DETERMINATION OF BIOAVAILABLE IRON AND VITAMIN A IN FORTIFIED BLENDED FOODS AND FATTY ACIDS AND PHYTOSTEROLS IN SAW PALMETTO SUPPLEMENTS

by

KAVITHA PENUGONDA

M.S., Oklahoma State University, 2009

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Human Nutrition
College of Human Ecology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2016

Abstract

Fortified blended foods (FBFs), in particular, corn-soybean blend (CSB), are food aid commodities widely used in infant and young children supplementary feeding programs. A United States Agency for International Development (USAID) Food Aid Quality Review report recommended developing novel FBFs using local alternative commodities such as sorghum and improving the nutritional quality of FBFs using extrusion processing.

Extruded sorghum-cowpea, sorghum-soy and corn-soy FBFs were developed and compared with the non-extruded FBFs corn-soy blend 13 (CSB13) and corn-soy blend plus (CSB+) using the in-vitro digestion/Caco-2 cell model. Dry FBFs' iron and vitamin A content ranged from 8.0 to 31.8 mg/100g and 0.54 to 1.67 mg/100g, respectively. Following in-vitro digestion, bioavailable iron and vitamin A levels were determined by measuring Caco-2 cell ferritin and vitamin A levels in response to 12-hour and 4-hour treatments, respectively, with aqueous fractions collected from digested FBFs. Most extruded FBFs' aqueous fraction iron levels were 2- to 7-fold higher ($p<0.05$) than CSB13 and CSB+. However, Caco-2 cell ferritin and vitamin A levels were not significantly different among FBFs. These results suggest that consumption of newly developed extruded sorghum-cowpea, sorghum-soy and corn-soy FBFs will result in bioavailable iron and vitamin A levels comparable to traditional non-extruded CSB13 and CSB+. Thus, extruded sorghum-cowpea FBF may be a suitable alternative to corn-soybean based FBFs.

Saw palmetto supplements are one of the most commonly consumed products by men with prostate cancer and/or benign prostatic hyperplasia (BPH). Some studies have found significant improvements in BPH with saw palmetto supplementation, whereas others found no benefits. The variation in the efficacy in these trials may be a result of differences in the putative

active components, fatty acids and phytosterols, of the saw palmetto supplements. We quantified fatty acids and phytosterols in 20 commercially available liquid, powder, dried berry, and tincture saw palmetto supplements. Liquid saw palmetto supplements contained significantly higher ($p<0.05$) concentrations of total fatty acids (908.5 mg/g), individual fatty acids, total phytosterols (2.04 mg/g), and individual phytosterols, than the other supplement categories. Our findings suggest that liquid saw palmetto supplements may be the best choice for individuals who want to take a saw palmetto supplement.

DETERMINATION OF BIOAVAILABLE IRON AND VITAMIN A IN FORTIFIED BLENDED FOODS AND FATTY ACIDS AND PHYTOSTEROLS IN SAW PALMETTO SUPPLEMENTS

by

KAVITHA PENUGONDA

M.S., Oklahoma State University, 2009

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Human Nutrition
College of Human Ecology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2016

Approved by:

Major Professor
Brian Lindshield

Copyright

Abstract

Fortified blended foods (FBFs), in particular, corn-soybean blend (CSB), are food aid commodities widely used in infant and young children supplementary feeding programs. A United States Agency for International Development (USAID) Food Aid Quality Review report recommended developing novel FBFs using local alternative commodities such as sorghum and improving the nutritional quality of FBFs using extrusion processing.

Extruded sorghum-cowpea, sorghum-soy and corn-soy FBFs were developed and compared with the non-extruded FBFs corn-soy blend 13 (CSB13) and corn-soy blend plus (CSB+) using the in-vitro digestion/Caco-2 cell model. Dry FBFs' iron and vitamin A content ranged from 8.0 to 31.8 mg/100g and 0.54 to 1.67 mg/100g, respectively. Following in-vitro digestion, bioavailable iron and vitamin A levels were determined by measuring Caco-2 cell ferritin and vitamin A levels in response to 12-hour and 4-hour treatments, respectively, with aqueous fractions collected from digested FBFs. Most extruded FBFs' aqueous fraction iron levels were 2- to 7-fold higher ($p<0.05$) than CSB13 and CSB+. However, Caco-2 cell ferritin and vitamin A levels were not significantly different among FBFs. These results suggest that consumption of newly developed extruded sorghum-cowpea, sorghum-soy and corn-soy FBFs will result in bioavailable iron and vitamin A levels comparable to traditional non-extruded CSB13 and CSB+. Thus, extruded sorghum-cowpea FBF may be a suitable alternative to corn-soybean based FBFs.

Saw palmetto supplements are one of the most commonly consumed products by men with prostate cancer and/or benign prostatic hyperplasia (BPH). Some studies have found significant improvements in BPH with saw palmetto supplementation, whereas others found no benefits. The variation in the efficacy in these trials may be a result of differences in the putative

active components, fatty acids and phytosterols, of the saw palmetto supplements. We quantified fatty acids and phytosterols in 20 commercially available liquid, powder, dried berry, and tincture saw palmetto supplements. Liquid saw palmetto supplements contained significantly higher ($p<0.05$) concentrations of total fatty acids (908.5 mg/g), individual fatty acids, total phytosterols (2.04 mg/g), and individual phytosterols, than the other supplement categories. Our findings suggest that liquid saw palmetto supplements may be the best choice for individuals who want to take a saw palmetto supplement.

Table of Contents

List of Figures

List of Tables

Acknowledgements

I thank my advisor Dr.Brian Lindshield for his immense support and guidance throughout the PhD program. He gave me the opportunity to be involved in different projects that helped me gain knowledge in multiple research areas. His constant support and critique helped me conduct the experiments that are otherwise difficult. It is a great educational and innovative research experience working with Dr. Lindshield.

I would like to thank Dr. Sajid Alavi for giving me the opportunity to be involved in Micronutrient Fortified Food Aid Products Pilot (MFFAPP) project and for the opportunity to visit extrusion facility and learn new research areas.

I would also like to thank Dr. Mark Haub for being on my committee and guiding me to work towards dissertation goals. I am always grateful for the motivation he provided and encouraging positive thinking.

I am thankful to Dr. Weiqun Wang for being on my committee and providing critique that helped me improve the dissertation. I would also like to thank Dr. Kelly Getty, who showed interest in my research and encouraged me to think about the benefits and applicability of this research in other areas. I would also like to thank Dr. Mark Failla, The Ohio State University, for providing me training in the experiments that we used to conduct research.

Finally, I would like to thank my family and friends for their unconditional love, support and motivation during this educational phase.

Preface

The dissertation includes two studies conducted to determine the bioavailable iron and vitamin A in extruded fortified blended foods (FBFs) and the third study that determined the fatty acid and phytosterol profiles of commercial saw palmetto herbal supplements. The organization of the dissertation is described below.

Chapter 1 provides information on FBF history, development and nutritional improvements. Data on global prevalence of iron and vitamin A deficiency is included. This chapter also provides information on methodological differences in the in-vitro digestion/Caco-2 cell mode literature, iron and vitamin A bioavailability, sorghum and cowpea production, antinutritional factors (ANF) and processing methods that reduce ANF.

Chapter 2 describes a study on bioavailable iron in newly developed extruded sorghum-cowpea, sorghum-soy and corn-soy FBFs. Here we compared the new FBFs with the traditional food aid FBFs; corn-soy blend 13 (CSB13) and corn-soy blend plus (CSB+). This study was limited by the fact that the FBFs were low in vitamins A, D, and E. As a result, chapter 2 results are considered preliminary.

Hence, we conducted another study on the new FBFs that contained the recommend levels of the vitamins that is described in chapter 3. Chapter 3 assessed both bioavailable iron and vitamin A in extruded sorghum-cowpea, sorghum-soy and corn-soy FBFs compared to the traditional FBFs; CSB13 and CSB+. Here we modified the methods by using cell lysis buffer for harvesting the Caco-2 cells, and by adding $FeSO_4$ positive controls in addition to basal salt solution negative control.

Chapter 4 describes a study on characterizing the fatty acid and phytosterol profiles of different commercially available saw palmetto herbal supplements. This study was conducted

first, before changing the focus of my dissertation. It was published in Nutrients in 2013 and has been cited multiple times.

Chapter 5 provides conclusions and future directions about iron and vitamin A bioavailability in FBFs and therapeutic effects of bioactive components of saw palmetto supplements.

Chapter 1 - Literature Review

Introduction

Globally, more than 2 billion individuals are at risk for multiple micronutrient deficiencies [1]. Africa and South-East Asia are highly affected regions with the greatest number of individuals with iron deficiency anemia (IDA) and vitamin A deficiency (VDA) [2, 3], the two major micronutrient deficiencies of tremendous public health significance.

This chapter will review the global prevalence of iron deficiency, vitamin A deficiency, fortified blended foods, United States Agency for International Development (USAID)-Food Aid Quality Review (FAQR) recommendations, antinutritional factors (ANF) in sorghum and cowpea, effect of extrusion on ANF and iron bioavailability, iron and vitamin A bioavailability using in-vitro digestion/Caco-2 cell model and methodological differences in in-vitro digestion/Caco-2 cell model.

Global prevalence of iron deficiency

Iron deficiency is one of the major micronutrient deficiencies in the world along with vitamin A, zinc and iodine deficiencies. Iron deficiency is primarily caused by diets low in iron, deficient in animal protein, and predominantly based on cereals and legumes that are low in iron and /or contain iron inhibitors [4, 5]. Increased iron demands during growth, pregnancy, lactation, malabsorption syndrome, malaria, genetic disorders, and blood loss due to intestinal parasites are some other causes for iron deficiency [5]. Iron deficiency anemia affects the physical growth and cognitive performance in children, immunity, physical capacity and work performance in all age groups. It also increases the risk of maternal and infant mortality [6, 7].

