2003) used both extrinsic and intrinsic labels to study absorption from two bean genotypes, containing normal (50.4 µg/g) or high (82.9 µg/g) iron content. They studied young women with low iron reserves and fed the beans in single meals. Iron absorption was less than 2% from both bean types, and total iron absorbed was not different between types. Petry et al., (2010) found iron absorption from whole red beans of 2.5%, which

more than doubled with removal of polyphenols and phytic acid. Petry et al. (2012) found higher iron absorption rates (7 and 7.4%) when the beans were fed as multiple composite meals (served with rice or potatoes) as compared to bean puree (3.4 and 4.7%) for red and white varieties respectively. Thus, when fed alone, the high polyphenol content of red varieties tended to reduce iron absorption, but the effect was dampened when the beans were fed with rice or potatoes. The same study showed that enhancing iron content of a red bean variety (91 ppm of iron) led to reduction in bioavailability (3.8%) and did not translate into increased total iron absorbed as compared to low iron variety (52 ppm of iron, 6.3% bioavailability), both meals fed as multiple composite meals. Beiseigel et al (2007) showed that iron bioavailability in beans ranged from 2-3% using stable isotopes. This group also showed that iron absorption was increased 3 fold in vivo by addition of ascorbic acid (molar ratio of 20:1 ascorbic acid: iron).

2.4 Phytoferritin

Iron in plants is essential for plant productivity but its homeostasis is critical to prevent reaction with oxygen, which reactions could have deleterious effects on cell integrity. Ferritin is one of the proteins involved in homeostasis of iron in plants. Phytoferritin is a 540-600 kDa protein made up of a 24 subunit, ~28 kDa each, protein shell surrounding an iron oxide core and can store up to 4500 iron ions (Harrison and Arosio, 1996). Ferritin is a ubiquitous iron storage protein found in all living kingdoms and is highly conserved, with plant and animal ferritin sharing between 39-49% amino acid sequence homology. In vertebrates, ferritin consists of two types of subunit: heavy (H) and light (L), with apparent molecular weights of 21 and 19.5 kDa, respectively (Harrison and Arosio, 1996) and share ~55% identity in amino acid sequence.

However, phytoferritins show only one subunit type which shares ~40% sequence identity with the animal H-subunit (Masuda et al., 2001). Ferritin from black beans, soybean and pea seed consists of two subunits of 26.5 and 28.0 kDa, which are designated H-1 and H-2, respectively, sharing ~80% amino acid sequence identity (Deng et al., 2011). The two subunits are synthesized as a precursor (32 kDa) with a unique two-domain N-terminal sequence, the transit peptide that is followed by an extension peptide. The transit peptide is presumed to facilitate transport of the ferritin precursor to plastids, upon which it is cleaved resulting in the formation of the mature subunit which assembles into a 24 subunit apoferritin within the plastids (Waldo et al., 1995). The mechanism of iron uptake and storage by apoferritin in plants has been described by Liu and Theil (2005) and regulation of its synthesis, which is very different from that in animals, is described by Briat et al. (1995). Though ferritin is the major iron storage protein in plants, its concentration may not necessarily correlate to iron content. A positive correlation between ferritin expression and iron content has been reported in transgenic tobacco, lettuce (Goto et al., 1998, 2000) and maize (Aluru et al., 2011) but not in transgenic rice (Qu et al., 2005). Also, speciation of iron in the plant seed varies anywhere between 18-90% depending on species (Marentes and Grusak, 1998; Hoppler et al., 2008; Cvitanich et al., 2010). In white and red kidney beans it was calculated that 20 and 25% of the total seed iron was bound to ferritin respectively (Hoppler et al., 2008; Cvitanich et al., 2010) with the largest portion found in the cytoplasm of cells surrounding the provascular tissue and cells near the epidermal layer. In contrast, iron in wheat grain is primarily bound to phytate (May et al., 1980).

2.4.1 Ferritin iron absorption

Iron associated with phyto ferritin has been shown to be as bioavailable as ferrous sulfate (Davila-Hicks, et al., 2004, Lonnerdal et al., 2006) and may be absorbed intact by endocytosis or micropinocytosis (San Martin et al., 2008). For this to happen, ferritin must reach the enterocyte intact. At physiological stomach pH (pH 2) ferritin is susceptible to pepsin digestion; though may escape at pH 4 (Martinez-Torres et al., 1986; Hoppler et al., 2008) and the released iron may interact with polyphenols and phytic acid; dietary factors that inhibit nonheme iron absorption. However, ferritin is consumed within a food matrix where pH within a bolus of food may not reach pH 2 and may be partially protected from complete digestion (Kalgaonkar and Lonnerdal, 2008). A recent study suggests that tannins in food may complex ferritin and reduce its digestion at pH 4 (Li et al., 2012). Earlier studies had reported reduced rates of animal ferritin proteolysis (pepsin, pH 2.5) as compared to that to animal apoferritin (Crichton, 1970). Thus, contribution of ferritin to iron bioavailability is still a subject of discussion and more work needs to be done to model its influence in a complex food matrix after in vivo digestion and absorption.

2.5 Extrusion cooking

Extrusion cooking is a high-temperature, short-time process in which moistened, expansive, starchy and/or high protein food materials are plasticized and cooked in a barrel by a combination of moisture, pressure, temperature and mechanical shear, resulting in diverse chemical reactions (Bredie et al., 1998; Ilo and Berghofer, 1999; Camire, 2001). It has emerged as the preferred technology for development of Ready To Eat (RTE) products such as breakfast cereals, baby foods, snacks, textured vegetable proteins, and offers numerous advantages over other heat treatments such as drum drying, roasting, cooking, etc. (Anderson et al., 1969; Mercier and Feillet, 1975; Guy, 2001). Compared to conventional heat treatments, extrusion cooking

improves digestibility and bioavailability of nutrients; offers versatility, high productivity, low operating costs, energy efficiency and shorter cooking times (Alonso et al., 2000; Alonso et al., 2001). It also offers an ability to develop a range of products with distinct textural advantages including expansion, crispiness and general mouth feel (Skierkowski et al., 1995; Berrios and Pan, 2001).

In addition, extrusion cooking denatures many undesirable enzymes; inactivates some antinutritional factors (trypsin inhibitors, haemagglutinins); sterilizes the finished product; and minimizes nutrient, color and flavor loss (Bredie et al., 1998; Ilo and Berghofer, 1999; Camire, 2001; Guy, 2001). Other effects include improved protein digestibility, increased soluble dietary fiber and reduction of lipid oxidation (Bredie et al., 1998; Ilo and Berghofer, 1999; Alonso et al., 2001). The most important structural effect of extrusion cooking is starch gelatinization and protein denaturation to give desired product texture (Mercier and Feillet, 1975; Skierkowski et al. 1990). Therefore, protein and starch content of the raw material, as well as chemical composition of these biopolymers are important factors (Tomas et al., 1997). On the other hand, extruder processing parameters including raw material moisture content and flow rate, die temperature and pressure; and specific mechanical energy significantly influence extrudate physicochemical and sensory properties (Lawton et al., 1972; Mercier and Feillet, 1975; Edwards et al., 1994, Ilo et al, 1996).

2. 5.1 Extruder types

An extruder consists of a screw(s) which conveys the food material from a hopper along the barrel and out through a die achieving heating by both mechanical and thermal processes. Two

types of extruders are in use today; single screw and twin screw extruders. Single screw extruders were initially developed for oil pressing but have since undergone various transformations (screw configurations/shape, heating jackets, etc.), to make them more versatile (Hauck, 1988). Twin screw extruders on the other hand consist of two screws of equal length placed inside the same barrel (Clark, 1978). They are generally categorized according to the direction of screw rotation (counter- or co-rotating) and to the position of the screw in relation to one another (intermeshing and non-intermeshing). Twin screw extruders have become important in the food industry due to greater control of both product and process parameters, can work on formulations with high fat (18-22%) and wide ranges of moisture content (Colonna et al., 1983; Yacu, 1985; Edwards et al., 1994).

2.5.2 Raw material for extrusion cooking

Extrusion cooking is characterized by low moisture content (10-40%) and high temperatures (100-180 °C) and a wide range of raw materials can be utilized. Guy (1994, 2001) has classified the different ingredients in the raw material according to their functional role. Texture is one of the most important sensory attributes of extruded products and is mostly influenced by food biopolymers; mainly starches and proteins (Mercier and Feillet, 1975; Skierkowski et al. 1990, 1995). At high temperatures and extrusion moisture levels, the biopolymers form a melt fluid and bubbles of water vapor blow into the fluid to form a foam. At the die end, the pressure inside the barrel reaches a maximum and as the melt is forced out of the die, the pressure is suddenly reduced to atmospheric pressure causing water to change from liquid to vapor causing it to puff. Amylose/amylopectin ratios and protein composition are thus important influencers of

product quality (Tomas et al., 1997; Guy, 1994, 2001). Corn, wheat, rice and soybean have provided the bulk of raw material for extruded products on the market (Mercier and Feillet, 1975; Skierkowski et al. 1995; Ilo et al., 1996; Bredie et al., 1998).

2.5.3 Extrusion cooking of beans

Extrusion cooking has been effectively used to reduce the cooking time, overcome effects of hard-to-cook defect, eliminate anti-nutritional factors, as well as improving the textural, nutritional, and sensorial characteristics of beans (Martín-Cabrejas et al., 1999; Ruiz-Ruiz et al., 2008; Rocha-Guzman et al., 2008). Hard-to-cook defect develops in beans that have been stored under adverse conditions of high temperature ($>25^{\circ}\text{C}$) and high humidity ($>65\%$) and is characterized by extended cooking times for cotyledon softening (Hincks and Stanley, 1986; Jones and Boulter, 1983a; Jones and Boulter, 1983b) and thus require increased energy (fuel) cost for preparation; are less acceptable to the consumer due to changes in flavor, color, and texture; and have decreased nutritive quality (Bressani, 1982; Molina et al., 1975). However, by pregelatinizing bean starch, extrusion cooking effectively reduces cooking time, bulk viscosity and increases nutrient density and consumer acceptability of extrudates (Edwards et al. 1994; Nyombaire et al., 2011). Numerous studies have evaluated effect of extrusion cooking on nutritional and physicochemical characteristics of beans but comparison of these effects is hampered by the diverse types of extruders used, the extrusion conditions employed and the bean variety studied. For this reason, it may be necessary to report extrusion conditions in order to aid interpretation. The main extrusion conditions that have been studied include barrel temperature, moisture content and Specific Mechanical Energy (SME; a function of feed flow rate and extruder screw speed). The overall effect of extrusion cooking depends on severity of operation

and the most severe conditions are high temperature, high SME and low moisture content (Edwards et al., 1994). Twin screw extruders offer more severe mechanical shear and screw configurations can be altered to control severity of processing. In the following sections, literature on effects of extrusion cooking on nutritional and physicochemical characteristics of beans will be reviewed.

2.5.3.1 Effect on nutritional properties

Extrusion cooking significantly alters the food matrix and influences nutritional value in many important ways (Camire, 2001). It brings about starch gelatinization even at the low moisture contents employed and breaks down amylose and amylopectin polymers to lower molecular weight polymers, increasing susceptibility of carbohydrates to enzymatic digestion (Wang et al., 1993). Effect of extrusion cooking on proteins is mainly due to loss of amino acids during Maillard reactions and improved digestibility due to protein denaturation (Della Valle et al., 1994). Lysine is most reactive amino acid due to presence of two available amino groups (O'Brien and Morrissey, 1989), though arginine, tryptophan, cysteine and histidine are also vulnerable (Iwe et al., 2001). The rate of Maillard reaction increases with severity of extrusion cooking i.e. high temperatures and low feed moisture content. Noguchi et al., (1982) showed loss of lysine during extrusion cooking of a cereal/soy-based mixture containing 20% sucrose ranged from 60-100% at 170 °C and 10–14% feed moisture. Lysine loss increases with increase in sugar content (Bates et al., 1994). Berrios and Pan (2001) studied effect of extrusion processing at different screw speeds (400-500 rpm) and flour particle size (0.85 -2.28 mm) on some nutritional properties of black bean flour. A Werner & Pfleiderer Continua twin-screw extruder was used at constant screw configuration, die temperature (160°C), feed rate (25 kg h⁻¹),

and feed moisture content (18%, wb). Raw material flour particle size and screw speed did not affect the proximate composition ($p > 0.05$), though extrusion cooking significantly reduced ($p < 0.05$) fat content by over 50%. Loss in lipid content can be attributed to complex formation with amylose which may reduce their extractability and eventual bioavailability (Bhatnagar and Hanna, 1994 a, b). Simons et al. (2012) also did not find a significant effect of extrusion on proximate composition, but showed a slight increase in resistant starch. The stability of vitamins depends on their chemical structure and composition as well as extruder parameters (moisture, temperature, light, oxygen, time and pH (Killeit, 1994; Camire 2001). Overall, the lipid-soluble vitamins (D and K) and vitamins B6 and B12 (65-96% retention) are fairly stable while thiamin and folate are the most susceptible (30-65% retention) (Killeit, 1994). As in amino acids, retention of vitamins decreases with severity of extrusion cooking i.e., increasing temperature, screw speed and specific energy input, as well as decreasing moisture content. Steel et al., (1995) studied effect of extrusion cooking on the inactivation of anti-nutritional factors of freshly harvested HTC brown Carioca SH bean cultivar. They used a Brabender bench top 19 mm single-screw extruder run at screw speed of 100 rpm, die temperature of 190 °C, and feed moisture content of 21.5% (wb). They reported that extrusion effectively reduced trypsin-chymotrypsin inhibitor and hemagglutinin activities 88% and 95%, respectively. Previously, Edwards et al. (1994) reported a reduction of 85-99.6% of trypsin inhibitor activity on extrusion of small white bean flour extruded using a Werner & Pfleiderer 37 mm Continua twin-screw extruder with varying SME (146-294 Wh/kg), die temperature of 114-177°C and moisture content of 15-25%. Using a Brabender bench top 19 mm single-screw extruder, Balandran-Quintana et al. (1998) studied effect of die temperatures (140-180°C), screw speed (150- 250

rpm) and feed moisture content (18- 22%, wb), on trypsin inhibitor activity in extruded pinto bean flours and reported complete inactivation for all experimental conditions. They also reported that extrusion processing increased the in vitro protein digestibility of the bean extrudate by 8.3%. Similar to trypsin inhibitor activity, inactivation of lectins has been subject of numerous studies. Karanja et al. (1996) used a Brabender bench top 19 mm single-screw extruder to evaluate different processing conditions for lectin inactivation capacity of HTC Canadian Wonder bean flours. Die temperatures (100-180°C), screw speeds (49 -107 rpm), and feed moisture content (25- 30%, wb) were varied. Lectin inactivation increased with increased extrusion temperature, moisture content and residence time. Using a 30 mm laboratory co-rotating twin-screw extruder and varying moisture content (25-36%) die temperature (120-130 °C), screw speed (118-253 rpm), and feed rate (80 -120 g/min), Nyombaire et al., (2011) achieved a 90% reduction in phytohemagglutinin (PHA) activity for all experimental conditions.

Alonso et al. (2000) studied the comparative effect of extrusion cooking, dehulling, soaking, and germination on protein and starch digestibility and reduction of anti-nutritional factors in kidney and faba beans. Extrusion processing was performed in a Clextral X-5 45 mm twin-screw extruder, operated at 100 rpm, feed rate set at approximately 383-385 g/min, 25% wb moisture content and die temperatures of 152-156°C. They reported that even though trypsin, chymotrypsin, α -amylase inhibitor activities of the two types of beans were decreased significantly by dehulling, soaking, and germination, haemagglutinating activity was not affected by the conventional processing methods. However, extrusion cooking effectively inactivated all the antinutrients in the two beans under study, without altering protein content. Additionally,

extrusion was the best method for improving in vitro protein and starch digestibility. The effect of extrusion processing on some oligosaccharides in beans has also been studied. Borejszo and Khan (1992) used a Wenger TX-52 twin-screw extruder to process pinto bean High Starch Fraction (HSFs) at die temperatures in the range of 110–163°C, screw speed of 300 rpm, and feed moisture of 18.8% (wb), to determine the effect of processing conditions on flatulence-causing sugars (raffinose and stachyose). Increase in temperature led to decrease in flatulence-causing sugars. Sucrose content decreased by 76% in samples extruded at 163°C, while raffinose and stachyose contents were reduced 47% to 60%, respectively. Berrios and Pan (2001) showed that total oligosaccharides in black beans were not affected by difference in particle sizes, but was reduced significantly with increase in screw speed. Addition of up to 2.0% NaHCO₃ did not further reduce individual free sugars (Berrios et al., 2002). The latter study also observed a decrease in insoluble fiber (IF) and an increase in soluble fiber (SF), showing that extrusion processing causes a redistribution of the IF to SF fractions. The authors attributed this fiber fraction redistribution to hemicellulose depolymerization leading to solubilization of arabinose, and uronic acids. This confirmed what had been earlier reported by Martin-Cabrejas et al. (1999). Thus, in inactivating anti nutritional factors, improving digestibility and reducing flatulence factors, extrusion cooking enhances nutritional value of beans.

2.5.3.2 Effect on starch pasting properties and extrudate structure

Starch gelatinization and protein denaturation are some of the most important reactions that occur during extrusion cooking. The qualitative features of the extruded products are thus

characterized by several functional properties such as water absorption index (WAI), water solubility index (WSI), expansion ratio (ER), bulk density (BD) and pasting profiles. These can be used to estimate extent of cooking, the functional characteristics of extrudates, predict how the materials may behave if further processed and to what end use the extrudate is most suited. Physicochemical and pasting properties of extrudates of different types of dry beans have been the topic of several studies. By pregelatinizing bean starch, extrusion cooking effectively reduces viscosity of gruels, thus increasing their nutrient density for malnutrition intervention foods (Edwards et al. 1994; Nyombaire et al., 2011). However, as noted by Oikonomou and Krokida (2011), it is difficult to determine overall effect of a single extrusion parameter on physicochemical characteristics owing to differences in the starting raw materials, the ranges of extrusion conditions and their combinations. Edwards et al. (1994) extruded small white beans using a Werner & Pfleiderer Continua twin-screw extruder, at constant screw speed of 225 rpm. SME (146-294 Wh/kg), raw material moisture content (15–25%, wb) and die temperatures (114–177 °C) were varied. As the energy intensity increased, expansion ratio, starting viscosity and hot viscosity increased, while bulk density and ending viscosity decreased. At low energy intensity, SME, die temperature and feed moisture content significantly increased expansion index but at higher energy intensity, expansion index depended mainly on SME. Peak and final viscosity decreased with increase in either variable. On the other hand, starting viscosity increased with increase in SME, die temperature and feed moisture content; showing severity of starch degradation. All extrusion properties could be mathematically modeled as a function of feed moisture, die temperature, and SME using quadratic equations. Karanja et al. (1996) used a Brabender bench top single-screw extruder run at screw speeds of 49-107 rpm and die

temperature (100- 180 °C) to evaluate expansion, WAI and WSI of Canadian Wonder bean flours at 25-30% moisture (wb). Expansion and WAI of the extrudate increased while WSI and bulk density decreased with increased extrusion temperature. Similar results were later reported by Martin-Cabrejas et al. (1999) for dry bean (cv. Horse-head) extrudates, processed in the same type of extruder, at die temperatures of (140-180°C). Simons et al. (2012) showed increasing screw speeds (a measure of SME) reduced expansion index and increased b values on the Hunter color scale in precooked bean flours. WAI and WSI were not significantly affected by screw speed/SME.

Balandran-Quintana et al. (1998) extruded pinto bean flours at various moisture contents (18-22%, wb), screw speeds (150- 250 rpm) and die temperatures (140-180°C) using a Brabender 19 mm single-screw extruder. They indicated that temperature and feed moisture conditions significantly ($p < 0.05$) increased bulk density, expansion, and WAI. Gujska and Khan (1990) extruded high starch fractions (HSFs) of navy beans, pinto beans, and chickpeas and generally showed an increase in WAI, WSI and expansion ratio with increase in temperature up to 132°C, but a decrease thereafter. Using a 30 mm laboratory co-rotating twin-screw extruder and varying moisture content (25-36%) die temperature (120-130 °C), screw speed (118-253 rpm), and feed rate (80 -120 g/min), Nyomba et al. (2011) reported that increasing moisture content significantly increased bulk density of light red kidney beans. However, none of the conditions studied significantly influenced WAI and WSI, though there was a general trend of increase with increase in moisture content and feed rate.

Berrios and Pan (2001) showed that expansion ratio of black bean flours increased with increase in screw speed and decrease in particle size. Nyombaire et al., (2011) however did not report any effect of die temperature, screw speed or moisture content on expansion ratio of light red kidney beans. Only feed rate significantly increased expansion ratio. Berrios et al. (2004) further improved expansion ratio two- fold by adding 0.5% NaHCO_3 to black bean flours prior to extrusion processing, an effect attributed to an increase in the number of air cells and a decrease in cell wall thickness. The combined effect of these two factors caused the collapse of the cell walls and the appearance of large void spaces within the extrudates causing increased expansion and reduced strength. In conclusion, increasing the severity of mechanical and thermal energy input as well as the amount of plasticizer favors starch breakdown and loss of viscosity.

2.5.3.3 Effect of extrusion cooking on phytates and polyphenols

Extrusion cooking has been proposed as promising strategy to reduce effect of anti-nutritional factors in beans. It generally reduces the higher inositol phosphates (IP5 and IP 6) while increasing lower phosphate fractions (IP2-IP4) (Sandberg et al., 1987; Alonso et al., 2000; El-Hady and Habiba, 2003). On the other hand, effect of extrusion cooking on polyphenols is variable and dependent on the polyphenolic profile of beans, decreasing oligomers with degree of polymerization (DP) of 4–9 while increasing fractions with DP of 1-2 (Korus et al., 2007; White et al., 2010). A slight reduction in isoflavone content and their decarboxylation, causing higher proportions of acetyl derivatives, on extrusion cooking has been reported (Mahungu et al., 1999). However, the consensus is that total polyphenolic content and anti oxidant activity are reduced by extrusion cooking (Camire, 2001). Korus et al. (2007) studied effect of extrusion

cooking on the phenolic composition and antioxidant activity of three variously colored (dark-red, black-brown and cream) Polish bean cultivars. They used a Brabender 20DN single screw extruder at constant feed rate and screw speed (90 rpm); variable moisture content (14 and 20%) and die temperature (120 and 180 °C). Polyphenol content of raw material ranged from 0.3-0.9 mg/g; highest in the dark red and lowest in the cream seed coat colored varieties. Kaempferol and quercetin content (the most potent iron absorption inhibiting polyphenols in beans) followed similar trend. Myricetin, cyanidin, chlorogenic acid, caffeic acid, ferulic acid and p-coumaric acid were the other phenolics identified in both raw and extruded beans. The effect of extrusion on the total phenolic content depended on the cultivar; increasing up to 28% in dark red but decreasing 30 and 38% in black-brown and cream cultivars respectively. High moisture and low temperature maximized increase in former while low moisture and high temperature minimized polyphenol content in the later. Also, effect of extruder parameters (barrel temperature and moisture content) on individual phenolic fraction varied; the biggest increase being by 84% in quercetin content of dark red variety. Some fractions decreased while others increased, depending on variety and extrusion conditions. Overall, total polyphenol content decreased with increase in temperature and decrease in moisture content. Both anti-oxidant and free radical scavenging activity reduced with extrusion cooking, reducing with increase in both temperature and moisture. Similarly, Delgado-Licon et al. (2009) and Anton et al. (2009) observed a significant decrease in the total polyphenols and antioxidant activity during extrusion of bean/corn mixture. The decrease in total polyphenols and antioxidant activity was dependent on process conditions and bean variety; ranging from 10-70% and 22-65% respectively. Alonso et al. (2000) studied the comparative effect of extrusion cooking, dehulling, soaking, and

germination on polyphenol, condensed tannins, and phytate content in kidney and faba beans. Extrusion processing was performed in a Clextral X-5 45 mm twin-screw extruder, operated at 100 rpm, feed rate set at 383-385 g/min, 25%, wb, moisture content and die temperatures of 152-156 °C. Raw bean composition was 1.6% phytate, 3.6 mg/g condensed tannins and 2.1mg/g total polyphenols. Germination for 72 hrs was most effective in removing phytates (30.2%) while dehulling eliminated up to 90% of polyphenols and condensed tannins. Extrusion cooking eliminated 21.4% phytates, 83.8% tannins and 45.9% polyphenols. Similar results were obtained in a later study (Alonso et al., 2001) which, after accounting for individual inositol phosphates, showed that reduction in total phytates was only 4% while the IP-6 fraction reduced by 26.8%; with a concomitant increase in IP-5 (fivefold) and IP-4. Thermal hydrolysis was credited for effects of extrusion cooking on phytates while reduced extractability (due to increased polymerization or complexing with proteins and other hydrophobic components) and altered chemical reactivity could explain effect on polyphenols. Earlier, Sandberg et al. (1987) had shown thermal hydrolysis of IP-6 to lower inositol phosphates on extrusion cooking on wheat bran and subsequent inactivation of phytase enzyme. El-Hady and Habiba (2003) studied effect of soaking and extrusion conditions on polyphenol, tannins and phytate content of peas, chickpeas, faba and kidney beans. Seeds were soaked at 30 °C for 16 h and extruded in a Brabender Laboratory Single-Screw extruder at variable moisture content (18% or 22% wb), barrel temperature (140°C or 180°C), constant screw speed (250 rpm); screw compression 4:1; feeding screw speed (160 rpm). Raw beans contained 1.1% phytic acid, 2.33 mg/g tannins and 0.64 mg/g polyphenols. All the anti-nutrients reduced with soaking (11.9, 2 and 4.5%); extrusion cooking (12.6, 16 and 15.7%) and a combination of the two (14, 26.6 and 35.4 %)

respectively. During extrusion cooking, a high temperature and high moisture content favored loss of phytates, tannins and polyphenols though the interaction was not significant. Overall, a decrease in polyphenol and phytic acid content is observed on extrusion cooking; which effect increases with severity of extrusion cooking process (i.e., increase in temperature and reduction in moisture content). The effect on polyphenol content is further complicated by the fact that their analysis is challenging since they may be bound to non-starch polysaccharides and are not easily released during extraction procedures (Camire, 2001). The increase in free phenolic compounds on extrusion cooking may thus be due to thermal liberation. However, decrease in polyphenol content may be due to complexing with non-starch polysaccharides though some phenolics, e.g. caffeic-, ferulic- and p-coumaric acids are heat-sensitive and susceptible to thermal breakdown (Dimberg et al., 1996). Therefore, careful characterization of both phytic acid and phenolic compounds and their breakdown products in extruded foods by mass spectrometry is essential in order to understand how extrusion alters these compounds and their potential health effects.

2.5.3.4 Effect on iron bioavailability

Compared to effect on polyphenol and phytic acid contents (factors known to inhibit iron absorption), relatively little work has been done on the effect of extrusion cooking on iron bioavailability. In most studies, reduction in polyphenol and phytate acid content has traditionally been considered as a proxy to increase in iron bioavailability. However, as Hurrell et al., (1992) showed, reduction in phytate ought to be as high as 95% of natural levels for appreciable effects in bioavailability to be realized. Indeed, studies of effect of extrusion

cooking on iron bioavailability have showed mixed results with some showing an increase while others do not. Alonso et al. (2001) studied effect of extrusion cooking on mineral bioavailability in pea and kidney bean seed meals. Finely ground flours were extruded in a Co-rotating twin screw extruder (100 rpm screw speed, 350 g/min flow rate, 25% moisture content and 150 °C die temperature). A significant increase in iron content was reported after extrusion cooking, an effect considered to be due to wear and tear of extruder metal parts. Alongside a reduction in total phytates (4%) and IP-6 (26.8%) and concomitant increase in IP-5 (fivefold) and IP-4 as well 70% reduction in tannin content, iron bioavailability increased by 40%. The increase in iron bioavailability due to iron contamination from extruder parts was not accounted for in this study. However, the change in phytate fractions, from higher phosphate to lower phosphate inositols (as well as tannin degradation) may partially explain increase in iron bioavailability. Inositol phosphates with less than 5 phosphate groups have been shown to have no iron absorption inhibition capacity (Lönnerdal et al., 1989, Sanberg et al., 1999). Hazell and Johnson, (1989) used iron diffusibility as a measure of iron bioavailability and showed that it increased from less than 3% in raw whole maize to over 20% in a refined and extruded product. However, they concluded that the extrusion cooking process in itself was responsible for only a small part of the increase in iron diffusibility, with the bulk of the increase in diffusibility attributed to pre-processing procedures and contamination from processing equipment.

Hurrell et al., (2002) studied effect of extrusion cooking, steam cooking and roller drying on phytate degradation and related it to improvement in iron bioavailability in rice, maize and wheat using the radio labeled iron extrinsic tag technique. Extrusion cooking was carried out at 25%

moisture content, 160 °C die temperature, 10 Mpa pressure and steam cooking at 135 °C. The study showed that iron absorption was relatively low from products made with the different cereal flours, ranging from 1.8-5.5% for rice, 2.5-3.5% for maize, 4.9-13.6% for low-extraction wheat, and <1% for high-extraction wheat foods. The phytic acid content remained high after extrusion cooking (1.2, 1.7, 3.2, 3.3 mg/g in low-extraction wheat, rice, high-extraction wheat and maize products respectively) and could explain the low iron absorption. There were little or no differences in iron absorption between the extruded and roller-dried cereals. Bread-making, however, degraded phytic acid to non-detectable levels in the low-extraction wheat flour and increased iron absorption to 13.6%. Similar results were obtained by Frontela et al. (2008) who showed that an industrial roasting process did not sufficiently reduce phytic acid and high inositol phosphates (IP-5 and IP-6) in infant cereals to effect increase in iron bioavailability. The phytate/iron molar ratio was >1.3, substantially higher than the <0.4:1 required in order to achieve, at least, a 2-fold increase in iron absorption (Hurrell, 2004). Earlier, Ummadi et al., (1995) showed that though lower forms of inositol phosphates (IP-3, IP-4 and IP-5) increased to 51-71% of total phytate in extruded navy beans, chickpeas, cowpeas and lentils (from 21-33% in raw legumes) and tannin content decreased, iron dialyzability was 1.2-2.7% and was not significantly improved by extrusion cooking. They concluded that protein fractions formed during extrusion processing may bind iron tightly and reduce its bioavailability. In conclusion, extrusion cooking is a promising strategy to improve iron bioavailability at constant iron content by modifying the food matrix and aiding release of iron as well as reducing the anti-nutritional factors.