Iron deficiency prevalence data is limited due to difficulty in its assessment. A combination of measurements such as, serum ferritin, serum transferrin receptor, transferrin saturation are used as indicators of iron status. As it is often not feasible to assess multiple indicators of iron status at population level the data on global iron deficiency is not available. Hence, anemia prevalence estimates assessed by blood hemoglobin concentration are used as a measure of IDA [6]. According to World Health Organization (WHO), blood hemoglobin concentration of < 11 g/dL for pre-school children and pregnant women, and < 12 g/dL for non-pregnant women are anemia thresholds. Anemia prevalence is highest among pre-school children (47%), pregnant women (42%), and non-pregnant women (30%, Table 1-1) [2]. Africa has the highest proportion of anemia, where more than half of pre-school children and pregnant women are anemic (Table 1-1). However, South-East Asia has the largest population of 315 million anemic individuals. With the exception of two to three countries, all of the WHO member countries have mild to severe public health significance for anemia in vulnerable groups. Globally, anemia is of severe public health significance in at least 68 countries [2].

Table 1-1 Anemia prevalence and number of individuals affected in pre-school-age children, pregnant women and non-pregnant women in each WHO region

WHO region	Pre-school-age children		Pregnant women		Non-pregnant women	
	Prevalence (%)	# affected (millions)	Prevalence (%)	# affected (millions)	Prevalence (%)	# affected (millions)
Africa	67.6	83.5	57.1	17.2	47.5	69.9
Americas	29.3	23.1	24.1	3.9	17.8	39
South-East Asia	65.5	115.3	48.2	18.1	45.7	182
Europe	21.7	11.1	25.1	2.6	19	40.8
Eastern Mediterranean	46.7	0.8	44.2	7.1	32.4	39.8
Western Pacific	23.1	27.4	30.7	7.6	21.5	97
Global	47.4	293.1	41.8	56.4	30.2	468.4

Source: Worldwide prevalence of anaemia 1993–2005: WHO global database on anaemia [2].

WHO estimates anemia prevalence in non-industrialized countries to be, 52% of pregnant women, 48% of children aged 5-14 years, 42% of all women and 39% of children under 5 years

[6]. Whereas, in industrialized countries 23% of pregnant women, 5.9% of children aged 5-14 years, 10% of all women and 20% of children under 5 years are anemic. It is notable that percent pregnant women with anemia in non-industrialized countries is 2-times higher than the prevalence in industrialized countries [6].

Global prevalence of vitamin A deficiency

Vitamin A is an essential micronutrient needed in smaller amounts for normal vision, immunity and reproductive functions. Vitamin A deficiency (VAD) is generally associated with low dietary intakes of preformed vitamin A [8]. Infants, young children and pregnant women are at greater risk of vitamin A deficiency disorder xerophthalmia, a condition characterized by dry conjunctiva and cornea. Vitamin A deficiency is assessed by clinical examination for night blindness and biochemical examination for low serum or plasma retinol [3].

Current VAD estimates are based on 1995-2005 WHO data available in the Vitamin and Mineral Nutrition Information System (VMNIS) [3]. WHO criteria for VAD include history of night blindness and serum/plasma retinol cut-off of < 0.70 μmol/l. A prevalence of $\geq 5\%$ night blindness in pre-school children below 5 years, and $\geq 20\%$ low serum/plasma retinol (< 0.70 μmol/l) in pregnant women is considered a severe public health problem.

Worldwide, night blindness has been estimated to affect 5.2 million pre-school children and 9.8 million pregnant women corresponding to 0.9% and 7.8% of the population at risk of VAD (Table-1-2). Similarly, low serum retinol concentration, also referred to as biochemical VAD, has been estimated to affect 190 million pre-school children and 19.1 million pregnant women corresponding to 33% and 15% of the population at risk of VAD, globally (Table 1-2).

Africa and South-East Asia are the most affected regions with highest number of pre-school children and pregnant women with VAD. Compared to number of individuals affected globally, Africa has around ½ of the pre-school children with night blindness and South-East Asia has around ½ of the pre-school children with low serum retinol and around 1/3rd of pregnant women with both night blindness and low serum retinol (Tables 1-2, 1-3).

Table 1-2 Prevalence of night blindness in WHO regions (1995-2005).

WHO region	Pre-school-age children		Pregnant women	
	Prevalence (%)	Number affected (millions)	Prevalence (%)	Number affected (millions)
Africa	2.0	2.55	9.8	3.02
Americas	0.6	0.36	4.4	0.50
South-East Asia	0.5	1.01	9.9	3.84
Europe	0.8	0.24	3.5	0.22
Eastern Mediterranean	1.2	0.77	7.2	1.09
Western Pacific	0.2	0.26	4.8	1.09
Global	0.9	5.17	7.8	9.75

Source: WHO. Global prevalence of vitamin A deficiency in populations at risk 1995–2005. WHO Global Database on Vitamin A Deficiency, Geneva, World Health Organization, 2009 [3].

Table 1-3 Prevalence of low serum retinol (< 0.70 μmol/l) in WHO regions (1995-2005).

WHO region	Pre-school-age children		Pregnant women	
	Prevalence (%)	Number affected (millions)	Prevalence (%)	Number affected (millions)
Africa	44.4	56.4	13.5	4.18
Americas	15.6	8.68	2.0	0.23
South-East Asia	49.9	91.5	17.3	6.69
Europe	19.7	5.81	11.6	0.72
Eastern Mediterranean	20.4	13.2	16.1	2.42
Western Pacific	12.9	14.3	21.5	4.9
Global	33.3	190	15.3	19.1

Source: WHO. Global prevalence of vitamin A deficiency in populations at risk 1995–2005. WHO Global Database on Vitamin A Deficiency. Geneva, World Health Organization, 2009 [3].

International food aid

The International Food Aid Information System (INTERFAIS), developed by World Food Programme (WFP) provides information on global food aid deliveries. According to INTERFAIS database, the total global food aid in 2012 was 5 million metric tons, of which 58% was donated by WFP (Table 1-4). The major recipient of food aid is Sub-Saharan Africa (63%), followed by Asia (22%) [9].

Table 1-4 Global food aid flows to different WHO regions (2008-2012).

	2008	2009	2010	2011	2012
	Food aid deliveries (million mt)				
Global food aid deliveries	6.5	6.3	6.9	5	5
WFP share of total	4	4	4.3	3.3	2.9
	Global food aid deliveries (%)				
Sub-Saharan Africa	63	64	60	62	63
Asia	24	23	27	21	22
Eastern Europe and CIS	2	2	1	0	0
Latin America and the Caribbean	5	5	8	7	4
Middle East and North Africa	6	6	4	10	11

CIS -Commonwealth of Independent States

Fortified blended foods

FBFs are food aid commodities distributed under Title II of Public Law 480 (P.L. 480) of the Food For Peace (FFP) program. FBFs, introduced in 1966, were donated by US government to combat hunger and malnutrition in food insecure countries. Initially, FBFs were developed as food aid commodities to improve child nutrition. Later, they were modified to supplement moderately malnourished children by adding sugar and vegetable oil. In the subsequent years, FBFs were upgraded to adjust the macronutrient and micronutrient composition and were supplemented to other primary beneficiary groups such as pregnant women, lactating women,

and individuals affected with human immunodeficiency virus (HIV) and tuberculosis (Table 1-5) [10].

Table 1-5 Primary beneficiary groups.

- Prevention of stunting (linear growth promotion) among children 6–24 months
- Management of moderate wasting among children 6–59 months
- Meeting the elevated protein and micronutrient needs of nutritionally-vulnerable pregnant and lactating women
- Management or prevention of moderate wasting among people (including adults) living with HIV or AIDS

Source: Webb et al (2011) [14].

Later, FBFs were used to supplement the overall diet of food insecure populations. They are commonly distributed in maternal and child health nutrition (MCHN) programs and school-meal programs. FBFs are also provided during emergency situations such as natural disasters and in refugee camps since the local food resources are severely limited [11].

FBFs are value-added foods composed of cereal and legume flour with added vegetable oil and vitamin-mineral mix. The grains and legumes are partially precooked to denature ANF, increase digestibility, palatability and decrease cooking time [12]. The major cereals that are used are corn and wheat, while soybean is the major legume. FBFs generally contain 75-80% cereals, 20-25% legumes and a micronutrient premix (Table 1-5) [13].

The initial FBF formulations; corn soy milk (CSM) and wheat soy milk (WSM) were developed in 1966. They were high in protein 17.9 mg/100g and low in fat 6.3 mg/100g compared to the current FBF CSB13 that contains 15.9 mg/100g protein and 8.7 mg/100g fat [12, 14]. US agricultural surpluses affect the quantity and composition of FBF formula. Because of the deficit in US milk supply during 1989, dried skim milk powder was removed from CSM and WSM and instead CSB and wheat soy blend (WSB) developed without dried skim milk powder. FBFs have been upgraded several times to meet the macronutrient and micronutrient

requirements of the target groups. For instance, in 1988 vitamin A levels were doubled and in 1998, magnesium was added and zinc levels were increased [15].

The main donors of CSB are the USAID, WFP and United Nations Children's Fund (UNICEF). Because these organizations have different product specifications, CSB differs in nutritional content from one source to another (Table 1-6). USAID CSB commodity requirements are regulated by the United States Department of Agriculture (USDA), whereas local variants of CSB are regulated by WFP and UNICEF [11, 13].

Table 1-6 Ingredient and macronutrient composition of CSB from three donor agencies.