2.5.3.5 Effect on bean sensory properties

Extrusion is used commercially to produce high value breakfast and snack foods based on cereals such as wheat or corn. Though promising, the technology is not being commercially used for legume pulse seeds due to low expansion ratio and poor sensory properties as compared to traditional wheat and corn extruded products. However, the rise in consumer demand for convenience and/or healthy foods has spurred significant interest in developing bean based extruded products. The variety of products made from common bean extrudates include porridges (Nyombaire et al., 2011); and expanded snacks, breakfast cereal type products (Berrios et al., 2008). Extruded bean products are marketed on the basis of superior healthful properties like high protein and dietary fiber, low-calorie, very low in sodium and fat, cholesterol-free and gluten-free. Extrusion cooking generates various flavor profiles depending on the raw material (Bredie et al., 1998). Temperature and moisture level are the most important variables influencing aroma generation, with higher temperatures and moisture contents favoring burnt and toasted aromas. However, there is a dearth of research on specific sensory properties of extruded beans. Berrios et al. (2008) developed barbeque, cheese, classic, sugar and plain coated breakfast cereal type snack from a range of legumes and showed an overall average liking percentage of 80%, independent of the type of coating used, and about 60% for the plain snack. Nyombaire et al. (2011) used a 30 mm laboratory co-rotating twin-screw extruder to study effect of moisture content (25-36 %), die temperature (120-130 °C), screw speed (118-253 rpm), and feed rate (80 -120 g/min) on sensory characteristics of extruded red kidney bean. A Rwandese consumer panel scored porridge from the flour at 7 on a nine point hedonic scale with over 75% of the panelists scoring it at 7 and above. Steel et al. (1995) used extrusion cooking to reverse the effects of hard to cook defect in beans and established sensory properties of the extrudates.

They used a Brabender single screw laboratory extruder, Model GNF 101412, at 190 °C die temperature; rotation of the screw, 100 rpm; die diameter, 3mm and compression ratio, 3 : 1. A trained panel was used to assess sensory characteristics. Hard to cook beans exhibited a significantly inferior flavor to fresh beans and on extrusion cooking, hard to cook beans exhibited a bitter taste which was more pronounced than that of extruded fresh beans.

Skierkowski et al. (1995) studied effect of extrusion cooking on textural properties of snacks and compared instrumental measurements to consumer ratings. They used a Wenger X-5 laboratory single screw with a length/diameter ratio was 10.5:1. The screw speed was set at 700 rpm, feed rate of 7 kg/hr, die temperature 110-150 °C and moisture adjusted by injecting water into the feed at a constant level of 1.9 L/hr. Increasing barrel temperature reduced sample stress and increased sensory score for crispness. Products processed below 121°C were unacceptable.

They also established effect of protein and fiber content and showed acceptable ranges of 13-16% protein or 17-21% fiber; outside of which extrudates were too hard. However, shear stress increased with increasing protein or fiber content. To better understand the effect of extruded bean flour on sensory characteristics, investigations looking at effect of flour substitution at various levels have been done. Anton et al. (2009) studied effect of 15, 30 and 45% substitution of corn flour with bean flour in an extrusion cooked fortified puffed snack. They used a laboratory scale twin screw extruder screw length to diameter (L/D) ratio of 25.0. Process variables (screw speed, moisture, and temperature of the final zones) were kept constant (150 rpm, 22% and 160 °C). Corn starch-bean extrudates were denser, less expanded and harder than corn starch controls. However starch fortified with 30% bean flour produced extrudates with crispness comparable to corn starch. In conclusion, though data on sensory properties of

extruded bean flour is scarce, the few studies that have been presented show that beans increase hardness of snacks and introduce beany/bitter tastes on extrusion. However, as most of the snacks are flavored, flavoring of bean snacks will go a long way in improving consumer acceptability.

2.6 Food product optimization

2.6.1 Rationale for product optimization

Food product optimization refers to the process of determining values of the independent variables that lead to an optimal value of the function that is to be optimized (Schutz, 1983, Stone and Sidel, 1985). The information obtained is important for product formulation efforts and quality control; and forms a basis for identifying those specific product variables that require monitoring before and during processing (Stone and Sidel, 1985). Computer programs capable of analyzing optimization models have been developed, and these graphically or numerically identify a combination of factor levels that simultaneously satisfy the requirements placed on each of the responses and factors. Contour plots are useful to study optimization data and determine optimal conditions (Rustom, 1991). Optimization of ingredients and processes during product development helps answer questions like; How does each process and ingredient affect product quality? How do they correlate to the other ingredients/processes? Which correlations are most important for product quality management? How does one choose the right combination of ingredients and their levels? All this will lead to development of a product with optimal quality indices and a better consumer acceptance.

2.6.2 Response surface methodology

Response surface methodology (RSM) is the most common statistical optimization method used in the food industry (Saguy et al., 1984). It is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems in which the response of interest is influenced by several variables and the objective is to optimize this response. RSM is an effective technique used for processes or formulations with minimal experimental trials when many factors and their interactions may be involved (Malcolmson et al., 1993). These designs provide information on direct effects, pair wise interactions and curvilinear variable effects. In RSM an experimental design is used to fit a model using least squares regression analysis whose adequacy is revealed by diagnostic checking provided by analysis of variance (ANOVA) and residual plots. There are many classes of RSM designs but Center Composite Design (CCD) and D-optimal are the most popular.

2.6.3 Desirability function approach

Several optimization methods can be used to optimize multi-response systems including conventional graphic method, the improved graphic method, the extended response surface procedure (based on a software program to search for optimum solutions) (Fichtali, et al., 1990) and the desirability function approach. The Desirability Function Approach (DFA) is an analytical technique for optimization of multiple responses simultaneously. It was first developed by Harrington (1965) and later modified and extended by Derringer and Suich (1980). DFA calculates individual desirability associated with each response and then an overall desirability can be calculated as the geometric mean of individual desirability. The characteristic of DFA as simple and easy to apply has allowed subjective judgment on the importance of response variables (Guillou and Floros, 1993). DFA method has been employed by major

statistical software such as StatEase and Minitab as the main method to optimize the design to targeted values.

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CHAPTER 3. WHITE COMMON BEANS (*PHASEOLUS VULGARIS*) HAVE HIGHER INVITRO IRON BIOAVAILABILITY THAN COLORED SEED COAT VARIETIES

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Abstract

Iron bioavailability of 16 Ugandan bean varieties was determined and modeled with respect to key influencing factors; phytate, polyphenol, ferritin and iron content. An in vitro digestion/Caco-2 cell culture model was used to determine iron bioavailability and ferritin quantification by western blot densitometry. Polyphenol content ranged from 0.2-1.8 mg/g; phytate, 0.2-1.6%; iron, 57-90 $\mu\text{g/g}$; ferritin, 285-495 $\mu\text{g/g}$, relative bioavailability 5.5-34.3% and were significantly different ($P<0.05$) across bean varieties. Iron bioavailability of white seed coat varieties (34.3%) was significantly higher ($P<0.0001$) than in colored seed coat varieties (5-10%). A fixed effects multiple regression model showed that polyphenol content, iron content and their interaction were significant model terms ($P<0.05$), explaining 68% of the variation in iron bioavailability. Linear effects of polyphenol and iron content decreased iron bioavailability while their interaction increased it. Our data suggest that iron bioavailability in beans is complex but can be indirectly screened for by seed coat color.

Key Words

Caco-2 cell culture, common beans, ferritin, iron bioavailability, phytates, polyphenol

3.1 INTRODUCTION

Iron deficiency is the most prevalent micronutrient deficiency in the world (WHO, 2010) and is the primary cause of anemia. Adverse effects of anemia include increased mortality and morbidity, decreased labor productivity, and impaired neurological/mental development (Stoltzfus, 2001). The main cause of iron deficiency is a dependence on low iron bioavailability staples and particularly affected are populations in developing countries. Strategies to control iron deficiency include supplementation, fortification, biofortification and dietary diversification (Zimmermann and Hurrell, 2007). However, these efforts have all fallen short of making any significant improvements in the iron status of the global population (Micronutrient Initiative, 2004) and new, complimentary strategies need to be devised.

Common beans (*Phaseolus vulgaris*) are an important staple in many parts of South America, Africa and Asia. They are the prime source of calories, proteins and iron; and in spite of high iron content (30-190 ppm) (Graham *et al.*, 1999; CIAT, 2008; Beebe *et al.*, 2000), its bioavailability is very low (<5%) (Lynch *et al.*, 1984; Donangelo *et al.*, 2003; Beiseigel *et al.*, 2007; Petry *et al.*, 2010; Petry *et al.*, 2012). Strategies to increase their contribution to iron nutrition should increase both iron concentration and bioavailability. Indeed, biofortification efforts have been successful in increasing the iron content of beans (Bouis, 2003; CIAT, 2008) but these efforts have not been matched with strategies to increase its bioavailability. To achieve at least 30% Estimated Average Requirements (EAR) of iron from beans for nonpregnant, non

lactating women (1,460 $\mu\text{g/day}$) and children 4-6 yrs (500 $\mu\text{g/day}$) at constant iron bioavailability of 5%, iron content of beans needs to be increased to 107 $\mu\text{g/g}$ (Bouis et al., 2011). Though some studies show an increase in total absorbed iron with increase in bean iron content (Tako et al., 2009; Tako et al., 2011) others have shown no such benefit (Donangelo et al., 2003; Petry et al., 2012).

Iron bioavailability varies widely among bean varieties and is mainly determined by polyphenol and phytic acid content, factors known to inhibit iron absorption (Welch et al., 2000; Hu et al., 2006; Petry et al., 2010; Petry et al., 2012). These act by forming insoluble complexes with dietary iron in the gastro-intestinal tract thus inhibiting its absorption. Polyphenols and phytic acid reduce iron bioavailability in a dose dependent manner but their combined effect is complex (Welch et al., 2000; Hu et al., 2006; Petry et al., 2010) and may be a function of type of polyphenols present (Brune et al., 1989; Hurrell et al., 1999). However, the general consensus is that breeding for low polyphenol/low phytates varieties (Mendoza, 2002; Campion et al., 2009); or their elimination through appropriate processing (Hurrell et al., 1992; Petry et al., 2010) significantly improves iron bioavailability. Factors that enhance iron bioavailability include ascorbic acid, animal tissue and certain amino acids (Carpenter and Mahoney, 1992).

Ferritin, on the other hand, is an iron storage protein whose associated iron has been shown to be highly bioavailable (Davila-Hicks et al., 2004; Lonnerdal et al., 2006) and may be absorbed intact by endocytosis or micro pinocytosis (San Martin et al., 2008). However, ferritin is susceptible to gastric digestion at physiological pH (pH 2) releasing the associated iron to interact with phytates and polyphenols (Hoppler et al., 2008). Regardless, there is a lot of

interest in ferritin content of beans and how it affects iron bioavailability (Lukac et al., 2009; Aluru et al., 2011). Therefore, it is important to understand iron bioavailability in a complex food system containing both inhibitors and potential modifiers.

This study, therefore, aimed at screening 16 Ugandan bean varieties for iron bioavailability and modeling it with respect to key influencing factors; iron, phytates, polyphenol and ferritin. The *in vitro* digestion/Caco-2 cell culture model was used as a cost effective technique to measure iron bioavailability (Glahn et al., 1998; Proulx and Reddy, 2006). It was hypothesized that modeling iron bioavailability in beans will establish relative contribution of each factor and provide guidelines for breeding programs.

3.2 MATERIALS AND METHODS

3.2.1. Materials

Table 3.1 shows the bean varieties used in this study and their seed coat color. Sixteen (16) bean varieties were obtained from National Crops Resources Research Institute (NaCRRI) in Kampala, Uganda. The seeds were washed in distilled water, dried and ground using a cyclone sample mill (UD Corporation, Boulder, CA) to pass through a 0.5 mm diameter particle size screen. Flours were then stored at 4°C until analysis. Flours for iron bioavailability studies were autoclaved (121 °C for 15 min) with sufficient deionized water. After cooling to room temperature, the samples were lyophilized, ground, and stored in airtight containers at 4 °C. Recombinant pea ferritin (rFerr) was kindly donated from the Institute of Food Science and Technology, Laboratory of Human Nutrition (Zurich, Switzerland). Primary anti-soy ferritin polyclonal anti body was kindly donated by Dr. Paul Scott, Department of Agronomy, Iowa state

University. All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO) and Fisher Scientific Co. (Fairlawn, NJ) unless otherwise stated.

3.2.2. Analytical methods

3.2.2.1. Moisture content

The moisture content was determined by AOAC method 925.09 using a convectional drying chamber (AOAC, 1999). Two grams of bean flour were weighed on to an aluminum moisture dish and dried at 125 °C for 3 h, cooled for 5 min in a desiccator and a final dry weight measurement made.

3.2.2.2. Phytic acid

Phytic acid content was determined according to procedures developed by Harland and Oberleas (1986). Phytic acid is isolated by anion exchange chromatography followed by acid digestion to release inorganic phosphorous, which is quantified by colorimetric assay. Briefly, phytic acid was extracted from flour samples with 2.4% HCl (ratio of flour: acid of 1:20 w/v) for 4 h at room temperature on an orbital shaker, centrifuged at 4000 rpm for 10 min and supernatant filtered through a No.1 Whatman filter paper. The supernatant was mixed with Sodium EDTA-NaOH, added to Anion exchange resin (AG1-X4, 100-200 mesh, Chloride form; Bio-Rad Laboratories, Richmond, CA) and eluted sequentially with distilled water, 0.1 M NaCl and 0.7 M NaCl. The eluent was digested with H₂SO₄ and HNO₃ on a Micro Kjeldahl digestion block and released phosphorous colorimetrically assayed with ammonium molybdate and sulfonic acid reagents. AACC Red Wheat bran was used as a control.

3.2.2.3. Polyphenols

Polyphenol content was measured colorimetrically as catechin equivalents by Folin-Ciocalteu reagent after methanol/water/acetic acid (75:30:5) extraction according to procedures by Zielinski and Kozłowska (2000) with minor modifications as below. One gram of sample was extracted with 20 mL Methanol/Water/Acetic acid solution for 2 h at 4°C on a mechanical shaker. The samples were then centrifuged at 10,000xg for 10 min at 4°C and the supernatant filtered through #1 Whitman filter paper. A 0.25 mL aliquot was then mixed with 0.25 mL of 1N Folin-Ciocalteu reagent and 2 mL distilled water. After 3 min at room temperature (but less than 8 minutes), 0.25 mL of a saturated sodium carbonate solution was added and the mixture placed at 37 °C in a water bath for 30 min. The absorbance was measured at 750 nm using a UV/vis spectrophotometer (Spectronic^R 21D, Spectronic Instruments). (+)-Catechin was used as the reference standard and the results were expressed as mg of catechin equivalents/g sample.

3.2.2.4. Iron bioavailability

Iron bio-availability was determined according to methods proposed by Proulx and Reddy (2006) using in vitro digestion/Caco-2 cell culture model. Ferritin synthesis by the Caco-2 cells was used as a measure of iron absorption. The procedures are briefly described below.

In vitro digestion of bean flours: Autoclaved and freeze dried bean flour was sequentially digested with pepsin (at pH 2) and pancreatin (at pH 6) to simulate gastric and duodenal digestion and enzyme activity stopped by heat treatment for 4 min in boiling water. A ferrous ascorbic acid (1:20 molar ratio) solution was included as a positive control. In a separate set of samples, 0.1mM ascorbic acid was added to increase ferritin response.

Iron Bioavailability in Caco-2 Cells: All reagents for cell culture work were from Sigma Aldrich (St Louis, MO) or Gibco BRL (Grand Island, NY) unless otherwise mentioned. Human Caco-2 cells were obtained at passage 19 from American Type Culture Collection (Rockville, MD) and experiments conducted at passages 39-43. The cells were seeded at a density of 2.85×10^5 cells/well in a 12 well collagen treated cell culture plates and maintained at 37° C and 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 1% v/v nonessential amino acids and 1% v/v antibiotic-antimycotic solution for 15 days. On day 15, DMEM was removed and serum free media (Glahn et al, 1998) together with supernatant of bean digest were incubated for 24 h and cells were harvested by sonication. Total cellular protein was determined in the lysates by the Bradford Coomassie Assay and cellular ferritin content determined by radioimmunoassay (Fer-Iron II, Ramco Laboratories, Stafford, TX). Relative Biological Availability (RBA) was determined as ferritin per gram of cell protein content and expressed as percentage of ferrous ascorbic acid control.

3.2.2.5. Ferritin content

The ferritin content was determined by western blot procedures using a polyclonal primary anti soybean ferritin anti-body followed by densitometry for ferritin quantification.