Ingredient	WFP % by weight	UNICEF % by weight	USAID % by weight
Corn	Whole maize (white or yellow): 75-80	Whole maize: 70–80	Cornmeal, extruded (dehulled, degermed): 69.55
Soy	Whole soy beans: 20–25	Whole soy beans: 20–30	Soy flour (dehulled, defatted): 21.85
Vegetable oil	–	–	Soybean oil: 5.5
Sugar	–	5–10	–
Energy, kcal (minimum)	380	400	377
Protein % (minimum)	14	14	16.7
Fat % (minimum)	6	6	6
Crude fiber % (max)	5	5	2

Source: Fleige et al, 2010 [11] CSB, corn soy blend; UNICEF, United Nations Children's Fund; USAID, US Agency for International Development; WFP, World Food Programme.

CSB distributed by WFP and UNICEF uses whole corn and whole soy. Whereas the corn-soy blend distributed by USAID has corn meal derived from dehulled, degermed corn and soy flour made from dehulled soy. USAID has introduced a new FBF, CSB+, with heat processed corn and soy and improved micronutrient formulation [16]. WFP has another FBF, CSB++ that contains additional ingredients such as skim milk powder, sugar and oil [13].

USAID-Food Aid Quality Review (FAQR) recommendations for FBF improvements

Resource constraints, reduced global food aid delivery and concerns on whether food aid products are meeting the needs of target groups led to a review of Title II food aid products. A USAID-FAQR committee reviewed the existing food aid products and made recommendations for their improvement. The FAQR includes 35 recommendations to improve USAID food aid products and programs. Recommendations related to the nutritional enhancement of FBFs were enhancing the macronutrient and micronutrient content, decreasing anti-nutritional factors that impair nutrient absorption using processing methods such as extrusion, including animal source protein, and developing novel FBFs using local alternative commodities such as sorghum.

The FAQR recommends sorghum as an alternative cereal because of its low-cost and acceptability in Africa. A sorghum–soy or sorghum–pea or other pulse blends, millet–soy, rice–soy, or other cereal, potato–soy (or other pulse) blends were suggested as alternative FBFs to CSB or WSB [14].

Sorghum

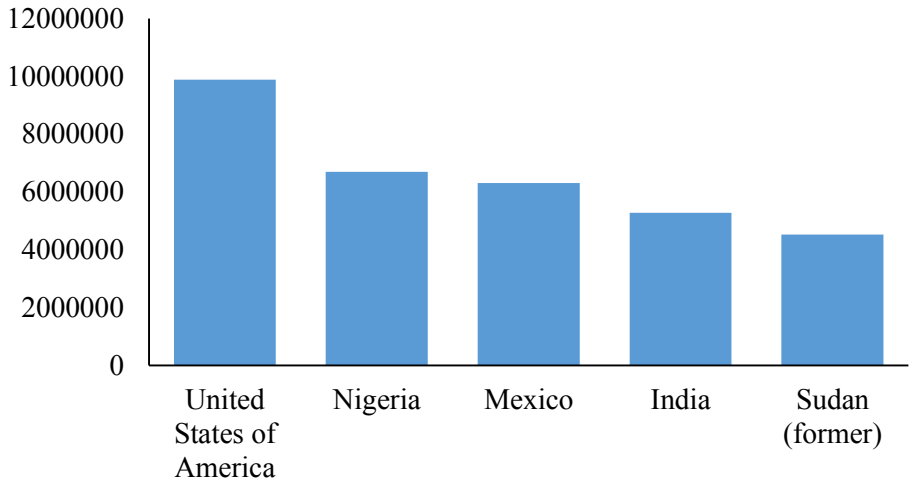
Sorghum (*Sorghum bicolor)* is an ancient crop and a fifth most consumed grain in the world after rice, wheat, corn and barley. It is a drought-resistant crop, hence it serves as a major food source in semi-arid regions of the world [17]. Sorghum is used in different African food preparations such as ogi (fermented weaning food) in Nigeria, injera (fermented pancake-like bread) in Ethiopia, nasha (infant porridge) and kisra (traditional bread) in Sudan, mahewu (fermented beverage) in Zimbabwe [18] and gowé (malted and fermented beverage) in Benin [19].

The total world sorghum production in 2013 was about 62 million metric tons (Figure 1-1). Thirty one million metric tons were used for food, alcohol, and industrial use. The United States ranks 1st and contributed around 17% of the world sorghum production. The other major producers of sorghum are Nigeria and Mexico. While majority of the countries consumed 100% of their produced sorghum, The US consumes around 54% of its production and exports the remaining grain to other countries. The US, sorghum is mainly used for animal feed and ethanol production [20, 21].

Cowpea

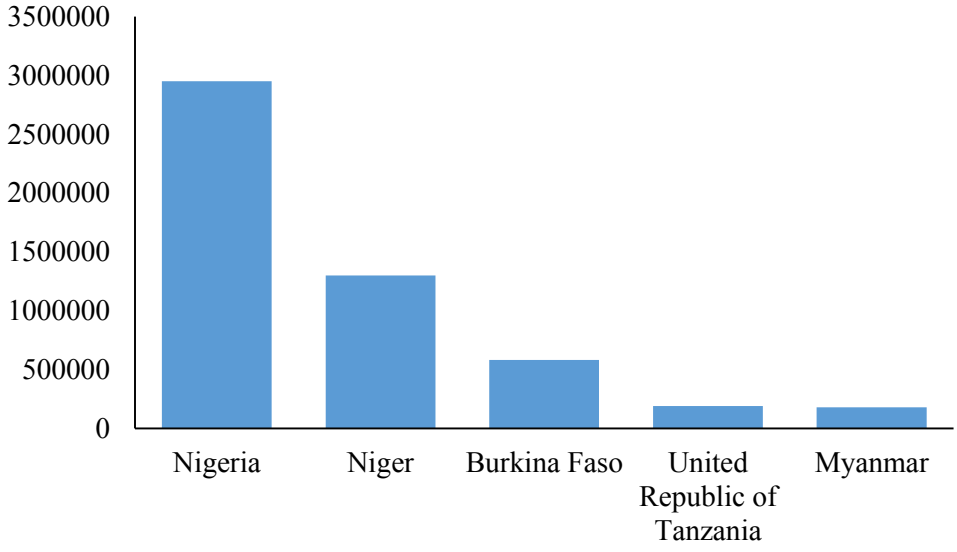
Cowpea (*Vigna unguiculata*), also called black-eyed pea, is a native crop of West Africa. Approximately 95% of world cowpea is cultivated in Africa. Cowpea is a drought-tolerant crop that adapts well even in the drier regions of tropics. Six million metric tons of cowpea were produced in 2013 (Figure 1-2). Nigeria and Niger are the two largest cowpea producers from the last 14 years. Together these two countries produce around 4 million metric tons while rest of the developing countries producing < 0.6 million metric tons [20, 22].

Figure 1-1 Sorghum production by country, tons, 2013.



Source: FAOSTAT, 2013 [20]

Figure 1-2 Cowpea production by country, tons, 2013.



Source: FAOSTAT, 2013 [20]

Antinutritional factors in sorghum and cowpea

Iron bioavailability has been shown to be poor form cereal and legume based diets [23]. Antinutritional factors such as phytates and tannins present in plant based diets form insoluble complexes with iron and reduce its bioavailability. ANFs also bind to proteins and reduce their digestibility and overall protein quality [24]. Food processing methods like soaking, germination, fermentation, cooking [25], dehulling [26], enzymatic treatments [27], chemicals [28], and extrusion [18] have been shown to decrease the ANF concentrations and improve iron bioavailability from cereals and legumes.

Phytates

Phytic acid or phytate (myoinositol hexakisphosphate, IP6) is widely present in cereals, legumes and oil seeds [29, 30]. Phytate is the major source of storage phosphorus for plants. During germination phytate is degraded, and released phosphorus is utilized for the plant growth. Phytate is mostly present in the aleurone layer in cereals and in cotyledons in legumes. Corn is different from other cereals as most of its phytate is present in germ [29].

Phytate content in a grain variety is influenced by its growing environment and genotype [31]. Phytate content in African sorghum cultivars varies greatly. When 76 locally grown sorghum varieties were analyzed, their phytate content ranged from 4.5 to 35.5 g/kg [31]. In another study, phytate content in white sorghum cultivars range from 5.6 to 6.0 g/kg [32]. Compared to other legumes, cowpea showed significantly lower levels of phytate (4.6 g/kg) compared to lima beans (9.0 g/kg), African yam bean (7.0 g/kg) and pigeon pea (6.0 g/kg). However, cowpea cultivars from India seem to have higher phytate content, the phytate content in four brown cowpea varieties ranged from 8 to 9 g/kg [33].

Tannins

Tannin is a naturally occurring polyphenol and a major ANF along with the phytate in cereal and legume based diets [34]. Tannins are mostly present in the seed coats, thus, colored varieties indicate a tannin concentration [26]. Tannin forms insoluble chelates with iron and inhibits its absorption. Tannin content of grains is also influenced by the soil where they grow [35]. When 198 Indian sorghum varieties were analyzed, the tannin content ranged from 10-2056 mg/100g. However, half of the analyzed samples had tannin values < 65 mg/100g and only 5% had values > 400 mg/100g. Indian native varieties tended to have more tannin than the hybrid and high-yielding varieties [35].

Tannin content of cowpea also varied between cultivars. Four different varieties of cowpea- blackeye, light brown, mottled brown and maroon-red cultivars had tannins ranging from 0.3-6.9 mg catechin equivalents/g. However, another variety of cowpea widely used in Egypt had higher tannin concentration of 210 mg/100g [36].