Protein and Ferritin Extraction Design: To optimize extraction of ferritin, NABE 6, a small white Ugandan bean variety, was used and two variables; bean matrix (whole bean and flour) and four pH buffers (pH 4.5, 6.8, 7.2 and 8) varied.

Protein extraction: Crude protein extracts were prepared from bean seeds and flours using methods described by Lukac et al. (2009) with some minor modifications as follows. Protein

was extracted from previously soaked bean seed with sodium phosphate buffer at pH according to experimental design. Likewise, the flours were soaked in extraction buffer for 4 h with shaking on orbital shaker at room temperature. The homogenate was centrifuged and supernatant termed initial extract. A semi purification procedure was followed by including sequential precipitation with 25% MgCl_2 and 50% sodium citrate and re-suspension of resultant pellet with 10 mM sodium phosphate buffer, at pH 7.2. This protein fraction was designated the final extract.

Protein analysis: The nitrogen content of bean flour was determined using the Dumas method (AOAC procedure 990.03) with a Rapid NIII Analyzer (Elementar Americas, Inc., Mt. Laurel, NJ) and a nitrogen-protein conversion factor of 6.25 used. The protein concentration of extracts was determined using the Lowry method modified to the micro plate (Fryer et al., 1986).

Gel electrophoresis: The protein content of all extracts was normalized to 3 mg/mL for western blotting and 1.5 mg/mL for SDS PAGE, to ensure a protein load of 60 and 30 μg protein per well, respectively. Where necessary, the proteins were concentrated by ultra-filtration using Amicon Ultra-0.5 mL Centrifugal Filters, with Nominal Molecular Weight Limit (NMWL) of 100 kDa (Millipore Corporation, Billerica, MA). Electrophoresis followed procedures developed by Laemmli (1979) using 4% mercaptoethanol as reducing agent and 1.5 mm thick, 15% sodium dodecyl sulfate (SDS) gels in MiniProtean II Electrophoresis Cell and Transfer Apparatus (Bio-Rad, Hercules, CA). The gels (1.5 mm) were either stained with Coomassie blue R-250 in fixative and destained with 40% methanol/10% acetate to detect separated protein bands or used for western blot.

Western blot: Proteins from the SDS gel were transferred to Immobilon-P^{SQ} (a 0.2 µm micro porous polyvinylidene fluoride (PVDF) transfer membrane (Millipore Corporation, Billerica, MA) according to the methods described by Towbin et al. (1979). Following transfer, the proteins were probed with anti-soy ferritin polyclonal antibody (1:500) and horseradish alkaline phosphatase (AP) -conjugated goat anti-rabbit secondary antibody (1:5,000) and color developed with standard chromogenic detection protocols using AP conjugate substrate kit (Bio-Rad Laboratories, Hercules, CA).

Densitometry: Blots and SDS PAGE gels, after color development and fixation respectively, were allowed to dry at room temperature (for blots) and a digital image captured using GS-800TM Calibrated Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA). The absorbance of bands at was measured using Image J free software (<http://rsbweb.nih.gov/ij/>). For western blot densitometry, observed absorbance values were converted to ng ferritin per gram of bean seed using known amount of rFerr as a standard.

Non- heme iron: Non Heme iron content in bean flours was measured colorimetrically after trichloacetic acid (TCA) digestion, using methods proposed by Swain et al., (2002). Flour (0.25 g) was mixed with 0.5 mL 10% TCA/3 N HCl in a 15 mL centrifuge tube, capped and incubated for 20 h at 65 °C. The sample was cooled and centrifuged at 3,000 rpm for 15 min. The supernatant was mixed with ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt] as a chromogen in a micro plate, incubated for 10 min and absorbance of color product measured at 563 nm using KC Junior software (version 1.14) and Micro Plate Reader

(ELx808; Bio-Tek Instruments, Inc., Winooski, VT). Iron concentration was measured against an iron standard curve (made from atomic absorption standard solution, 1mg/mL). All the water used was 18M Ω dd water and all glassware had been soaked overnight in 1N HCl and rinsed three times in 18M Ω water.

3.3. Statistical analysis

The results were expressed as means (\pm SD) and Analysis of Variance (ANOVA) was done to determine the significant differences among means followed by Tukey-Kramer multiple comparison test when the F-test demonstrated significance of differences among means. To assess the interactive effect of bean matrix and extraction buffer pH, two-way ANOVA was used. Multiple linear regression with interactions was used to model relationships between bean composition and iron bioavailability. Data from each assay (n=3-6) were averaged prior to statistical modeling. The differences were considered statistically significant at $P \leq 0.05$. Analyses were performed using SAS software, version 9.2 (SAS Institute Inc. Cary, NC).

3.4. RESULTS AND DISCUSSION

3.4.1. Modeling iron bioavailability

Table 3.1 shows iron, polyphenol, ferritin and phytic acid content of beans as well as the Relative Biological Availability (RBA) of bean iron. There were significant differences ($P < 0.05$) in the bean composition and iron bioavailability. Polyphenol content of raw beans (reported as catechin equivalents) ranged from 0.2 to 1.76 mg/g and was lowest in the white seed coat varieties. The Folin's reagent assay used in this study quantified total polyphenol content while

the acidified methanol used for extraction has been shown to maximize yield of total polyphenols from foods (Ju et al., 2003; Ranilla et al., 2009). Total phytic acid content ranged from 0.19 to 1.6 g/100g while iron content ranged from 57 to 91 µg/g and ferritin content varied from 285 to 495 µg/g. The phytic acid content of beans used in this study compared well with results reported in literature for beans (0.2-2.8%) (Reddy 2001). Iron content was also in the range (30-90 µg/g) reported for market classes (Graham *et al.*, 1999; CIAT, 2008; Beebe *et al.*, 2000) of bean seed and was significantly ($P < 0.001$) different among bean varieties, lowest in NABE2, the small black variety and highest in NABE14, a red variety. The RBA of autoclaved beans ranged from 5.5-34.3% and was significantly different ($P < 0.0001$) among bean varieties. White seed coat varieties had the highest RBA (34.3%) and were significantly different from colored seed coat varieties (5.5-9.8%). Table 3.2 shows significant model terms relating iron bioavailability and bean composition. A fixed effects multiple regression model ($P\text{-value} = 0.0028$, $R^2 = 0.68$) showed negative effects of polyphenol and iron, while their interaction was positive (Table 3.2).

Numerous studies have reported a polyphenol content of beans in the range of 0.19 to 0.48 mg/g seed and shown that bean seed coat color is a function of polyphenol content (Elias et al., 1979; Bressani and Elias, 1980; Hu et al., 2006, Luthria and Pastor-Corales, 2006). Bean seed coat color is determined by the presence and amounts of different types of flavonoids (flavonol glycosides, anthocyanins, and condensed tannins/proanthocyanidins) (Takeoka et al., 1997; Beninger et al., 1998). Though the inhibiting effect of polyphenols on iron absorption has largely been demonstrated, the capability of complex formation with iron depends on their structure. Colored bean seed coats contain polyphenols with ortho-dihydroxy (catechol) or

trihydroxybenzene group (galloyl) such as proanthocyanidins (catechol groups) and hydrolysable tannins (galloyl groups) which are the most potent iron absorption inhibitors (Brune et al., 1989; Hurrell et al., 1999; Hu et al., 2006). On the other hand, white seed coat beans contain phenolic acids, but not anthocyanins or condensed tannins (Choung et al., 2003; Espinosa-Alonso et al., 2006; Hu et al., 2006) which may explain the observed high iron bioavailability. Numerous previous workers have shown significantly higher iron bioavailability of white seed coat varieties as compared to colored ones (Welch et al., 2000; Hu et al., 2006; Ariza-Nieto et al., 2007; Tako and Glahn, 2010).

The inhibitory effect of phytic acid on iron absorption was not significant in this study. This is regardless of the fact that phytic acid has been shown to be a strong inhibitor of iron absorption in a dose dependent manner (Hallberg, 1989; Hurrell et al., 1992; Pertry et al., 2010). In the presence of polyphenols, phytic acid effect may not be significant; but once polyphenols are removed from the same food matrix, the effect of phytic acid becomes significant (Pertry et al., 2010). The effect of ferritin was also not significant in this study. This could be explained by the fact that in vitro digestion was carried out at physiological pH (pH 2) at which ferritin is susceptible to degradation (Hoppler et al., 2008; Deng et al., 2011; Li et al., 2012). The associated iron is released to interact with phytates and polyphenols; just like any other non-heme iron. Also, the fraction of total bean seed iron stored by ferritin may have been low. Hoppler et al. (2008) reported that only 20 and 25% of the total seed iron in white and red kidney beans was bound to ferritin respectively, a result which was confirmed by Cvitanich et al. (2010). The latter authors showed that the largest portion of iron is found in the cytoplasm of cells

surrounding the provascular tissue and cells near the epidermal layer. Further, the range of ferritin used in this study was too narrow (285-495 $\mu\text{g/g}$) and the standard deviation too high, which may explain the non-significant contribution of the factor to the model. A wider composition range and a more reliable assay are required to further study this parameter. The negative effect of iron on iron bioavailability observed in this study shows that increasing iron content in presence of inhibitors may not necessarily increase percentage absorbable iron (Donangelo et al., 2003; Hu et al., 2006; Petry et al., 2012) though total iron absorbed may increase (Tako et al., 2009; Tako et al., 2011). Thus, to optimize iron bioavailability, future biofortification programs ought to breed for low polyphenol and low phytic acid as well as high iron bioavailability.

Effect of ascorbic acid: Ascorbic acid is usually added to Caco-2 cell culture models to amplify iron uptake and its effect is assumed to be uniform across food matrices. This study aimed at establishing validity of this assumption in a bean food matrix. Table 3.3 shows relative bioavailability of beans with or without added 0.1mM ascorbic acid. We added ascorbic acid to digested bean samples to compare the beans with improved ferritin response. Ascorbic acid increased iron bioavailability in colored varieties but not in white seed coat ones. However, the relative bioavailability of white seed coat variety was still significantly higher ($P < 0.0001$) than that of colored ones even in the presence of ascorbic acid. Ascorbic acid is a known enhancer of iron bioavailability (Hallberg, 1989). However, its effect may be dependent on the food matrix. Hu et al., (2006) reported enhancement of iron bioavailability of white seed coat varieties but not the colored seed coat ones in a Caco-2 cell culture model. The iron uptake from colored seed coat varieties was not significantly different from the control blank and 0.1mM ascorbic acid did

not significantly enhance bioavailability, an effect they related to the polyphenol content of colored seed coat varieties. The authors also showed that dehulling significantly reduced polyphenol content and enhanced bioavailability. Thus, the effect of ascorbic acid on iron uptake from beans in Caco-2 cell culture may be dependent polyphenol content, which in turn is genetically controlled. However, it is not clear why ascorbic acid did not enhance iron bioavailability of white seed coat varieties in the current study.

3.4.2. Optimization of ferritin extraction

The methodology used to determine ferritin content in this study is novel (Lukac et al., 2009) and we aimed to optimize it with respect to extraction buffer pH and the form of seed used (whole seed vs. bean flour). We hypothesized that a pH near the pI of phaseolin (the major bean storage proteins with a pI of 4-5) would eliminate this fraction and reduce competition for ferritin (pI of 6.0) binding to anti-body in the western blot assay. Figure 3.1 shows the percentage of total seed protein that was extracted from the seed and flour fractions (designated initial protein extract); and that retained after the semi-purification procedure (final protein extract) of NABE 6. Total protein content of the bean variety used was 23.3%. Of this, the percent protein extracted with buffer (initial protein extract) ranged from 41 ± 2 to $74 \pm 5\%$. Table 3.4 shows two-way analysis of ANOVA showing effect of pH, seed matrix and their interaction on initial and final protein extraction. pH, seed matrix and pH*seed matrix interaction significantly ($P < 0.05$) affected amount of protein extracted, increasing with increase in pH and in flour matrix. The final protein extract after semi-purification ranged from 0.002 ± 0.0006 to $0.009 \pm 0.0012\%$. Extraction buffer pH significantly ($P < 0.05$) increased final protein extracted where as neither seed matrix nor seed matrix*pH interaction were significant.

Figure 3.2 shows SDS-PAGE gels of initial and final extracts. Five bands with molecular weight ranging from 30 to 47kDa (probably globulins, 7S and 11S) dominated in all samples, accounting for over 80% of the total proteins. Phaseolin (7S) is soluble at all pH and its N-glycosylation may contribute to further increased solubility at neutral and weak alkaline pH (Kimura et al., 2008). On the other hand, bean 11S globulins are poorly soluble at a low pH (<pH 5) but are fairly soluble near pH 2 mainly due to high content of acidic amino acids (Kimura et al., 2008). Solubility of albumins (represented by the 27kDa band of Phytohemagglutinins) is independent of pH mainly due to the high content of carbohydrates (Mundi and Aluko, 2012) and yield was constant in the initial extract.

Figure 3.3 shows western blot comparing effect of buffer pH and semi-purification procedure on ferritin yield. The polyclonal anti-soy ferritin primary antibody used in this study gave two bands with bean extracts; a distinct band with a molecular weight of 27-28kDa corresponding to the molecular weight of bean ferritin subunits and double band corresponding to the molecular weight of phaseolin (47-52 kDa). The latter band was ignored in all ferritin quantification procedures as it was considered a cross reaction of the less specific polyclonal antibody. A similar banding pattern was seen in higher concentrations of the rFer standard. Yield of ferritin increased with increase in pH (Figure 3.3) and was maximum at pH 8. The ferritin content of the initial, discarded supernatant and final extract of the flour matrix extracted at pH 8 was 230, 134 and 28µg/g of seed respectively, showing significant loss of ferritin in the discarded supernatant

fraction. Therefore, all further ferritin quantification procedures made use of the initial extract from bean flour, eliminating the need for semi-purification procedure.

Our results for percentage protein and ferritin extracted at pH 7.2 were similar to those obtained by Lukac et al. (2009) from a red bean whole seed with a similar protein content and extraction buffer concentration. However, pH 8 and flour matrix were found to be the optimal protein extraction conditions in the bean variety studied. Alkali pH is more efficient in solubilizing protein from beans because phaseolin (pI of 4-5) and ferritin (pI of 6.0) have pI in the acidic range (Danielsson, 1949; Sathe and Salunkhe, 1981a,b; Kimura et al., 2008). Of the parameters known to affect protein solubility, pH, temperature, ionic strength and type of salt were shown to be the most important (Sathe et al., 1984). These authors recommended use of low concentration alkalis at pH >7.5 for better protein solubilization efficiency. Also, the compact structure of globulins is thought to influence solubility. Hydrophilic groups on these proteins may be buried in the interior of the molecule, thus not exposed under neutral pH environment, but may be exposed and ionized with increasing pH (Sathe et al., 1984).

Ferritin content from the semi purified fraction was similar to results obtained by Lukac et al. (2009) and that from the initial extract matched results obtained by Deng et al. (2011) for beans and Laulhere et al. (1988) for soybean. The polyclonal antibody used by Lukac et al. (2009) was developed against a highly conserved ferritin sequence from maize ferritin and could have been less immunogenic than the one used in this study (developed against soy ferritin) explaining the failure of the former to detect ferritin in the initial extract. The anti-ferritin primary antibody

used in this study was able to detect ferritin from the crude extract and we believe gave better quantification. This study showed that extracting protein at a pH near the pI of phaseolin (pI 4.5) eliminated this particular fraction from the extract but also limited extraction of ferritin. However, both total protein and ferritin yield increased with increase in buffer pH and were higher in flour than seed matrix.

3.5 Conclusions

The study showed that in the complex bean matrix containing inhibitors (polyphenols and phytic acid) and possible promoters (iron and ferritin), contribution of individual factors is complex and difficult to model. In this study, only iron and polyphenol content were significant model terms and iron bioavailability could be indirectly screened by seed coat color; with white seed coats showing higher bioavailability than colored ones. Polyphenol, a known inhibitor of iron absorption significantly decreased iron bioavailability while interaction with iron increased it. Effects of ferritin and phytic acid were not significant in the bean varieties studied. Contribution of ferritin to the model could have been hindered by the narrow composition range and high residual error associated with western blot optical densitometry.

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Table 3.1: Iron, phytate, ferritin and total polyphenol content of 16 Ugandan bean varieties^a

Variety	Color	Total polyphenol (mg/g)	Iron (µg/g)	Phytate (%)	Ferritin (µg/g) ^b
NABE 1	Red mottled	1.25 ± 0.07 ^{bc}	78.9 ± 9.2 ^b	1.12 ± 0.16 ^{cde}	357 ± 34 ^{bcdef}
NABE 2	Black	0.79 ± 0.05 ^d	62.6 ± 0.8 ^{efg}	0.50 ± 0.01 ^{def}	285 ± 36 ^f
NABE 3	Red	1.61 ± 0.02 ^{ab}	75.3 ± 1.3 ^{bc}	0.46 ± 0.03 ^{def}	444 ± 56 ^{abcd}
NABE 4	Red mottled	1.39 ± 0.26 ^b	64.5 ± 1.5 ^e	0.55 ± 0.13 ^{def}	479 ± 113 ^{ab}
NABE 5	Cream/Red	1.66 ± 0.02 ^a	70.5 ± 2.0 ^{cd}	0.28 ± 0.01 ^{ef}	384 ± 35 ^{abcde}
NABE 6	White	0.20 ± 0.02 ^e	58.9 ± 0.1 ^{fg}	0.80 ± 0.02 ^{cd}	353 ± 102 ^{bcdef}
NABE 7	Red	1.42 ± 0.07 ^b	57.4 ± 0.6 ^g	0.30 ± 0.03 ^{ef}	293 ± 47 ^{ef}
NABE 8	Red	1.26 ± 0.07 ^{bc}	79.9 ± 1.2 ^b	0.60 ± 0.05 ^{cdef}	390 ± 113 ^{abcdef}
NABE 9	White/Black	1.06 ± 0.01 ^c	67.0 ± 1.8 ^{de}	0.25 ± 0.03 ^{ef}	344 ± 9 ^{cde}
NABE 10	Red	1.60 ± 0.11 ^{ab}	64.7 ± 1.8 ^e	0.60 ± 0.37 ^{cdef}	293 ± 31 ^{ef}
NABE 11	Cream/Red	1.25 ± 0.04 ^{bc}	65.4 ± 2.2 ^{de}	0.19 ± 0.06 ^f	325 ± 29 ^{def}
NABE 12	Cream/Red	0.99 ± 0.10 ^c	87.3 ± 4.2 ^a	0.66 ± 0.01 ^{cde}	313 ± 79 ^{ef}
NABE 13	Red	1.10 ± 0.03 ^c	63.5 ± 3.4 ^{ef}	1.61 ± 0.07 ^a	419 ± 29 ^{abcde}
NABE 14	Red	1.76 ± 0.29 ^a	90.6 ± 3.1 ^a	1.01 ± 0.12 ^{bc}	495 ± 96 ^a
K131	Brown	1.76 ± 0.15 ^a	67.8 ± 2.7 ^{de}	1.01 ± 0.20 ^{bc}	304 ± 95 ^{ef}
K132	Red mottled	1.06 ± 0.08 ^c	75.0 ± 2.7 ^{bc}	1.31 ± 0.12 ^{ab}	470 ± 120 ^{bc}

^aValues are means±SD; n=4-6; ^b Ferritin extracted from bean flour. Within each column, mean values with a letter in common are not significantly different from one another (P<0.05).

Table 3.2: Significant model terms relating iron bioavailability and bean composition^a

Independent variable	Parameter estimate	p-value	R ²
Model	95.6	0.0028	0.68
Polyphenol	-64.7	0.0008	
Iron	-1.1	0.0166	
Polyphenol*iron	0.8	0.0133	

^aFixed effects multiple linear regression modeling was used

Table 3.3: Relative biological availability of iron from 16 Ugandan bean varieties with or without added ascorbic acid^a

Variety	RBA (%)	
	No added ascorbic acid	With 0.1mM ascorbic acid
NABE 1	7.6 ± 2.7 ^b	13.2±2.6 ^b
NABE 2	5.5 ± 1.0 ^b	13.7±2.8 ^b
NABE 3	6.2 ± 0.8 ^b	13.8±2.0 ^b
NABE 4	7.6 ± 1.6 ^b	13.9±3.2 ^b
NABE 5	8.4 ± 1.7 ^b	10.1±0.8 ^b
NABE 6	34.3 ± 10 ^a	38.5±3.2 ^a
NABE 7	8.1 ± 1.8 ^b	11.3±1.2 ^b
NABE 8	9.1 ± 0.8 ^b	13.5±4.0 ^b
NABE 9	5.5 ± 0.9 ^b	13.1±5.8 ^b
NABE 10	6.4 ± 1.9 ^b	15.0±7.4 ^b
NABE 11	8.7 ± 0.9 ^b	15.8±5.0 ^b
NABE 12	8.4 ± 1.0 ^b	16.2±3.7 ^b
NABE 13	9.8 ± 1.0 ^b	18.4±4.4 ^b
NABE 14	8.8 ± 0.7 ^b	14.6±1.6 ^b
K131	6.2 ± 0.3 ^b	10.2±0.8 ^b
K132	6.1 ± 2.4 ^b	11.7±2.9 ^b

^aValues are means ± SD; n=6; Within each column, mean values with a letter in common are not significantly different from one another (P<0.05).

Table 3.4: Two way analysis of variance (ANOVA) showing P-values relating independent variables (pH and seed matrix) to percentage initial and final protein content extracted

Source of variation	P value	
	Initial	Final extract
pH	<.0001	0.7502
Matrix	0.0046	<.0001
pH*Matrix	0.0291	0.2036

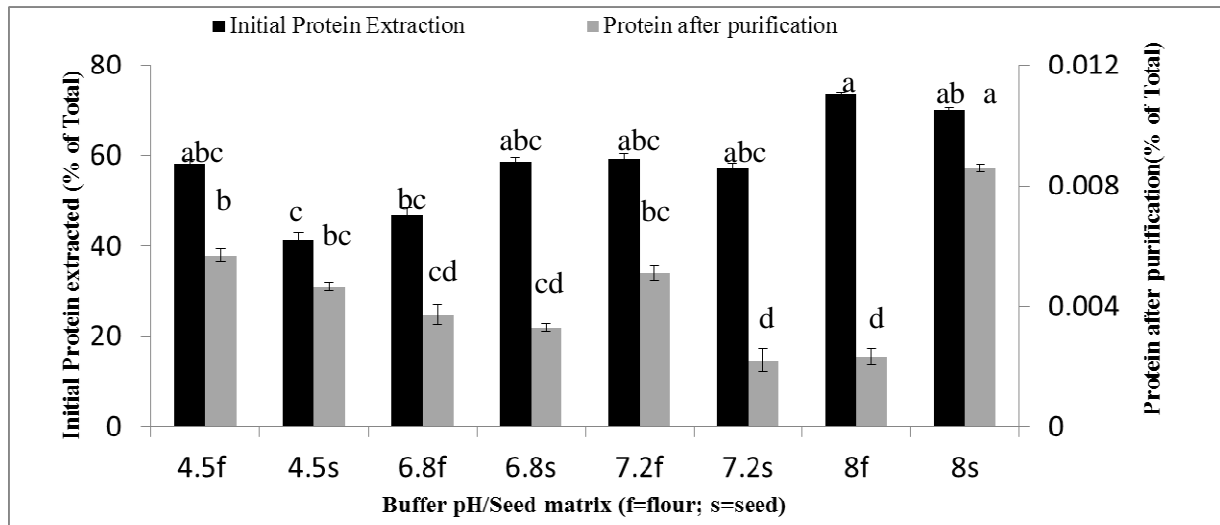


Figure 3.1: Percentage of total seed protein retained at different levels of purification (initial protein extraction and after the semi purification process). Mean \pm SD, n=3. Bars with no common letter were significantly different at $P < 0.05$ within each treatment (initial protein extraction and protein after purification).

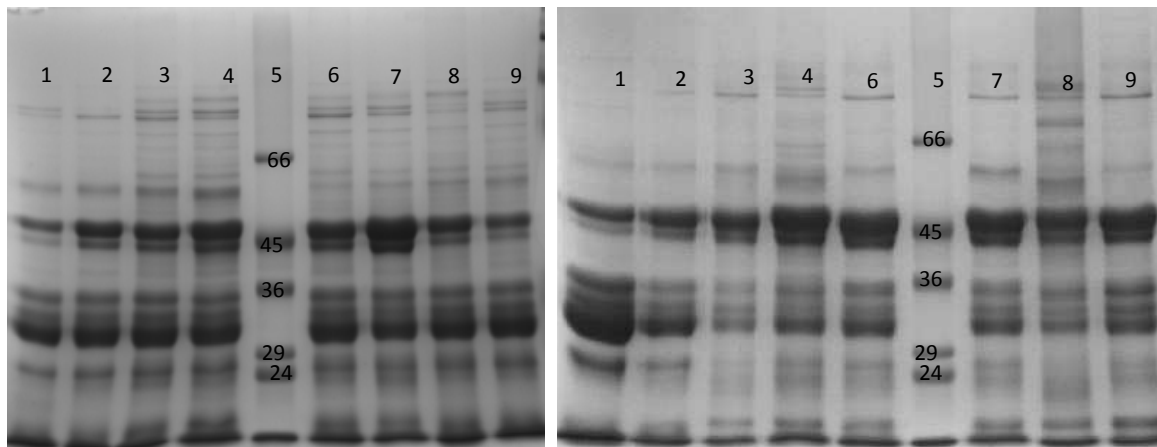


Figure 3.2: SDS-PAGE gels of initial (left) and final (right) protein extract (30 μ g protein/lane) under reducing conditions.

Key: **Lane 1:** pH 4.5 seed; **Lane 2:** pH 4.5 flour; **Lane 3:** pH 7.2 seed; **Lane 4:** pH 7.2 flour; **Lane 5:** MW Marker, **Lane 6:** pH 8 seed, **Lane 7:** pH 8 flour; **Lane 8:** pH 6.8 seed; **Lane 9:** pH 6.8 Flour

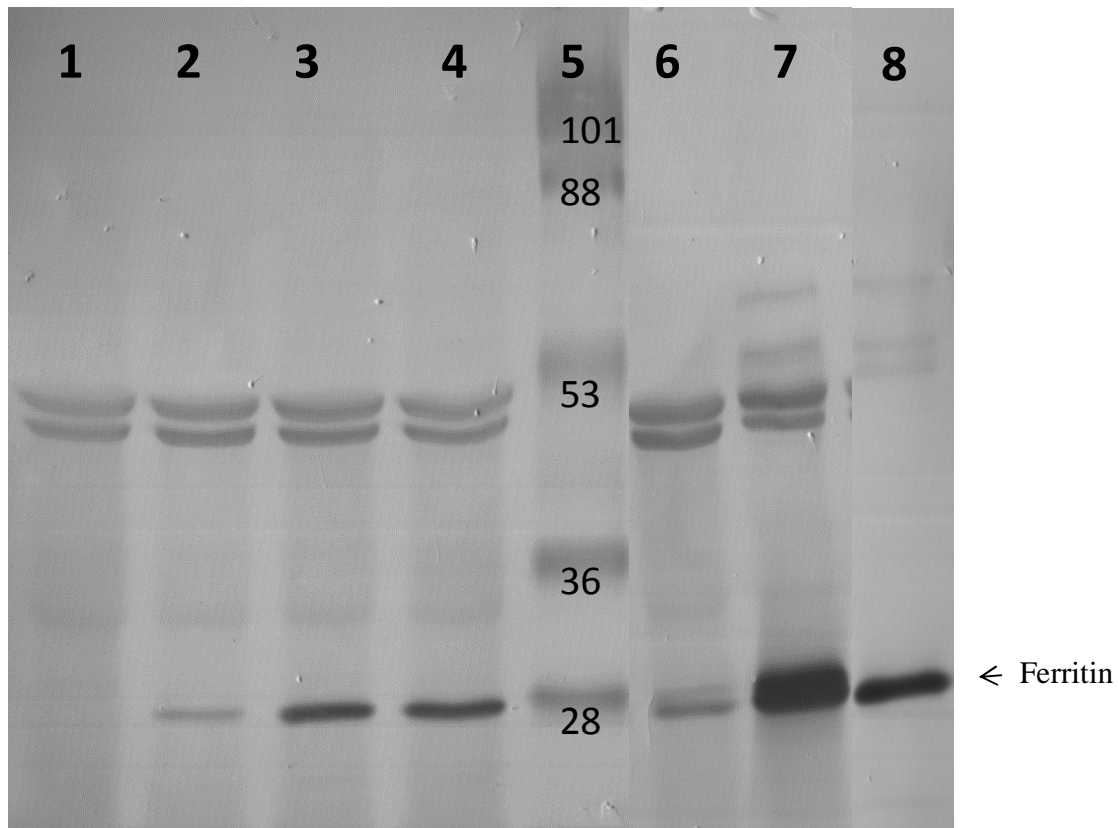


Figure 3.3: Western blot showing ferritin (27kDa band) content of initial and final extract as well as loss in discarded supernatant (protein load, 60 μ g/lane for samples).

Key: Lane 1: Initial extract pH 4.5; Lane 2: Initial extract pH 6.8; Lane 3: Initial extract pH 7.2; Lane 4: Initial extract pH 8; Lane 5: MW Marker; Lane 6: Ferritin lost to semi-purification (pH 8); Lane 7: Final extract pH 8; Lane 8: Standard rFer (320ng/lane)

CHAPTER 4. OPTIMIZATION OF WHITE COMMON BEANS (*PHASEOLUS VULGARIS*) EXTRUSION COOKING PROCESS

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Abstract

Extrusion cooking process variables; raw material moisture content (15-35 % wb), extruder die temperature (120-175 °C) and feed flow rate (1.8-3 kg/h) were optimized with respect to iron bioavailability, final viscosity and overall consumer acceptability of a small white bean variety. An in vitro digestion/Caco-2 cell culture model was used to determine iron bioavailability as Relative Biological Availability (RBA) and Response Surface Methodology (RSM) techniques used for process optimization. RBA of extruded beans ranged from 54 to 389%, a 1.5-10 fold increase on that of autoclaved beans (34.3%). However, there was an increase in iron content of extrudates indicating possible contamination from extruder parts; partly explaining the increase in bioavailability. The analysis of variance for the thirteen responses studied indicated that predictive models were significant for seven of the responses with the three variables explaining 32 - 78% of the variability. The coefficients of each response showed extruder die temperature had a significant effect on each significant response, decreasing all sensory properties, expansion ratio and pasting viscosities while increasing iron bioavailability. Linear effects of moisture content and flow rate reduced pasting viscosity but increased iron bioavailability while their

interaction had opposite effects. The optimal combination of extrusion variables was 15% moisture content, 120 °C die temperature and 3 kg/h feed flow rate. Model validation experiments' results revealed that expansion ratio, peak and final viscosity could be reliably predicted but RBA was below the lower limit of prediction.

Key words

Common beans, extrusion cooking, iron bioavailability, optimization, RSM

4.1 INTRODUCTION

Common beans (*Phaseolus vulgaris*) are an important staple in many parts of South America, Africa and Asia where they are a prime source of iron. In spite of the high iron content (30-190 ppm) (CIAT, 2008), its bioavailability is very low (<5%) due to high polyphenols and phytic acid content (Donangelo et al., 2003; Petry et al., 2012). These act by forming insoluble complexes with dietary iron in the gastro-intestinal tract thus inhibiting its absorption. The dependence on low iron bioavailability crop staples is the major nutritional cause of iron deficiency, the most prevalent micronutrient deficiency in the world (WHO, 2010). Iron deficiency is the primary cause of anemia, whose effects include increased mortality and morbidity, decreased labor productivity, and impaired neurological/mental development (Stoltzfus, 2001).