Preparation methods that reduce antinutritional factors

Household food preparation methods such as soaking, germination, fermentation and pressure cooking have been shown to decrease phytates [36]. As phytate is water soluble, soaking the grains for extended period of time leaches of this ANF. The beneficial effects of these processing methods have been related to activation of native and bacterial phytase enzyme [25].

When sorghum samples were soaked for 20 hours, phytate content was reduced by 32%. Also, germination of sorghum for 72 hours caused a similar 35% decrease in phytate content [32]. Duration of soaking and germination had an effect on extent of phytate reduction. In a sorghum-based infant porridge, phytate content decreased with increased soaking and

germination time. The phytate lowering effect was further enhanced when germination was combined with fermentation [38]. In a cowpea sample, germination for one week caused a significant 5-fold increase in native phytase enzyme activity and a 5-fold decrease in phytate content [37].

Other methods such as acid-salt washing with ultracentrifugation and phytase enzyme treatments have also been shown to be effective in reducing the phytate content to negligible amounts. When soy protein isolate was treated with continuous acid-salt washing, phytate content reduced from 8.4 to 0.2 mg/g (98%), and furthermore, a decrease from 6.5 to < 0.01 mg/g (99.8%) was observed with phytase treatment [28].

Mechanical processing such as decortication in cereals [18] and dehulling in legumes [26] has been shown to reduce the tannin content. As tannins are highly localized in the outer layers of grains [26], mechanical removal of pericarp and seed coats lowers tannin concentrations. In three Zimbabwean tannin-sorghum varieties, decortication decreased tannin levels 79 to 92% [18]. Dehulling legumes, such as cowpea, also reduced tannin content by 98% [26]. Other methods such as boiling and pressure cooking also caused 2-3 times significant reduction in the tannin content [39].

Similar to the effects on phytate, germination and fermentation reduce tannin content in cereals and legumes. Tannin content decreased from 429.5 to 174.1 mg/100 g dry matter during sorghum germination [19]. More tannin reduction was observed when germination was combined with cooking. When cowpea seeds were cooked by either ordinary or pressure cooking, tannin content reduced by approximately 40%, whereas, more than 60% reduction was observed when germinated cowpea was cooked [36].

Most of the traditional preparation methods activate native and bacterial phytase enzyme and give modest decreases in phytate content. However, complete removal of phytate is recommended to achieve higher iron bioavailability. Even though it is not possible with the existing phytate removal methods, a commercial phytase enzyme can degrade phytate completely. When phytase was added to aquatic slurry of cereals, maintained at optimum pH and temperature, it can completely degrade phytate in approximately 1-2 hours. However, the cost of 1-2 dollar cents per kg food to be dephytinized and safety regulations limit this enzyme usage. As commercial phytase is produced from *Aspergillus Niger*, which is a genetically modified organism (GMO), its usage differs among countries depending on their safety regulations [27]. In addition to this, phytase has GRAS (Generally Regarded As Safe) status only for individuals aged 3 years and above [13]. De Pee & Bloem (2009) recommends exploring phytase treatment options to improve the mineral bioavailability from FBFs by adding it during production such that the heat processing destroys the enzyme and final product that reaches the consumers will be free of phytase [13].

Effect of antinutritional factors on iron bioavailability

It is unlikely that phytate will be decreased to < 10% or completely removed to achieve maximum iron absorption with traditional methods such as soaking, germination, and fermentation. Two approaches that have achieved this affect include acid-salt washing, and phytase enzyme treatments, which were shown to reduce phytate content by 98% and 99.8%, respectively. While the difference in phytate content between the two treatments is slight, their effect on iron bioavailability is great. Acid-salt washing increased iron bioavailability by 2-fold, whereas phytase treatment increased the bioavailability by 5-fold [28].

Iron bioavailability was most influenced by phytate-to-iron molar ratio (Phyt/Fe molar ratio) rather than phytate content. Iron bioavailability was impaired at Phyt/Fe molar ratio of greater than 14 [31]. Better iron absorption was observed with Phyt/Fe molar ratio 1:1, and the absorption was much better with 0.4:1 [27]. Iron bioavailability was similar when iron fortified white cowpea meal with low polyphenol (PP) (74 mg/serving), and red cowpea meal with high PP (158 mg/serving) were administered to young women. Both cowpea varieties contained similar Phyt/Fe molar ratio. It seems that iron bioavailability of cowpea is mostly influenced by Phyt/Fe molar ratio rather than polyphenol content [40].

Extrusion

Extrusion is a high-temperature process that cooks food under high pressure in combination with moisture and mechanical shear [41]. During this heat treatment, phytates may be degraded to lower forms of inositol phosphates with lower than 5 phosphates. Extrusion technology has been shown to decrease the ANF in legumes [25] and oil seeds [42]. The desirable effects of this cost-effective method include increased starch and protein digestibility, increased palatability, decreased viscosity, and decreased ANF that results in improved iron bioavailability [25, 43-45].

Lipid levels in the food effect the extrusion process and final product texture. During extrusion starch and proteins present in the food act as structure forming biopolymers that provide necessary viscosity and elasticity for good expansion of the extrudate. Whereas, lipid ingredients or added oils provide lubrication to the flour particles and smooth flow of extrudate. However, lipid levels above a critical point, such as more than 5%, decreases the expansion ratio [46]. Hence, extrusion of high fat foods is not advisable, as they interfere with extruder

performance. As defatting improves extrusion process, degermed corn and defatted soy are commonly used in extruded food products [41, 47].

In addition to the starch, protein and lipid levels in the feed, extruder barrel temperature, screw speed and feed moisture affect the expansion ratio and bulk density of the extrudate. A higher expansion ratio, an indicator of puffiness; and a lower bulk density, an indicator of porosity, are desirable physicochemical and sensory characteristics in the final product [48]. Also, mild extrusion conditions such as low barrel temperature and high feed moisture favor nutrient retention and decrease lipid oxidation, whereas severe extrusion conditions such as, high temperature (≥ 200º C) and low moisture (≤ 15%) can cause nutrient destruction [49].

Effect of extrusion on antinutritional factors and iron bioavailability

El-Hady & Habiba (2003), studied the effect of extrusion conditions such as barrel temperature and feed moisture in reducing ANF from soaked legumes [25]. Faba beans, pea seeds, chickpeas and kidney beans were soaked for 16 hours, extruded and analyzed for ANF. Both soaking and extrusion significantly reduced phytate and tannin levels. The higher barrel temperature and higher feed moisture content of 180º C and 22% were more effective in decreasing phytate and tannin levels than lower temperature and moisture content of 140º C and 18% [25]. A maximum tannin reduction of 99% in sorghum [18] and 97% in peas and kidney beans [44] were observed with extrusion.

Extrusion processing has shown increased iron availability and protein digestibility compared to roasting [50]. Extruded and roasted African infant formulas composed of pearl millet (65%), peanut (15%) and cowpea (10%) with and without skim milk powder (4%) were subjected to in-vitro digestion and dialysates were analyzed. Extruded infant formulas showed

3.5-6.5 times higher iron availability and 10% improved protein digestibility compared to roasted formulas.

The effect of extrusion on chickpea iron bioavailability was assessed by the hemoglobin regeneration method in anemic rats and compared with home-cooking [48]. Both the methods caused improvements in iron bioavailability compared to control diets containing ferrous sulfate, with no significant difference between extrusion-cooked and home-cooked chickpea groups. The total phytate and total tannin content were not different between home-cooked and extrusion-cooked chickpeas, but IP5 levels were significantly decreased in extrusion-cooked chickpeas. Considering the observations that both IP6 and IP5 inhibit iron absorption [51], a decrease in IP5 fraction of phytate in extruded chickpea could have caused proportional increase in iron absorption compared to home-cooked. However, the small amount of phytate in chickpeas compared to other legumes may be the reason for not causing detectable improvements in iron absorption. Also, extrusion conditions such as 14% feed moisture and 130ºC barrel temperature may not be sufficient to cause a profound destruction in ANF [48].

Alonso et al (2001), studied the effect of extrusion on mineral bioavailability in pea seed and kidney bean seed meals fed to male rats. A significant increase in iron bioavailability was observed in extruded seed meals. Also, a significant reduction in total phytate (4-21%) and total tannin (70-92%) content was observed in extruded seed meal. Most importantly, extrusion cooking caused a significant decrease and partial hydrolysis of inositol hexaphosphate (IP6) to low-molecular weight phytates such as penta (IP5) and tetraphosphates (IP4) [44]. This shift towards lower phytate fractions in synergy with a maximum of 92% tannin reduction, may have caused increased iron bioavailability.

Iron fortificants

Some iron compounds are highly bioavailable, but associated with drawbacks such as development of off colors and flavors in the fortified food. Iron fortification is not effective if the food vehicle contains iron inhibitors. Under these circumstances iron salts that do not change the organoleptic properties of fortified food, and overcome the inhibitor's effect, seem to be more effective in improving the intake and iron bioavailability [52].

Elemental iron is the most commonly used commercial fortificant, and iron salts such as ferrous sulfate ($FeSO_4$) can cause undesirable organoleptic changes in the food during storage. However, there is limited information on the bioavailability of elemental iron in humans. Hoppe et al (2005), evaluated five commercially available and two developmental elemental iron powders in a randomized, double blind, cross-over study in male blood donors [53]. Subjects were given wheat rolls fortified with 100 mg $FeSO_4$ or 100 mg elemental iron powders. The relative bioavailability, measured by serum iron area under curve (AUC) values compared to $FeSO_4$, of all the seven elemental powders, ranging from 36-65%, was significantly lower than $FeSO_4$. However, when one of the elemental iron powders was administered along with 50 mg ascorbic acid (AA), the relative bioavailability was significantly increased and the absorption was similar to $FeSO_4$. This study indicates the poor bioavailability of elemental iron, hence, co-fortifying the elemental iron powders with ascorbic acid may enhance the iron bioavailability [53].