House hold and commercial processing technologies (soaking, germination/malting, heat treatment) have been shown to reduce the concentration of anti-nutritional factors and increase iron bioavailability (Alonso et al., 2001). Extrusion cooking has been shown to increase iron bioavailability through hydrolysis of polyphenols and phytates (Alonso et al., 2001; Hurrel et al., 2002). Extrusion cooking is a high temperature-short time processing technology that effects

product cooking by a combination of moisture, pressure, temperature and mechanical shear. In addition to enhancing iron bioavailability, extrusion cooking reduces pasting viscosity and anti-nutrition factor content, and improves consumer acceptability (Edwards et al., 1994; Nyombaire et al., 2011). Reduced pasting viscosity is important for sauces and porridges as it maximizes flour rate and increases nutrient density.

Literature on effect of extrusion cooking on nutritional, physicochemical and sensory properties of beans is limited and there is thus need to optimize system parameters in order to maximize product characteristics. System parameters (e.g. mechanical and thermal energy inputs, and the residence time distribution) unlike operating conditions, are valid for different machines and are preferred in optimization studies (Meuser et al., 1992). However, in this study, two processing conditions (raw material moisture content and feed flow rate) and one system parameter (extruder die temperature) were varied. The mechanical energy input (defined as Specific Mechanical Energy-SME) was not determined since the machine did not have provision for measuring %Torque; and feed flow rate was preferred to screw speed as a proxy to SME because, from trial runs, it gave a wider operating range.

This study therefore aimed at optimizing extrusion cooking process variables with respect to iron bioavailability, pasting viscosity and consumer acceptability of small white bean flour.

Response surface methodology (RSM) was used as a tool for optimizing factor combinations to achieve specific pre-set product outcomes. We hypothesized that optimizing a combination of extrusion cooking parameters will significantly improve iron bioavailability, pasting viscosity and consumer acceptability of beans through elimination of anti-nutritional factors and enhancement of desirable flavor.

4.2 MATERIALS AND METHODS

4.2.1. Materials

NABE6, a small white seed coat bean variety from Uganda, was used. Seed from National Crops Resources Research Institute (NaCRRI), Kampala, Uganda was multiplied at the Iowa State University Horticulture Farm in Gilbert, IA in the summer of 2011. Clean seed was rough milled to pass through US standard 1531 0125 sieves using a Fitzpatrick Impact (comminuting) mill (Fitzpatrick, Elmhurst, IL) at 4500rpm. Flours were stored at 4°C until processing. All chemicals and reagents were purchased from Aldrich Chemical Co Inc. (Milwaukee, WI) and Sigma Chemical Co (St. Louis, MO), unless specified and the water used for processing and analysis was 18Ω deionized distilled water.

4.2.2. Extrusion design

Extrusion of the bean flour was performed in a Brabender twin screw co rotating extruder (Model CTSE-Y, Brabender Instruments Inc., S. Hackensack, NJ) with a screw length to diameter ratio of 13:1 and four heating regions. Table 4.1 shows the three independent variables that were selected for optimization; raw material moisture content, die temperature and feed flow rate and their ranges. The temperature in each of the four heating regions was increased by 20 °C from the preceding region along the barrel towards the die end. In this study, the temperature of the extruder barrel at the die end was varied according to the study design and that of each of the preceding 3 regions reduced by 20°C respectively. The ranges of the independent variables were established experimentally by running different combinations of the variables to determine the possible working range applicable to the material and the extruder. The extruder was operated at 50 rpm and an attached feeder (Screw speed hopper, Type 15-31-00, Brabender

Instruments Inc., S. Hackensack, NJ) was set to deliver 1.8-3 kg/hr of raw material according to the research design. Moisture content of the raw material varied between 15-27.5% wb according to the design. The extrudates were allowed to cool to room temperature, dried at 50°C overnight in conventional oven and milled in a comminuting Fitzpatrick Impact mill (Fitzpatrick, Elmhurst, IL) at a speed of 4500 rpm to pass through a 0.5 mm sieve.

Table 4.2 shows the D-Optimal experimental design generated by Design Expert Statistical Software (Design expert 7.1.6, Stat-Ease Inc., Minneapolis, MN; 2007), consisting of 10 model points, 4 points to estimate lack of fit, 4 replicates and 2 additional center points.

4.2.3. Analytical methods

4.2.3.1. Moisture content

The moisture content was determined by AOAC method 925.09 using a convectional drying chamber (AOAC, 1999). Two grams of bean flour were weighed on to an aluminum moisture dish and dried at 125 °C for 3 h, cooled for 5 min in a desiccator and a final dry weight measurement made.

4.2.3.2. Polyphenols

Polyphenol content was measured colorimetrically as catechin equivalents by Folin-Ciocalteu reagent after methanol/water/acetic acid (75:30:5) extraction according to procedures by Zielinski and Kozłowska (2000) with minor modifications as below. One gram of sample was

extracted with 20 mL Methanol/Water/Acetic acid solution for 2 h at 4°C on a mechanical shaker. The samples were then centrifuged at 10,000xg for 10 min at 4°C and the supernatant filtered through #1 Whitman filter paper. A 0.25 mL aliquot was then mixed with 0.25 mL of 1N Folin-Ciocalteu reagent and 2 mL distilled water. After 3 min at room temperature (but less than 8 minutes), 0.25 mL of a saturated sodium carbonate solution was added and the mixture placed at 37 °C in a water bath for 30 min. The absorbance was measured at 750 nm using a UV/vis spectrophotometer (Spectronic^R 21D, Spectronic Instruments). (+)-Catechin was used as the reference standard and the results were expressed as mg of catechin equivalents/g sample.

4.2.3.3. Iron bioavailability

Iron bio-availability was determined according to methods proposed by Proulx and Reddy (2006) using in vitro digestion/Caco-2 cell culture model. Ferritin synthesis by the Caco-2 cells was used as a measure of iron absorption. The procedures are briefly described below.

In vitro digestion of bean flours: Extruded bean flour was sequentially digested with pepsin (at pH 2) and pancreatin (at pH 6) to simulate gastric and duodenal digestion and enzyme activity stopped by heat treatment for 4 min in boiling water. A ferrous ascorbic acid (1:20 molar ratio) solution was included as a positive control.

In a separate set of samples, 0.1mM ascorbic acid was added to increase ferritin response.

Iron bioavailability in Caco-2 cells: All reagents for cell culture work were from Sigma Aldrich (St Louis, MO) or Gibco BRL (Grand Island, NY) unless otherwise mentioned. Human

Caco-2 cells were obtained at passage 19 from American Type Culture Collection (Rockville, MD) and experiments conducted at passages 39-43. The cells were seeded at a density of 2.85×10^5 cells/well in a 12 well collagen treated cell culture plates and maintained at 37° C and 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 1% v/v nonessential amino acids and 1% v/v antibiotic-antimycotic solution for 15 days. On day 15, serum free media (Glahn et al, 1998) and supernatant of bean digest were incubation for 24 h and cells harvested by sonication. Total cellular protein was determined in the lysates by the Bradford Coomassie Assay and cellular ferritin content determined by radioimmunoassay (Fer- Iron II, Ramco Laboratories, Stafford, TX). Relative Biological Availability (RBA) was determined as ferritin/g protein of samples expressed as percentage of ferrous ascorbic acid control.

4.2.3.4. Non-heme iron

Non Heme iron content in bean flours was measured colorimetrically after trichloacetic acid (TCA) digestion, using methods proposed by Swain et al., (2002). Flour (0.25 g) was mixed with 0.5 mL 20% TCA/6 N HCl in a 15 mL centrifuge tube, capped and incubated for 20 h at 65 °C. The sample was cooled and centrifuged at 3,000 rpm for 15 min. The supernatant was mixed with ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt] as a chromogen in a micro plate, incubated for 10 min and absorbance of color product measured at 563 nm using KC Junior software (version 1.14) and Micro plate Reader (ELx808; Bio-Tek Instruments, Inc., Winooski, VT). Iron concentration was measured against an iron standard curve (made from atomic absorption standard solution, 1mg/mL). All the water used

was 18M Ω dd water and all glassware had been soaked overnight in 1N HCl and rinsed three times in 18M Ω water.

4.2.3.5. Pasting properties of raw and extruded samples

Extruded and raw bean flour pasting properties were analyzed using an RVA (RVA-4, Newport Scientific, Sydney, Australia) according to the manufacturer's methodology. Briefly, an 11% (w/w) flour suspension was prepared by weighing 3.23 g (dry basis) bean flour into an RVA canister and making up the total weight to 30 g with distilled water. The sample suspension was equilibrated at 30 °C for 1 min, heated at a rate of 6.0 °C/m to 95°C, maintained at that temperature for 5.5 min, and then cooled to 50°C at a rate of 6.0 °C/min. A constant rotating speed of the paddle (160 rpm) was used throughout the analysis.

4.2.3.6. Expansion ratio

After drying extrudates, the average diameter of 10 randomly selected extrudates were measured for each treatment using a Vanier caliper. The ratio of the mean diameter of extrudates to the diameter at die end of extruder barrel was calculated as the expansion ratio.

4.2.3.7. Sensory evaluation of extruded bean flour porridges

An untrained consumer panel comprising of 60 students and staff members, aged 18-65 years, from Makerere University, School of Food Technology, Nutrition and Bio-engineering tasted bean flour porridges over a two day period. Two sessions were conducted per day (9am-1pm and 2-5pm) and each panelist attended one session per day. Panel members were chosen basing on their availability and willingness to participate and were required to sign a consent form (refer to Appendix I) consistent with the Institutional Review Board of Iowa State University and Uganda National Council of Science and Technology. Demographic information on sex, age,

porridge consumption patterns was provided by the panelists (See Appendix II). A 150 mm unstructured line scale anchored at dislike extremely and like extremely was used for evaluating the products (Meilgaard et al., 1991; Moskowitz, 1994; See Appendix III). Porridges were prepared by thoroughly mixing 5 table spoonfuls of extruded flour with half a cup of cold water until all lumps were broken and the slurry smooth. One liter of hot water was added and stirred in to make a smooth paste which was then simmered over an open flame for 10 minutes. A little hot water was added to the simmering gruel until a consistency typical of traditional Ugandan hot maize porridge was obtained. The products were stored in vacuum flasks until served to panelists. An aliquot of 10-20 mL of porridge were served in transparent disposable plastic cups, which were in turn placed on serving trays along with a bottle of mineral water, an empty spit cup and four crackers to act as palate cleansers in between samples. Each panelist received sequentially seven coded samples in a randomized order according to a balanced incomplete block design (Plan 11.35, $t = 21$, $k = 7$, $r = 10$, $b = 30$, $\lambda = 2$, $E = 0.9$, type III) described by Cochran and Cox (1957). Where t =number of treatments, k =number of samples per panelist, r =replicates, b = number of panelists. This design was replicated four times to achieve a replication rate of 40 analyses per sample. The panelists then evaluated organoleptic attributes with respect to taste, color, flavor, texture and overall acceptability in individual booths with white fluorescent lights, using the above line scale. Panelist ratings from each ballot were measured off in mm with a metric ruler and recorded as unit less measures of consumer acceptability (Table 4.2).

4.2.3.8. Validation experiments

Two different processing conditions were selected to test the validity of the regression model as shown in Table 4.4. The extrusion cooking process was identical to that described in the previous experiment. Measurements of nutritional and physicochemical characteristics of extruded bean flour were performed as described earlier.

4.2.4. Statistical analysis

Response surface methodology procedures were used to develop regression models relating product quality characteristics to the processing variables. The following second order polynomial model for the dependent variables was developed;

$Y = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_4x_1x_2 + \beta_5x_1x_3 + \beta_6x_2x_3 + \beta_7x_1^2 + \beta_8x_2^2 + \beta_9x_3^2$ to fit the experimental data (where Y=dependent variable, β =coefficient, x=independent variable).

ANOVA was used to determine significant model terms. Desirability Function Approach (DFA) was used for multiple response optimization. Design expert statistical software (Design expert 7.1.6, Stat-Ease Inc., Minneapolis, MN; 2007) was used to analyze the data.

4.3. RESULTS AND DISCUSSION

The objective was to optimize extrusion cooking process variables; raw material moisture content, extruder die temperature and feed flow rate, with respect to sensory, nutritional and physicochemical properties of common bean flour. The small white seed coat bean variety from Uganda (NABE 6) was used in this study because it showed significantly higher iron bioavailability in earlier studies and also gave an appreciable yield of all Ugandan bean varieties that were multiplied at the Iowa State University Horticulture Farm, Gilbert, IA. Though a

balanced incomplete block design was used to assign treatments to panelists in order to reduce panelist effect, sensory data was further corrected for this source of error by using adjusted means (taking into account panelist effect) using SAS statistical software.

Effect of independent variables: Table 4.2 shows chemical composition, %RBA and sensory acceptability of extruded beans. Polyphenol content of extruded bean flour ranged from 0.51-1.81 mg/g, iron (57-91 µg/g), expansion ratio (1-1.8), peak viscosity (69-388 cP), final viscosity (83-671 cP), %RBA (54-349) and overall acceptability (59-117 on a 150mm line scale). ANOVA was used to determine significant model terms relating extruder processing variables to sensory, nutritional and physicochemical characteristics of extruded bean flour/porridge. Table 4.3 shows significant model terms ($P < 0.05$) for sensory, physicochemical and nutritional properties of extruded bean flour/porridge. Linear effects of moisture content (x_1) decreased all pasting properties and increased iron bioavailability and extrudate expansion ratio. Die temperature (x_2) had a significant linear effect on all significant models, decreasing consumer acceptability of appearance, color and overall acceptability; peak and final viscosity but increasing RBA and extrudate expansion ratio. Linear effects of feed flow rate (x_3) decreased peak and final viscosity but increased RBA. Interaction effects between moisture content and feed flow rate increased peak and final viscosity but decreased RBA. Interactions between die temperature and flow rate were significant for RBA, decreasing it. There were no significant linear effects of moisture content and flow rate on sensory attributes and neither were any interactive or quadratic effects. Figures 4.1-4.4 show contour surfaces describing the relationship between extrusion variables and sensory properties of resultant porridge, pasting properties and nutritional properties. Only

contours showing curvature i.e. interactive effects are shown. Contour surfaces of all sensory properties lacked curvature, showing a decrease in consumer rating of porridge with increase in temperature (contours not shown). Contour surfaces for pasting properties showed curvature to depict the interaction between moisture content and flow rate, maximizing pasting viscosities at upper limits of both variables, at constant die temperature. Response surfaces for iron bioavailability showed curvature due to interaction between flow rate and die temperature; which interactions maximized iron bioavailability at upper temperature limit and lower flow rate limit.

One of the experimental runs (Run 8: 21.25% moisture content, 175 °C die temperature and 1.8 kg/hr feed flow rate) was an outlier in all responses measured and was ignored in subsequent data analyses. The extrudate from this run appeared burnt and had a very high RBA (349%); three times the second highest value recorded in the experiment. Also, the extrudate did not gain any appreciable viscosity on pasting suggesting it had undergone significant starch degradation.

Focus group panelists described porridge from this extrudate as tasting burnt and with a consistence more of a soup than porridge. A replicate of the same run gave results in agreement with the rest of the design justifying its elimination from further data analysis. However, this data point shows that it is possible to increase iron bioavailability 10 fold and obtain bean flour with no swelling characteristics on pasting.