Sodium iron EDTA (NaFeEDTA) is a protected iron compound that prevents the fortificant iron from reacting with phytate present in the food or diet. NaFeEDTA not only provides supplemental iron, it also protects native iron in the diet from phytates and tannins [54]. Cowpea meal fortified with NaFeEDTA significantly increased hemoglobin levels, serum

ferritin, body iron stores and significantly decreased IDA by 47% in Ghanian school children compared with nonfortified flour [55]. This indicates the effectiveness of NaFeEDTA in improving iron absorption even in the presence of iron inhibitors inherent to cowpea. Another study among adolescent children also found significant increase in iron bioavailability (9%) with NaFeEDTA compared to $FeSO_4$ (5.5%) [56].

A recent study found increased (6.4% vs. 4.1%) iron absorption from a millet porridge fortified with 1:1 mixture of NaFeEDTA: $FeSO_4$ compared to $FeSO_4$ alone in children aged 2 to 2.5 years [57]. Using NaFeEDTA in combination with other iron compounds may be a good strategy for iron fortification of food vehicles, as NaFeEDTA increases the native iron bioavailability and the combination with other iron salts keeps the EDTA levels under acceptable limits.

Vitamin A bioavailability

The dietary forms of vitamin A are provitamin A carotenoids such as α-carotene, β-carotene and β-cryptoxanthin found in plant foods, and preformed vitamin A found in animal food sources. The vitamin A value of dietary β-carotene and α-carotene and β-cryptoxanthin is $1/12^{th}$ and $1/24^{th}$, respectively, that of retinol [58]. Insufficient intake of dietary vitamin A and or inefficient absorption can lead to vitamin A deficiency.

The bioavailability of the fat-soluble nutrients may depend on micellarization in the intestine and incorporation into chylomicrons in the enterocyte. Dietary fat stimulates pancreatic and biliary secretions necessary for lipid digestion and micelle formation. While dietary fat improves mixed micelle formation, dietary fiber may interfere with it by partitioning bile salts and fat [59]. However, soluble fiber increases vitamin A bioavailability by promoting post-absorptive conversion of β-carotene to vitamin A in Mongolian gerbils [61].

Iron bioavailability

Iron bioavailability is influenced by several factors such as iron concentration, enhancers and inhibitors of iron absorption, interaction with other dietary factors and host-related factors such as iron deficiency, iron adequacy, physiological status and disease state [67]. Dietary iron exists in two forms, as heme iron in animal foods and as non-heme iron in plant foods. Non-heme iron exists either as ferric (Fe^{3+}) or ferrous (Fe^{2+}) form. In order to be absorbed into the enterocyte iron should be in the ferrous form. While heme iron is absorbed directly into the enterocyte by endocytosis, non-heme iron is absorbed through a non-specific divalent metal transporter 1 (DMT1) expressed on the apical side of the enterocyte.

In vivo iron bioavailability assessed by human balance studies and animal absorption studies are expensive and time consuming. Differences between animal and human iron metabolism limit the use of animal models for iron bioavailability studies. Human studies using radiolabeled iron provide accurate information on iron bioavailability, however, the risk of radiation exposure limits this kind of studies [67].

In-vitro digestion/Caco-2 cell model

The parental Caco-2 cells were isolated from the colon adenocarcinoma of a 72 year old Caucasian patient during 1970 (ATCC). Even though they are originated from colon, after differentiation, usually within 2-weeks post-confluence, Caco-2 cells behave like mature enterocytes by developing brush border and expressing the digestive enzymes such as sucrase, maltase, lactase and alkaline phosphatase. Similar to enterocytes, differentiated Caco-2 cells express iron transporter DMT-1 [75]. Due to the nature of the model, it is important to use fully

differentiated Caco-2 cells to study the nutrient uptake as it affects the expression of transporters and consequent nutrient uptake by Caco-2 cells [76].

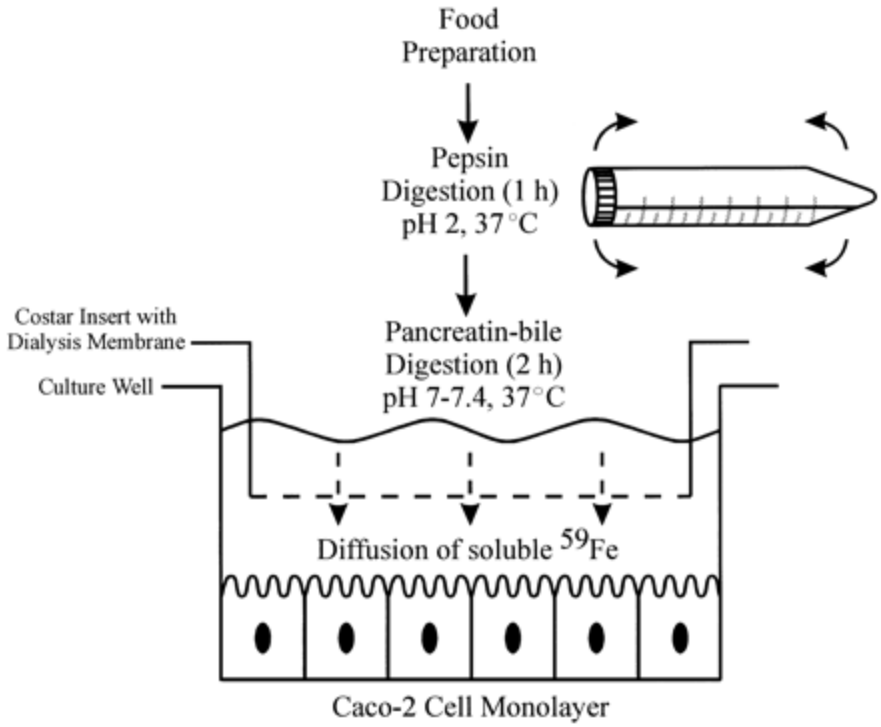
In-vitro digestion combined with Caco-2 cell model is an advanced method over other in-vitro iron availability techniques such as solubility and dialyzability. The solubility method involves exposing the food to lower pH conditions, like in stomach, and measuring the soluble iron in the digest. In the dialyzability method, simulated gastrointestinal digestion is conducted using pepsin, pancreatin and bile extracts. Then the intestinal digest is incubated in the presence of a dialysis bag. The amount of iron that moves into the bag is used to estimate iron bioavailability. Iron in the dialysate represents low molecular weight soluble iron (< 10,000) that can pass through a dialysis membrane [67].

Observations of no correlation between iron solubility and iron bioavailability lead to adding a living component of Caco-2 cells to in-vitro digestion technique to study beyond the digestion, i.e. uptake by enterocytes [77]. While in-vitro digestion determines iron availability, this technique combined with the Caco-2 cell model allows for measurement of the complete digestion and bioavailability of a nutrient.

In-vitro digestion predominantly consists of two phases. 1) Gastric or pepsin-HCl digestion- during which food samples are incubated with pepsin-HCl solution for 2 hours at pH 2 in a shaking water bath at 37°C. 2) Intestinal digestion- during which aliquots of gastric digests are incubated with pancreatin-bile extracts for 2 hours at pH 6 in a shaking water bath at 37°C, with a dialysis bag added to the beaker. At the end of the digestion, the dialysis bag is removed and the contents collected for analysis [67]. However, Glahn et al (2009), modified this method to accommodate the coupling of in-vitro digestion and Caco-2 cell model (Figure 1-3). In this new method, gastric digestion is conducted on a rocking platform shaker kept in an incubator at

37°C for 1 hour. Intestinal digestion is carried out in the top compartment of the two-chamber system in a 6-well plate with Caco-2 cells in the bottom chamber. Inserts fitted with dialysis membrane with 15 K Da molecular weight cut-off are placed in 6-well plates to create a two-chamber system. Aliquots of gastric digest are added to the top compartment of the 6-well plates kept on a rocking platform shaker with digestion continued for 2 hours. This model allows the simultaneous intestinal digestion and nutrient uptake by Caco-2 cells, similar to the intestinal physiological conditions [78]. Iron bioavailability is measured either by assessing Caco-2 cell iron uptake using atomic absorption spectrophotometer (AAS) or by determining caco-2 cell ferritin synthesis, an indirect measure of iron bioavailability.

Figure 1-3 In-vitro digestion/Caco-2 cell culture model



Source: Glahn et al, 1998 [78]

Earlier iron bioavailability studies using in-vitro digestion/Caco-2 cell model were conducted using radiolabeled iron added either intrinsically or externally to the food samples [78]. Due to the possible radiation exposure risks, later studies used stable isotopes. In-vitro digestion/Caco-2 cell model is, a cost-effective, time saving technique and also an effective method to screening foods for better bioavailable varieties [77].

In-vitro digestion/Caco-2 cell model has demonstrated similar results compared to human iron bioavailability studies [68] such as promoting effects of ascorbic acid [69], inhibitory effects of phytates and polyphenols [70] and higher iron bioavailability from animal foods such as beef and fish than from corn and green beans [71]. This model has been successfully used in studying the effects of seed coat removal [69] on iron bioavailability and for screening different cereal and legume genotypes [69, 72].

Seed coat removal has been shown to increase iron bioavailability in legumes. Eight genotypes of common bean were studied for the effect of seed coat removal and ascorbic acid on iron bioavailability [69]. Most bean varieties had poor iron bioavailability of below baseline ranging from 1.1-11.5 ng ferritin/mg protein. However, when seed coats were removed iron bioavailability increased to 10.8-31.9 ng ferritin/mg protein. Several fold increase was observed in ferritin response (68.0-148.0 ng ferritin/mg protein) when ascorbic acid was added to beans without their seed coat. Iron bioavailability may have been enhanced because most polyphenols are located in seed coats [69]. Another study conducted with 24 varieties of cooked legumes with green, red and brown seed coat color found a 2-fold increase in iron bioavailability in dehulled lentils compared to whole lentils [73].

Glahn et al (2002) demonstrated the inhibitory effect of phytic acid and tannic acid on iron uptake by Caco-2 cells in the absence of food matrix [70]. An equimolar ratio of 1:1

Fe:phytic acid resulted in 70% decrease in iron uptake, and 1:10 ratio of Fe:phytic acid Inhibiting iron absorption by 85%. Whereas tannins caused a 92% inhibition in iron uptake even at lowest ratio of 1:0.1, indicating that tannins are more potent inhibitors of iron absorption than phytic acid. However, other studies observed no correlation between iron bioavailability and phytate content [73].

Ascorbic acid has been shown increasing effect on iron bioavailability in Caco-2 uptake studies [69]. The possible explanation for this enhancing effect is that ascorbic acid promotes the reduction of ferric iron to ferrous form. Ferrous iron has low affinity to bind and form complexes with other molecules such as proteins and also has low tendency to polymerize. Hence, ascorbic acid promotes not only the soluble iron but also soluble iron with low-molecular weight that can easily be taken up by Caco-2 cells [74].

There has been only one study that examined the vitamin A bioavailability in fortified foods using in-vitro digestion/Caco-2 cell model [63]. Six different foods; minced pork, beef and turkey, apple sauce, bread and mayonnaise were supplemented with vitamin A, cooked and subjected to in-vitro digestion/Caco-2 experiment. All three meat samples retained retinol well after cooking than the other food samples. Turkey had 4-fold higher retinol in micellar fraction than the other meats, which was reflected in the Caco-2 cell uptake. Retinol content in the micellar fraction has correlated well ($R^2 = 0.99$) with Caco-2 cell uptake [63].

Methodological differences in iron bioavailability studies using in-vitro digestion/Caco-2 cell model

The in-vitro digestion/Caco-2 cell model is a widely used and well-accepted method. However, differences in digestion technique and culture conditions between laboratories make

comparison of results difficult. Due to the lack of standardization of the technique, researchers have modified the method according to the experimental design and resources. The major methodological differences are described below. A total of 17 studies using the in-vitro digestion/Caco-2 model to assess the iron bioavailability in different foods are described under this section.

Passage number

While the Caco-2 parental cell line, HTB37, is supplied at a lower passage by ATCC, most studies used the cells between passages 20 to 40. However, a few studies used cells with high passage number of 53-95 [79, 62].

Seeding density

Most authors seed cells at a density of 5 x 10^4 cell/cm^2 . Higher seeding density promotes all the areas of the Caco-2 cell monolayer reaching confluence at the same time [80].

Inserts with dialysis membrane

Iron bioavailability studies, primarily from Glahn's laboratory, developed a modified in-vitro digestion/Caco-2 model with dual chamber system. In this method, plastic inserts fitted with a 15 K Da molecular weight cut-off dialysis membrane are placed in 6-well plates to create two compartments with Caco-2 cells in the bottom one [71]. The second part of the digestion, intestinal, was conducted in the top chamber. During this time, soluble or bioaccessible iron passes through the dialysis membrane and is taken up by Caco-2 cells in the bottom chamber [69, 71]. The dialysis membrane protects the Caco-2 cells from digestive enzymes and bile salts added during digestion [81]. This two chamber arrangement, with inserts and dialysis membrane, allows for the simultaneous intestinal digestion and uptake, similar to human intestinal digestion.

The major limitation of this method is that, ferritin and heme-iron complexes cannot pass through the dialysis membrane because their molecular weight is greater than15 K Da [77].

Table 1-7 In-vitro digestion/Caco-2 cell method details of different iron bioavailability Studies.

Reference	Food sample	Passage No	Day Caco-2 cells used	Type of 6-/12-well plates / T-75-flasks	Cell density (cells/cm^2)	Two chambers/ cells treated directly	Treatment duration	Aqueous fraction (AF) /digesta	Ferritin /cell iron method
[71]	Nonradiolabeled food	25-33	13	Collagen treated	50000	Two chamber w/ dialysis membrane	24 hrs	1.5 ml digesta	IRA
[69]	Bean genotypes	25-33	13	Collagen treated	50000	Two chamber w/ dialysis membrane	25 hrs	1.5 ml digesta	IRA
[79]	Milk- based infant formulas	56-95	14-16	NI	50000	Two chamber w/ dialysis membrane	22 hrs	1 ml dialysate	ELISA
[72]	Chinese polished rice genotypes	20-43	21	Collagen coated	50000	Two chamber w/ dialysis membrane	23 hrs	1.5 ml AF	IRA
[81].	Chinese wheat flours	25	13	Collagen treated	50000	Two chamber w/ dialysis membrane	24 hrs	1.5 ml digesta	IRA
[82]	Beverage premixes	NI	14	NI	50000	Two chamber w/ dialysis membrane	24 hrs	2.0 ml digesta	ELISA
[83]	Low and high Fe bioavailable maize lines	29	13	Collagen treated	50000	Two chamber w/ dialysis membrane	24 hrs	1.5 ml digesta	IRA
[84]	Maize hemoglobin	29	15	Collagen treated 12-well plates	50000	Cells treated directly	24 hrs	0.5 ml heat treated AF	IRA
[85]	Fortified fruit beverages	30-36	14-16	NI	50000	Cells treated directly	24 hrs	1.0 ml AF	ELISA
[79]	Milk based infant formulas	56-95	14-16	NI	50000	Cells treated directly	22 hrs	1.0 ml of dialysate	ELISA
[73]	Lentils	25-33	13	Collagen treated	50000	Two chamber w/ dialysis membrane	24 hrs	1.5 ml digesta	IRA

[86]	Wheat	27-34	13	Collagen treated	47500	Two chamber w/ dialysis membrane	25 hrs	10% of the AF	ELISA
[60]	Fruit beverage	30-40	14-16	NI	50000	Cells treated directly	24 hrs	1.0 ml AF	ELISA
[62]	Infant formula	53-55	15-18	T-75 plastic flasks	50000	Cells treated directly	1 hr	18-22.0 ml AF	AAS
[64]	Micromilled wheat	28	13	NI	50000	Cells treated directly	24 hrs	1.5 ml digesta	ELISA
[65]	Soy root nodules	20-26	15	Collagenized 12-well plates	50000	Cells treated directly	24 hrs	0.5 ml AF	IRA
[66]	Complementary foods	46-47	14	Collagen treated	10000	Two chamber w/ dialysis membrane	2 hrs	1.5 ml AF	LSC

AF, aqueous fraction; IRA, immunoradiometric assay; ELISA, enzyme-linked immunosorbent assay; AAS, atomic absorption spectrophotometer; LSC, liquid scintillation counter; NI, no information whether the 6-well plates were collagen treated or not.

Incubation duration

In studies that used aqueous fraction instead of digesta, all the in-vitro digestion phases were conducted in a shaking water bath. Aliquots of digesta were centrifuged and collected supernatant aqueous fraction, usually 0.5-1.0 ml, was used to treat the Caco-2 cells. The usual treatment duration for iron bioavailability experiments was 22-24 hours. However, Latunde-Dad et al (2014) and Cilla et al (2007), allowed the food treatments on Caco-2 cells only for 2 hours, after that, treatment was replaced with Minimum Essential Medium (MEM) and the incubation was continued for additional 22 hours [85, 64]. Higher iron concentration and higher iron bioaccessibility of up to > 95% in fruit beverage samples [85], and micromilled wheat flour with reduced particle size [64] may be the reasons for choosing the short incubation time in the above studies. Another modification of incubation duration is diluting the food treatment after few hours of exposure to Caco-2 cells. In a soy root nodule iron bioavailability study, Caco-2 cells were treated with 0.5 ml of aqueous fraction for 2 hours then followed by addition of 1 ml DMEM and continued incubation for further 22 hours [65].

Dialysis bag

In studies that used dialysis bag method [79, 62], gastric digestion was conducted in shaking water bath and a dialysis bag was added during intestinal digestion. Dialysate collected into the bag at the end of the digestion was used to treat the Caco-2 cells for ferritin synthesis.

6-well or 12-well plates/T-75 flasks

While all the reviewed studies grew cells on 6-well or 12-well plates, Jovani et al (2001) cultured cells on T-75 cm^2 flasks using the similar cell density of 50000 cells/cm^2. However, they treated the cells with larger amounts of 18-22 ml of dialysate for a short period of 1 hour [62].

Heat treatment

Another method heat-treated the digesta before extracting the aqueous fraction that goes on to the cells. The major purpose of heat treating the digesta [72, 62, 65], or aqueous fraction [84], is to inactivate the proteolytic enzymes that were used during in-vitro digestion that might be harmful to the Caco-2 cells during the 24 hour long incubation. Even though the aqueous fraction was heat treated, Jovaní et al. (2001) reduced the Caco-2 cell incubation time to 1 hour [62].

Post-seeding treatment time

In majority of the studies iron bioavailability experiment was conducted on 13th day post-seeding. This time was used due to acceptable Transepithelial Electric Resistance (TEER) values that indicate Caco-2 cell monolayer integrity, consistency of well protein content [71], and full differentiation of Caco-2 cells indicated by maximal sucrase and alkaline phosphatase activity [62]. However, experiments have been conducted ranging from 13 to 21 days post-seeding in the reviewed studies. As the Caco-2 cell monolayer integrity was lost between 20 and 25 days [62], 13-20 day post-seeding seems like an optimum time period for the uptake experiment.