Die temperature was the only significant model parameter in sensory properties of porridge from extruded bean flour. An increase in die temperature led to a decrease in all consumer ratings. Application of high temperatures at low moisture content (<30 %) could have led to brown color

pigment formation and development of caramelization products which may reduce consumer acceptability of the product (Steel et al., 1995; Bredie et al., 1998). The darkening may be due to reducing sugars that promote non enzymatic browning and the Maillard reaction (Iwe et al., 2001). Burnt/beany taste and flavor were also developed.

All three independent variables showed significant negative linear effects on peak and final viscosity. Significant interactions between moisture content and raw material flow rate were however positive for both pasting properties. Extrusion cooking achieves starch modification in two important ways; gelatinization and molecular breakdown; which are functions of moisture content, temperature and mechanical shear. Gelatinized starch is characterized by a lack of gelatinization peak, a continuous decline in viscosity with shear, and lack of retrogradation. The effect of increasing die temperature on decreasing paste viscosity may be attributed to increased thermal and mechanical degradation of starch; which extruded starch shows no further gelatinization and retrogradation. Increase in moisture content and flow rate decreased peak and final viscosity though their interaction increased it. Nyombaire et al. (2011) made a similar observation which can be explained by the fact that high moisture content plasticizes the raw material under extrusion, reducing effects of mechanical shear. This will ultimately lead to reduced gelatinization of starch and thus a higher paste viscosity. Likewise, increasing the flow rate increases amount of feed in the barrel and reduces effects of mechanical shear.

Table 4.2 shows that though the model for polyphenol content was not significant, extrusion cooking increased polyphenol content. This is in spite of susceptibility of some polyphenolic fractions e.g. caffeic-, ferulic- and p-coumaric acids to thermal breakdown (Dimberg et al., 1996) and complexing with non-starch polysaccharides rendering them inextractable. Increase in

polyphenol content can be explained by the fact that white beans are low in polyphenols and heat application could have enhanced their extraction. However, this result is not isolated. Korus et al. (2007) reported an increase (28%) in the total phenolic content in dark red bean cultivar but a decrease (30 and 38%) in black-brown and cream cultivars respectively with extrusion cooking. The increase in total phenolic content was accompanied by an 84% increase in quercetin content of the dark red variety. Overall, some phenolic fractions decreased while others increased, depending on variety and extrusion conditions. Other studies have however reported reduction in total phenolics and tannins with extrusion cooking (Alonso et al., 2001; El-Hady and Habiba, 2003; Delgado-Licon et al., 2009; Anton et al., 2009). El-Hady and Habiba (2003) showed that soaking followed by extrusion had a greater impact on polyphenol reduction than extrusion cooking alone. Overall, literature suggests that extrusion causes a slight reduction, usually less than 25%, in phenolic contents of beans. Table 4.2 also shows that extrusion cooking significantly increased iron content of the extrudates. Contamination from extruder parts has been implicated in increasing iron content of extruded bean flour (Hazell and Johnson, 1989; Alonso et al., 2001). Contamination with iron can also be explained by the fact that the extruder used was not used to handling such rough and fibrous material. Further studies need to be carried out in extruders regularly used for beans (or similar material). Increase in iron bioavailability on extrusion cooking could be accounted for by; contamination from wear and tear of extruder parts, thermal modification of polyphenolic profiles, thermal hydrolysis of polyphenols and phytates and thermal modification of the food matrix to liberate bound iron. Korus et al. (2007) reported a decrease in antioxidant and free radical scavenging activity of beans on extrusion; which can also be said of metal chelating effect. Extrusion cooking has been

shown to reduce total phytic acid content and to modify distribution of phytic acid profiles.

Alonso et al. (2001) showed reduction of only 4% in total phytates but after accounting for individual inositol phosphates showed that the IP-6 fraction reduced by 26.8%; while both IP-5 and IP-4 fractions increased. Tannin content reduced by 70% while iron bioavailability increased by 40%. Earlier, Sandberg et al. (1987) had shown thermal hydrolysis of IP-6 to lower inositol phosphates on extrusion cooking on wheat bran. Inositol phosphates with less than 5 phosphate groups have been shown to have no iron absorption inhibition capacity (Lönnerdal et al., 1989; Sandberg et al., 1999).

Expansion ratio of extrudates reduced with increase in moisture content but increased with increase in temperature. Expansion ratio is the ratio of the cross sectional area of the extrudate relative to the diameter of the die. It is an important measure of texture as highly expanded products are softer to the bite and easy to mill. Expansion of extrudate occurs due to sudden exit of molten mass from a very high pressure in the barrel, via a restricted die, to the atmosphere resulting in intensive flash-off of internal moisture and the subsequent expansion of the extrudate. The elastic character of the molten extrudate creates a die swell, which controls the overall expansion of the extrudate (Guy, 2001). Determinants of extent of expansion are complex, but chemical composition of the raw material, particle size, temperature and moisture, among others are key (Guy, 2001). The expansion ratio attained in this study was very low. Berrios et al., (2004) attributed the low expansion ratio of extruded black bean to formation of a limited number of air cells. These authors showed that extruded bean air cell walls are composed mainly of gelatinized starch matrices, cooked protein inclusions, cotyledon bean cell wall components and intact cells and seed coat fragments. Fiber from seed coat at low

concentration acts as an air cell nucleating agent and may aid in expansion while at high concentration may rupture air cells and reduced expansion. The high protein content (Guy, 2001) and coarseness of flour used (Berrios and Pan, 2003) may also be responsible for the low expansion observed. Co-extrusion with sodium bicarbonate has been shown to increase the number of air cells with a resultant increase in expansion volume. The increase in expansion ratio with increase in temperature obtained in this study may be due to the plasticizing effect of higher temperatures. With increase in temperature, the extrudate inside the barrel is less viscous and easily expands on exiting. The reduction in expansion ratio with increase in moisture content is harder to explain. Water as a plasticizer contributes to the molten state of the extrudate and ought to have increased expansion ratio.

Optimal extrusion conditions: Table 4.4 shows optimal solutions for maximum consumer acceptability and iron bioavailability at minimal paste viscosity while Figure 4.5 is an overlay plot showing the region at which the dependent variable is not significantly different from the optimum. The optimal combination of extrusion variables was 15% moisture content, 120 °C die temperature and 3 kg/h feed flow rate.

4.4 Validation of regression models and optimum solution

The adequacy of the predictive models at optimum or near-optimum conditions was tested by performing 2 validation experiments. The experiment settings and results are shown in Table 4.5. The results showed that expansion ratio, peak and final viscosity could be reliably predicted but RBA was below the lower limit of prediction. Thus, RBA, the most important nutritional quality of extruded bean flour studied, could not be predicted by using the regression model developed.

This could be due to the fact that the validation cell culture experiment was performed apart from the others (different passage numbers), which could have introduced cell batch related differences.

4.5 Conclusions

Extrusion cooking significantly increased iron bioavailability and reduced pasting profile of beans, giving consumer acceptable extruded bean flours. The linear effect of die temperature was significant in all significant models. Only die temperature was significant in sensory responses. All three independent variables were significant in pasting properties and iron bioavailability. Significant interactions between raw material moisture content and feed flow rate increased pasting properties but reduced iron bioavailability. Increasing iron bioavailability at minimum pasting viscosity maximizes nutrient density of porridges made from extruded bean flour significantly increasing their contribution to iron nutrition of vulnerable population groups.

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Table 4.1. Independent variables used in the study design and their upper and lower limits

Independent Variables	Notation	Lower limit	Upper limit
Moisture content (%)	x_1	15	27.5
Die temperature (°C)	x_2	120	170
Flow rate (kg/h)	x_3	1.8	3

Table 4.2. Optimization experiment design showing sensory and nutritional properties of extruded bean flour

Run No.	Independent variables*			Dependent variables											
	Moisture content (%)	Die Temperature (°C)	Flow rate (Kg/hr)	^a Iron (µg/g)	^b Polyp-phenol (mg/g)	^c Expansion ratio	^d Peak viscosity (cP)	^e Final viscosity (cP)	^f Appearance	^f Color	^f Taste	^f Flavor	^f Texture	^f Overall acceptability	^g Relative biological availability (%)
1	21.25	120	1.8	84	0.54	1.1	271	439	108	114	94	91	105	98	98
2	21.25	175	3	63	0.82	1.6	217	343	85	92	79	80	76	85	94
3	21.25	120	3	75	0.61	1.0	247	455	118	112	97	87	102	97	111
4	21.25	175	3	65	0.65	1.4	208	360	95	102	155	102	98	95	57
5	15	147.5	2.4	71	0.89	1.4	203	356	98	100	80	83	103	84	98
6	21.25	175	1.8	59	0.99	1.4	177	289	95	96	94	91	129	99	109
7	21.25	147.5	2.4	57	0.80	1.3	254	435	103	104	82	96	91	99	69
8	21.25	175	1.8	80	1.81	1.8	69	83	87	86	64	64	83	72	349
9	21.25	120	3	67	0.55	1.1	247	453	107	110	97	106	110	109	106
10	21.25	120	1.8	65	0.51	1.0	253	432	113	108	90	94	103	104	59
11	21.25	147.5	2.4	65	0.67	1.3	235	422	99	96	77	116	94	96	69
12	15	120	2.4	87	0.67	1.1	306	563	101	106	93	96	100	98	72
13	27.5	147.5	3	64	0.66	1.3	332	554	102	97	92	100	100	99	55
14	15	175	2.4	91	1.12	1.8	156	237	61	78	57	64	70	59	79
15	27.5	147.5	1.8	68	1.09	1.4	214	347	95	93	72	95	92	81	112
16	27.5	147.5	2.4	75	1.52	1.3	241	393	94	103	75	83	85	87	73
17	15	147.5	1.8	65	0.76	1.2	388	671	109	108	82	85	97	90	54
18	15	147.5	3	67	0.84	1.5	215	357	77	87	71	83	80	81	104
19	27.5	175	2.4	61	0.88	1.4	247	426	82	81	74	81	84	84	81
20	27.5	120	2.4	67	0.53	1.1	315	536	113	113	109	108	110	117	68
Raw flour				58.9	0.20	ND	ND	ND	ND	ND	ND	ND	ND	ND	34.3

[#]Sensory scores (appearance, color, taste, flavor, texture and overall acceptability) are unitless scores showing consumer ratings measured as mm on a 150 mm unstructured line scale anchored at dislike extremely(0) and like extremely (150mm), ^an=4; ^bn=6; ^cn=10; ^dn=3; ^en=3; ^fn=40; ^gn=6, ND=Not determined

Table 4.3: Significant model terms relating extrusion independent variables to sensory, physicochemical properties and RBA of resultant flour/porridge

Independent variable	Model*	R ²	Lack of fit [#]
Sensory attributes			
Appearance	167.5-0.5x ₂	0.55	NS
Color	155-0.37 x ₂	0.59	NS
Overall acceptability	146-0.4 x ₂	0.38	NS
Pasting properties			
Peak viscosity	1413-45x ₁ -1.3x ₂ -420x ₃ +19.4x ₁ x ₃	0.64	S
Final viscosity	2560-82x ₁ -2.6x ₂ -739x ₃ +35x ₁ x ₃	0.66	S
Nutritional properties			
Relative Biological Availability	-583+17x ₁ +2x ₂ +280x ₃ -7x ₁ x ₃ -0.9x ₂ x ₃	0.57	NS
Physical property			
Expansion ratio of extrudate	0.36-0.1 x ₁ +0.008x ₂	0.78	NS

*Models and model terms significant at p<0.05; x₁-moisture content; x₂-Die temp; x₃-flow rate;

[#]NS-Not Significant; S- Significant

Table 4.4: Optimization results showing solutions maximizing desirability^a

Solutions	Independent Variables			Dependent Variables			Desirability
	Moisture content (%)	Die temperature (°C)	Flow rate (kg/h)	Final viscosity (cp)	Overall Acceptability	%RBA	
1	15	120	3	356	102	126	0.81
2	15	121	3	354	102	125	0.81
3	15	120	3	355	102	125	0.81
4	15	123	3	349	101	124	0.81
5	15	124	3	346	101	124	0.81
6	15	130	3	330	99	121	0.81
7	15	130	3	330	99	121	0.81
8	15	134	3	318	97	118	0.81

^aSelection criteria for the optimization procedure; minimize final viscosity, maximize overall acceptability and %RBA while keeping independent variables in range

Table 4.5: Validation experiments results

Run No.	Independent variables			Dependent variables*							
	Moisture content (%)	Die Temperature (°C)	Flow rate (Kg/hr)	^a Expansion ratio		^b Peak viscosity (cP)		^c Final viscosity (cP)		^d RBA (%)	
				Predicted	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted	Experimental
1	15	120	3	1±0	1.1 ±0	197±29	329±52	356±52	600±81	126±13	70±17 [#]
2	19	120	2.8	1.1±0	1.1±0	254±19	291±0	453±33	523±0	103±9	60±8 [#]

^an=10; ^bn=3; ^cn=3; ^dn =6; *Predicted=Mean±SEM; Experimental=Mean±SD; [#]Experimental value is significant different from predicted value at P<0.05.

Design-Expert® Software

peak viscosity

X1 = C: Flow rate (kg/h)
X2 = A: Moisture Content (%)

Actual Factor
B: Die Temperature(°C) = 120.00

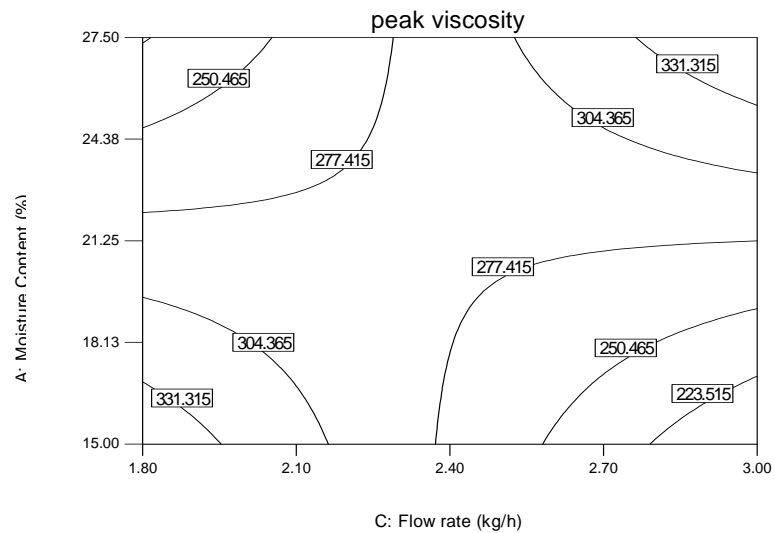


Figure 4.1: Contour surface showing relationship between moisture content, feed flow rate and peak viscosity of extruded bean flour at the optimal die temperature (120 °C)

Design-Expert® Software

Final viscosity

X1 = A: Moisture Content (%)

X2 = C: Flow rate (kg/h)

Actual Factor

B: Die Temperature(oC) = 120.00

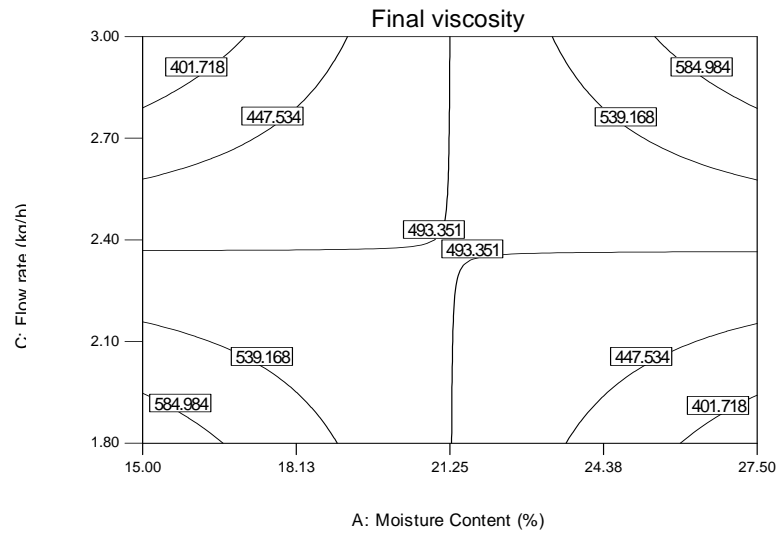


Figure 4.2: Contour surface showing relationship between moisture content, feed flow rate and final viscosity of extruded bean flour at the optimal die temperature (120 °C)

Design-Expert® Software

Relative Biological Value (%)

X1 = A: Moisture Content (%)

X2 = C: Flow rate (kg/h)

Actual Factor

B: Die Temperature(oC) = 148.24

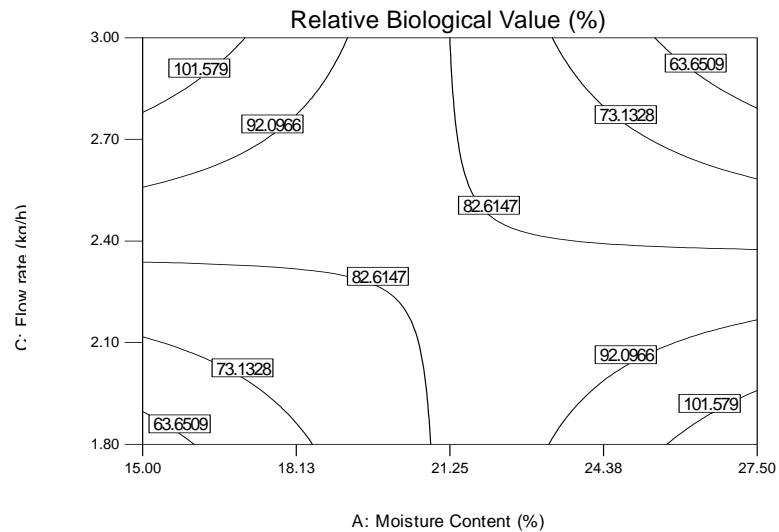


Figure 4.3: Contour surface showing relationship between moisture content, feed flow rate and RBA of extruded bean flour at constant die temperature (148.24 °C).