Biomarkers of iron bioavailability

Most studies measured Caco-2 cell ferritin, an indirect measure of iron bioavailability by using either immunoradiometric assay or enzyme-linked immunosorbent assay (ELISA). However, Jovani et al (2001), measured cell iron uptake by AAS [62], and Pynaert et al (2006) measured radiolabeled iron uptake by liquid scintillation counter [66]

The in-vitro digestion/Caco-2 technique with varied cell and culture conditions makes the comparison of nutrient bioavailability results difficult. Standardization of this technique using

reference foods and standard protocols will ensure reproducibility of results and enable inter-laboratory comparisons.

References

1. Bhutta ZA, Salam RA. Global nutrition epidemiology and trends. Ann Nutr Metab. 2012;61 Suppl 1: 19-27.

2. World Health Organization. Worldwide prevalence of anaemia 1993-2005: WHO global database on anaemia. 2008.

3. World Health Organization. Global prevalence of vitamin A deficiency in populations at risk 1995-2005: WHO global database on vitamin A deficiency. 2009.

4. Huma N, Salim-Ur-Rehman FM, Anjum MA, Murtaza M, Sheikh. Food fortification strategy--preventing iron deficiency anemia: a review. Crit Rev Food Sci Nutr. 2007;47: 259-65.

5. Pettit K, Rowley J, Brown N. Iron deficiency. 2011;21: 339-343.

6. World Health Organization. Iron deficiency anaemia: assessment, prevention and control: a guide for programme managers. 2001.

7. Ahmed T, Hossain M, Sanin KI. Global burden of maternal and child undernutrition and micronutrient deficiencies. Ann Nutr Metab. 2012;61 Suppl 1: 8-17.

8. Akhtar S, Ahmed A, Randhawa MA, Atukorala S, Arlappa N, Ismail T, et al. Prevalence of vitamin A deficiency in South Asia: causes, outcomes, and possible remedies. J Health Popul Nutr. 2013;31: 413.

9. World food programme, The International Food Aid Information System. 2012 food aid flows. 2013: 1. Available at http://documents.wfp.org/stellent/groups/public/documents/newsroom/wfp262299.pdf. Accessed 11 November, 2015.

10. Pérez-Expósito AB, Klein BP. Impact of fortified blended food aid products on nutritional status of infants and young children in developing countries. Nutr Rev. 2009;67: 706-18.

11. Fleige LE, Moore WR, Garlick PJ, Murphy SP, Turner EH, Dunn ML, et al. Recommendations for optimization of fortified and blended food aid products from the United States. Nutr Rev. 2010;68: 290-315.

12. Rosenberg I, Tilahun J, Schlossman N, Bagriansky J, Johnson Q, Webb P, et al. Nutritional enhancement of US Title II food aid products. . 2011;32: 134S-151S.

13. de Pee S, Bloem MW. Current and potential role of specially formulated foods and food supplements for preventing malnutrition among 6-to 23-month-old children and for treating moderate malnutrition among 6-to 59-month-old children. 2009;30: S434.

14. Webb P, Rogers B, Rosenberg I, Schlossman N, Wanke C, Bagriansky J, et al. Delivering improved nutrition: recommendations for changes to US food aid products and programs. 2011.

15. Marchione TJ. Foods provided through U.S. Government Emergency Food Aid Programs: policies and customs governing their formulation, selection and distribution. J Nutr. 2002;132: 2104S-2111S.

16. USDA Commodity Requirements. Corn-Soy Blend Plus for Use in International Food Aid Programs. Available at < https://www.fsa.usda.gov/Internet/FSA_File/csbp2.pdf>. Accessed on July 29, 2014.

17. Gourichon H. Analysis of incentives and disincentives for sorghum in Nigeria. Technical notes series, MAFAP, FAO, Rome. 2013.

18. Dlamini NR, Taylor JR, Rooney LW. The effect of sorghum type and processing on the antioxidant properties of African sorghum-based foods. Food Chem. 2007;105: 1412-1419.

19. Kayodé AP, Mertz C, Guyot J, Brat P, Mouquet-Rivier C. Fate of phytochemicals during malting and fermentation of type III tannin sorghum and impact on product biofunctionality. J Agric Food Chem. 2013;61: 1935-1942.

20. UN, Food and Agricultural Organization. Sorghum. Crops.FAOSTAT Domains. Prodcution. 2013. Available at http://faostat3.fao.org/browse/Q/*/E. Accessed on 27, November 2015.

21. USDA FAS. World Sorghum Production, Consumption, and Stocks, 2015. Available at http://apps.fas.usda.gov/psdonline/psdReport.aspx?hidReportRetrievalName=World+Sorghum+Production%2c+Consumption%2c+and+Stocks&hidReportRetrievalID=462&hidReportRetrievalTemplateID=7. Accessed on 27 November, 2015.

22. Akibode S, Maredia M. Global and regional trends in production, trade and consumption of food legume crops. 2011: 87.

23. Sandberg A. Bioavailability of minerals in legumes. Br J Nutr. 2002;88: 281-285.

24. Gilani GS, Cockell KA, Sepehr E. Effects of antinutritional factors on protein digestibility and amino acid availability in foods. J AOAC Int. 2005;88: 967-987.

25. El-Hady EA, Habiba R. Effect of soaking and extrusion conditions on antinutrients and protein digestibility of legume seeds. . 2003;36: 285-293.

26. Plahar W, Annan N, Nti C. Cultivar and processing effects on the pasting characteristics, tannin content and protein quality and digestibility of cowpea (Vigna unguiculata). 1997;51: 343-356.

27. Hurrell. Phytic acid degradation as a means of improving iron absorption. 2004;74: 445-452.

28. Hurrell RF, Juillerat MA, Reddy MB, Lynch SR, Dassenko SA, Cook JD. Soy protein, phytate, and iron absorption in humans. Am J Clin Nutr. 1992;56: 573-578.

29. Hídvégi M, Lásztity R. Phytic acid content of cereals and legumes and interaction with proteins. 2003;46: 59-64.

30. Helfrich A, Bettmer J. Determination of phytic acid and its degradation products by ion-pair chromatography (IPC) coupled to inductively coupled plasma-sector field-mass spectrometry (ICP-SF-MS). J Anal At Spectrom. 2004;19: 1330-1334.

31. Kayodé AP, Linnemann AR, Hounhouigan JD, Nout MJ, van Boekel MA. Genetic and environmental impact on iron, zinc, and phytate in food sorghum grown in Benin. J Agric Food Chem. 2006;54: 256-262.

32. Afify AEM, El-Beltagi HS, El-Salam SMA, Omran AA. Bioavailability of Iron, Zinc, Phytate and Phytase Activity during Soaking and Germination of White Sorghum. 2011.

33. Punia KP, Darshan. Proximate composition, phytic acid, polyphenols and digestibility (in-vitro) of four brown cowpea varieties. Int J Food Sci Nutr. 2000;51: 189-193.

34. Roos N, Sørensen JC, Sørensen H, Rasmussen SK, Briend A, Yang Z, et al. Screening for anti-nutritional compounds in complementary foods and food aid products for infants and young children. 2013;9: 47-71.

35. Radhakrishnan MR, Sivaprasad J. Tannin content of sorghum varieties and their role in iron bioavailability. J Agric Food Chem. 1980;28: 55-57.

36. Ibrahim S, Habiba R, Shatta A, Embaby H. Effect of soaking, germination, cooking and fermentation on antinutritional factors in cowpeas. 2002;46: 92-95.

37. Azeke MA, Elsanhoty RM, Egielewa SJ, Eigbogbo MU. The effect of germination on the phytase activity, phytate and total phosphorus contents of some Nigerian-grown grain legumes. J Sci Food Agric. 2011;91: 75-79.

38. Kayodé AP, Nout MJ, Bakker EJ, Van Boekel MA. Evaluation of the simultaneous effects of processing parameters on the iron and zinc solubility of infant sorghum porridge by response surface methodology. J Agric Food Chem. 2006;54: 4253-4259.

39. Deol JK, Bains K. Effect of household cooking methods on nutritional and anti nutritional factors in green cowpea (Vigna unguiculata) pods. 2010;47: 579-581.

40. Abizari A, Moretti D, Schuth S, Zimmermann MB, Armar-Klemesu M, Brouwer ID. Phytic acid-to-iron molar ratio rather than polyphenol concentration determines iron bioavailability in whole-cowpea meal among young women. J Nutr. 2012;142: 1950-1955.

41. Ilo S, Schoenlechner R, Berghofe E. Role of lipids in the extrusion cooking processes. Grasas Y Aceites. 2000;51: 97-110.

42. Imran M, Anjum FM, Butt MS, Sheikh MA. Influence of extrusion processing on tannin reduction and oil loss in flaxseed (Linum usitatissimum L.) meal. J Food Process Preserv. 2014;38: 622-629.

43. Batista KA, Prudêncio SH, Fernandes KF. Changes in the Functional Properties and Antinutritional Factors of Extruded Hard-to-Cook Common Beans (Phaseolus vulgaris, L.). J Food Sci. 2010;75: C286-C290.

44. Alonso R, Rubio L, Muzquiz M, Marzo F. The effect of extrusion cooking on mineral bioavailability in pea and kidney bean seed meals. Anim Feed Sci Technol. 2001;94: 1-13.

45. Tran QD, Hendriks WH, van der Poel, Antonius FB. Effects of extrusion processing on nutrients in dry pet food. J Sci Food Agric. 2008;88: 1487-1493.

46. Harper JM. Food extrusion. CRC Crit Rev Food Sci Nutr. 1978;11: 155-215.

47. Janes D. Methods of assessing raw materials and extrudates. 1993;12: 9-34.

48. Poltronieri F, Areas J, Colli C. Extrusion and iron bioavailability in chickpea (Cicer arietinum L.). Food Chem. 2000;70: 175-180.