Design-Expert® Software

Expansion ratio

X1 = A: Moisture Content (%)

X2 = B: Die Temperature(°C)

Actual Factor

C: Flow rate (kg/h) = 2.40

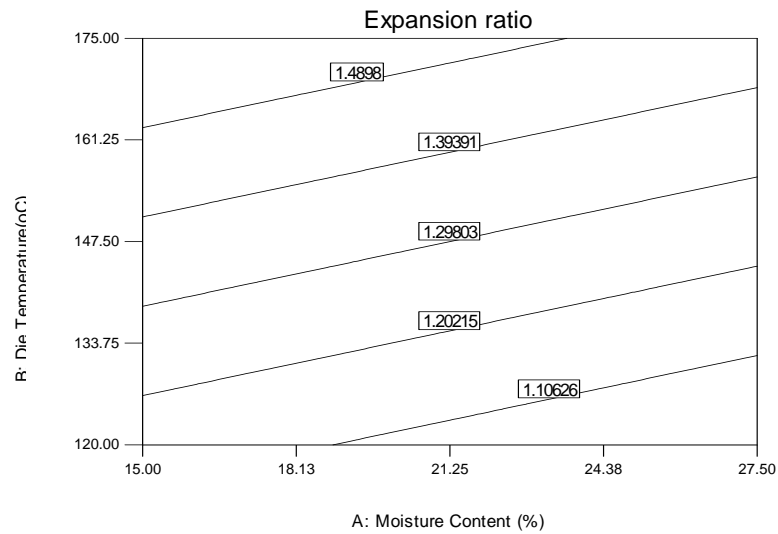


Figure 4.4: Contour surface showing relationship between moisture content, die temperature, and expansion ratio of bean flour at constant feed flow rate (2.4 kg/hr).

Design-Expert® Software

Overlay Plot

Final viscosity

Overall

FeBA

X1 = A: Moisture Content (%)

X2 = B: Die Temperature(°C)

Actual Factor

C: Flow rate (kg/h) = 3.00

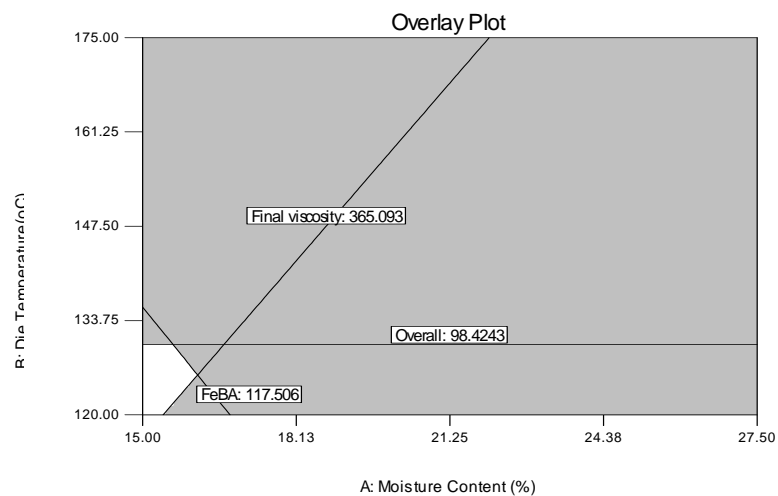


Figure 4.5: Overlay plot showing optimal (un-shaded) region maximizing overall acceptability and RBA at minimum final paste viscosity

CHAPTER 5. GENERAL CONCLUSIONS

The current study was carried out with the objectives of screening Ugandan bean varieties for iron bioavailability and to determine effect of extrusion cooking on iron bioavailability. The first part of the study demonstrated that iron bioavailability, as determined by in vitro Caco-2 cell culture, varied widely in the bean varieties, with white seed coat varieties having significantly higher iron bioavailability than colored seed coat ones. Multiple regression modeling showed that only polyphenol and iron content were significant factors influencing iron bioavailability in this particular set of beans. Similar results have been shown in other studies (Tako and Glahn, 2010; Hu et al., 2003). Linear effects of polyphenol and iron content decreased iron bioavailability while their interaction increased it. The negative effect of iron content on iron bioavailability highlights challenges in breeding for high iron bean varieties in the presence of high polyphenol and phytates content (Donangelo et al., 2003; Petry et al., 2010). Ferritin and phytic acid were not significant factors in this study but could potentially influence iron bioavailability. Over expression of ferritin in plants is a current area of interest with encouraging results showing a positive correlation with iron content and bioavailability (Aluru et al., 2012). Western blot quantification of ferritin used in this study showed very high variation and may have led to this lack of significance. Therefore, more accurate assays need to be explored to further study this factor. Effect of phytates was not significant in this study but phytates are well known inhibitors of iron bioavailability. The wide variation in the composition of the various factors studied shows potential for breeding for these traits. However, it is evident that iron

biofortification should aim at reducing polyphenol and phytates and maximizing ferritin content.

Low phytate varieties have already been developed (Campion et al., 2009).

The second study aimed at optimizing extrusion cooking process variables with respect to iron bioavailability of NABE6, the small white seed coat variety from Uganda with the highest iron bioavailability (from study one). Extrusion cooking increased iron bioavailability of NABE6 bean variety 1.5-10 fold, reduced pasting viscosity and produced consumer acceptable flours. The linear effect of die temperature was significant in all significant models while the effect of moisture content and feed flow rate were only significant in pasting viscosity and iron bioavailability. Optimal processing conditions were 15% moisture content, 120 °C die temperature and 3 kg/hr feed flow rate to give a relative bioavailability score. The optimal extrudate had RBA (124%) which was 3.5 fold higher than that of the conventionally cooked NABE6. However, a significant increase in iron content with extrusion showed probable contamination from extruder parts which may have contributed to increase in iron bioavailability (Alonso et al., 2001). Extrusion cooking did not reduce polyphenol content of beans thus maintaining bioactive properties of beans (Korus et al., 2007). On the other hand, household processing technologies (soaking, germination, malting, etc.) have been shown to enhance iron bioavailability but at the expense of polyphenolic compounds. However, if iron bioavailability can be enhanced while maintaining functionality of beans, extrusion cooking will go a long way in enhancing nutritional value of beans. The importance of extrusion cooking in enhancing iron bioavailability shouldn't be seen to replace biofortification efforts since as an energy intensive process, it may not reach the rural poor of the world in most need of improved iron intake. Also,

it is not clear if this technology will be valid for all bean types (especially colored seed coat ones) and if products will be acceptable across different populations. However, it can be expected that modification of bean matrix to more readily release iron on digestion, change in polyphenolic profile and reduction in higher inositol phosphates could improve iron bioavailability of these beans as well. More work is warranted to elucidate these issues, and also determine if heat modified polyphenols possess bioactive properties. Extruders that are regularly used for extruding beans ought to be used in subsequent studies to limit contamination from iron containing parts. Finally, stable isotope studies and human feeding trials are necessary to corroborate the beneficial effects of extrusion cooking. In conclusion, this study has provided proof-of concept of the considerable potential of extrusion cooking for combating iron deficiency in populations dependent on beans for their iron nutrition.

The two studies taken together showed that screening for high bioavailability bean varieties and applying appropriate processing conditions could significantly improve the contribution of common beans to iron nutrition.

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APPENDICES

APPENDIX I. INFORMED CONSENT FORM PROVIDED TO EACH PANELIST AND APPROVED BY THE INSTITUTIONAL REVIEW BOARD OF IOWA STATE UNIVERSITY AND UGANDA NATIONAL COUNCIL OF SCIENCE AND TECHNOLOGY

Informed Consent Form

“Sensory Evaluation of Extruded Bean Porridge and Soup”

Researcher: Martin Mutambuka **Project Co-Principal Investigators:** Dr. Manju Reddy, Dr. Suzanne Hendrich, and Dr. Patricia Murphy, Department of Food Science and Human Nutrition, ISU; Dr. Dorothy Nakimbugwe, Makerere University.

Participation

I am asking for your voluntary participation in a project that involves sensory evaluation of bean based porridge and soups. Please read the information below about the project and feel free to ask any questions. If you agree to participate, please indicate by signing below. You will be given a copy of this consent form to keep. Participation in this study is completely voluntary. If you decide to participate, you may decide not to answer any specific question that makes you feel uncomfortable and you may stop participating at any time. If you decide to not participate in the study or leave the study early, it will not result in any penalty. There are no foreseeable risks from participating in this study. There will be no direct benefits to you from participating in this study. However, information gained may benefit society by being utilized to improve the nutritional quality of bean based meals.

Procedures

If you decide to participate, you will be part of a 140 people consumer panel. Each panelist will be presented with a maximum of 6 porridge or soup samples to taste and responses will be recorded on a nine point hedonic scale provided at the time of tasting. All testing sessions will be held in sensory evaluation booths housed in the Department of Food science and Technology, Makerere University. Bottled mineral water will be provided for rinsing between samples.

Confidentiality

Your identity will be kept anonymous. Information obtained about this study will be kept confidential to the extent permitted by applicable laws and regulations and will not be made publicly available. The information will be stored in a closed cabinet and only accessed by members on the research team.

“Federal government regulatory agencies, the Collaborative Research Support Program, Auditing departments of Iowa State University Institutional Review Board (a committee that reviews and approves research studies with human subjects) may inspect and/or copy your records for quality assurance and analysis. These records may contain private information.”

Questions

If you have any questions about this study, feel free to contact; Dr. Dorothy Nakimbugwe (0782246089) or the graduate student, Martin Mutambuka (0782367118).

Signature

By signing this form, I am attesting that I have read and understood the information above and I freely give my consent/assent to participate.

Signature:_____ Date_____

APPENDIX II. DEMOGRAPHIC INFORMATION QUESTIONNAIRE

PROVIDED TO ALL SENSORY PANELISTS

CONSUMER QUESTIONNAIRE

Please check the appropriate answer for the following demographic information:

Panelist No.....

1. Sex ☐ male ☐ female

2. Age group

☐ 18 – 25 years

☐ 26 – 35 years

☐ 36 – 45 years

☐ 46 – 55 years

☐ 56 – 65 years

3. Do you have any **food allergies** to beans? ☐ yes ☐ no

Please answer the following questions. There are no right or wrong answers. We want to know about you and what you think. Please ask if you have any questions!

4. Do you purchase any flours for making porridge? ☐ yes ☐ no

5. How often do you consume porridges?

☐ I do not consume porridges

☐ Occasionally

☐ At least once per month

☐ At least 2-3 times per month

☐ At least once per week

☐ Two to three times per week

☐ Four or more times per week

6. If you consume porridges, what products do you eat? Check all that apply:

☐ Maize ☐ Millet ☐ Soya ☐ Rice ☐ composites (mixtures of flours)

☐ Other (specify) _____

7. What factors influence your choice of porridge flour? Check all that apply:

☐ Tradition ☐ Price ☐ Texture ☐ Flavor ☐ Health

☐ Availability

APPENDIX III. SENSORY EVALUATION BALLOTS USED FOR CONSUMER ACCEPTABILITY STUDIES

Sensory evaluation ballot

Panelist No. _____ Sample No. _____

You are provided with a sample of porridge. Please observe and record your liking for the appearance and color on the line scale below. Use provided spoon and place sufficient sample in your mouth. Taste the porridges and rate them against the given scale by placing a vertical mark at the appropriate position on the line scale for taste, aroma/flavor, texture and overall acceptability. Please evaluate the products in the order in which they are presented. Use the water and biscuits provided to rinse your mouth before and after tasting each sample and between samples.

ANSWER ALL QUESTIONS. We want to know what you think!!

If you have any questions, please ask the study coordinators.

NB: if you have any further comment about the product, please note it on the space provided below the line.

1. Appearance

	<div style="position: absolute; left: 0; top: -5px; width: 5px; height: 10px; border-left: 1px solid black;"></div> <div style="position: absolute; right: 0; top: -5px; width: 5px; height: 10px; border-left: 1px solid black;"></div>	
Dislike extremely		Like extremely

Comment.....

2. Color

	<div style="position: absolute; left: 0; top: -5px; width: 5px; height: 10px; border-left: 1px solid black;"></div> <div style="position: absolute; right: 0; top: -5px; width: 5px; height: 10px; border-left: 1px solid black;"></div>	
Dislike extremely		Like extremely

Comment.....

3. Taste

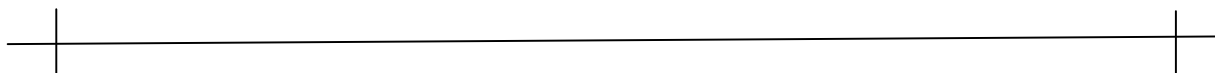
	<div style="position: absolute; left: 0; top: -5px; width: 5px; height: 10px; border-left: 1px solid black;"></div> <div style="position: absolute; right: 0; top: -5px; width: 5px; height: 10px; border-left: 1px solid black;"></div>	
Dislike extremely		Like extremely

Comment.....

4. Flavor/aroma

	<div style="position: absolute; left: 0; top: -5px; width: 5px; height: 10px; border-left: 1px solid black;"></div> <div style="position: absolute; right: 0; top: -5px; width: 5px; height: 10px; border-left: 1px solid black;"></div>	
Dislike extremely		Like extremely

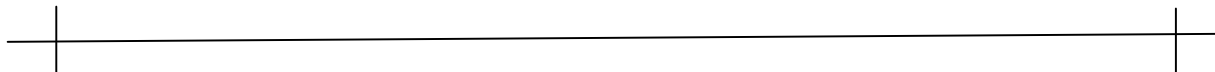
Comment.....

5. Texture

Dislike extremely

Like extremely

Comment.....

6. Overall acceptability

Dislike extremely

Like extremely

Comment.....