49. Singh S. Nutritional aspects of food extrusion: a review. Int J Food Sci Tech. 2007;42: 916-929.

50. Cisse D, Guiro A, Diaham B, Souane M, Doumbouya N, Wade S. Effect of food processing on iron availability of African pearl millet weaning foods. Int J Food Sci Nutr. 1998;49: 375-381.

51. Sandberg A, Carlsson N, Svanberg U. Effects of Inositol Tri-, Tetra-, Penta-, and Hexaphosphates on In-vitro Estimation of Iron Availability. J Food Sci. 1989;54: 159-161.

52. Fairweather-Tait SJ, Teucher B. Iron and calcium bioavailability of fortified foods and dietary supplements. Nutr Rev. 2002;60: 360-367.

53. Hoppe M, Hulthén L, Hallberg L. The relative bioavailability in humans of elemental iron powders for use in food fortification. Eur J Nutr. 2006;45: 37-44.

54. Gibson RS, Bailey KB, Gibbs M, Ferguson EL. A review of phytate, iron, zinc, and calcium concentrations in plant-based complementary foods used in low-income countries and implications for bioavailability. 2010;31: 134-146.

55. Abizari AR, Moretti D, Zimmermann MB, Armar-Klemesu M, Brouwer ID. Whole cowpea meal fortified with NaFeEDTA reduces iron deficiency among Ghanaian school children in a malaria endemic area. J Nutr. 2012;142: 1836-1842.

56. Davidsson L, Dimitriou T, Boy E, Walczyk T, Hurrell RF. Iron bioavailability from iron-fortified Guatemalan meals based on corn tortillas and black bean paste. Am J Clin Nutr. 2002;75: 535-539.

57. Chang S, Huang Z, Ma Y, Piao J, Yang X, Zeder C, et al. Mixture of ferric sodium ethylenediaminetetraacetate (NaFeEDTA) and ferrous sulfate: an effective iron fortificant for complementary foods for young Chinese children. 2012;33: 111-116.

58. Barua. Absorption and conversion of a single oral dose of beta-carotene in corn oil to vitamin A in Sprague-Dawley rats with low reserve of vitamin A. 2003;73: 267-273.

59. Reboul E. Absorption of vitamin A and carotenoids by the enterocyte: focus on transport proteins. 2013;5: 3563-3581.

60. García-Nebot MJ, Cilla A, Alegría A, Barberá R, Lagarda MJ, Clemente G. Effect of caseinophosphopeptides added to fruit beverages upon ferritin synthesis in Caco-2 cells. Food Chem. 2010;122: 92-97.

61. Deming DM, Boileau AC, Lee CM, Erdman JW,Jr. Amount of dietary fat and type of soluble fiber independently modulate postabsorptive conversion of beta-carotene to vitamin A in mongolian gerbils. J Nutr. 2000;130: 2789-2796.

62. Jovaní M, Barberá R, Farré R, Martín de Aguilera E. Calcium, iron, and zinc uptake from digests of infant formulas by Caco-2 cells. J Agric Food Chem. 2001;49: 3480-5.

63. O'Callaghan Y, O'Brien N. Bioaccessibility, cellular uptake and transepithelial transport of α-tocopherol and retinol from a range of supplemented foodstuffs assessed using the caco-2 cell model. Int J Food Sci Tech. 2010;45: 1436-1442.

64. Latunde-Dada GO, Li X, Parodi A, Edwards C, Ellis P, Sharp PA. Micromilling enhances iron bioaccessibility from wholegrain wheat. J Agric Food Chem. 2014;62: 11222-11227.

65. Proulx AK, Reddy MB. Iron bioavailability of hemoglobin from soy root nodules using a Caco-2 cell culture model. J Agric Food Chem. 2006;54: 1518-1522.

66. Pynaert I, Armah C, Fairweather-Tait S, Kolsteren P, Van Camp J, Henauw SD. Iron solubility compared with in-vitro digestion–Caco-2 cell culture method for the assessment of iron bioavailability in a processed and unprocessed complementary food for Tanzanian infants (6–12 months). Br J Nutr. 2006;95: 721-726.

67. Miller DD, Schricker BR, Rasmussen RR, Van Campen D. An in-vitro method for estimation of iron availability from meals. Am J Clin Nutr. 1981;34: 2248-2256.

68. Siegenberg D, Baynes RD, Bothwell TH, Macfarlane BJ, Lamparelli RD, Car NG, et al. Ascorbic acid prevents the dose-dependent inhibitory effects of polyphenols and phytates on nonheme-iron absorption. Am J Clin Nutr. 1991;53: 537-541.

69. Ariza-Nieto M, Blair MW, Welch RM, Glahn RP. Screening of iron bioavailability patterns in eight bean (Phaseolus vulgaris L.) genotypes using the Caco-2 cell in-vitro model. 2007;55: 7950-7956.

70. Glahn R, Wortley G, South P, Miller D. Inhibition of iron uptake by phytic acid, tannic acid, and ZnCl2: studies using an in-vitro digestion/Caco-2 cell model. J Agric Food Chem. 2002;50: 390-5.

71. Glahn RP, Lee OA, Yeung A, Goldman MI, Miller DD. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in-vitro digestion/Caco-2 cell culture model. J Nutr. 1998;128: 1555-61.

72. He W, Feng Y, Wei Y, Yang X, Shi C, He Z, et al. Differential iron-bioavailability with relation to nutrient compositions in polished rice among selected Chinese genotypes using Caco-2 cell culture model. Int J Food Sci Nutr. 2013;64: 822-9.

73. DellaValle D, Vandenberg A, Glahn R. Seed coat removal improves iron bioavailability in cooked lentils: studies using an in-vitro digestion/Caco-2 cell culture model. J Agric Food Chem. 2013;61: 8084-9.

74. Miller DD, Berner LA. Is solubility in-vitro a reliable predictor of iron bioavailability? Biol Trace Elem Res. 1989;19: 11-24.

75. Tandy S, Williams M, Leggett A, Lopez-Jimenez M, Dedes M, Ramesh B, et al. Nramp2 expression is associated with pH-dependent iron uptake across the apical membrane of human intestinal Caco-2 cells. J Biol Chem. 2000;275: 1023-1029.

76. Sharp P. Methods and options for estimating iron and zinc bioavailability using Caco-2 cell models: benefits and limitations. 2005;75: 413-421.

77. Glahn R, McClements D, Decker E. The use of Caco-2 cells in defining nutrient bioavailability: application to iron bioavailability of foods. 2009: 340-361.

78. Glahn RP, Wien EM, Van Campen DR, Miller DD. Caco-2 cell iron uptake from meat and casein digests parallels in vivo studies: use of a novel in-vitro method for rapid estimation of iron bioavailability. J Nutr. 1996;126: 332-339.

79. Viadel B. Ferritin synthesis by Caco-2 cells as an indicator of iron bioavailability: Application to milk-based infant formulas. Food Chem. 2007;102: 925-931.

80. Sambuy Y, De Angelis I, Ranaldi G, Scarino M, Stammati A, Zucco F. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biol Toxicol. 2005;21: 1-26.

81. Lei J, Zhang Y, Chen X, Zhang M, Bai L, Huang C, et al. Assessment of iron bioavailability in ten kinds of Chinese wheat flours using an in-vitro digestion/Caco-2 cell model. Biomed Environ Sci. 2012;25: 502-8.

82. Pullakhandam R, Nair KM, Pamini H, Punjal R. Bioavailability of iron and zinc from multiple micronutrient fortified beverage premixes in Caco-2 cell model. J Food Sci. 2011;76: H38-42.

83. Tako E, Hoekenga O, Kochian L, Glahn R. High bioavailability iron maize (Zea mays L.) developed through molecular breeding provides more absorbable iron in-vitro (Caco-2 model) and in vivo (Gallus gallus). Nutr J. 2013;12: 3.

84. Bodnar A, Proulx A, Scott MP, Beavers A, Reddy M. Iron bioavailability of maize hemoglobin in a Caco-2 cell culture model. J Agric Food Chem. 2013;61: 7349-56.

85. Cilla A, Perales S, Lagarda M, Barbera R, Farre R. Iron bioavailability in fortified fruit beverages using ferritin synthesis by Caco-2 cells. J Agric Food Chem. 2008;56: 8699-703.

86. Eagling T, Wawer A, Shewry P, Zhao F, Fairweather Tait S. Iron bioavailability in two commercial cultivars of wheat: comparison between wholegrain and white flour and the effects of nicotianamine and 2'-deoxymugineic acid on iron uptake into Caco-2 cells. J Agric Food Chem. 2014;62: 10320-5.

Chapter 2 - Determination of Bioavailable Iron in Sorghum, Cowpea, Corn and Soybean Fortified Blended Foods

Abstract

Background: Fortified blended foods (FBFs), particularly corn-soybean blend (CSB), are food aid products distributed in developing countries. In order to improve FBFs, a United States Agency for International Development (USAID) Food Aid Quality Review report recommended developing novel FBFs by using local alternative commodities such as sorghum and improving the nutritional quality of FBFs by using extrusion processing.

Objective: The objective of the study was to determine the bioavailable iron levels in novel, extruded sorghum-cowpea, sorghum-soy and corn-soy FBFs compared to the traditional food aid FBFs; corn-soy blend 13 (CSB13) and corn-soy blend plus (CSB+).

Methods: Fourteen extruded FBFs; sorghum-cowpea (n=7), sorghum-soy (n=4) and corn-soy (n=3), two non-extruded FBFs; CSB13 and CSB+ were prepared as porridges and bioavailable iron levels were assessed by measuring Caco-2 cell ferritin (ng ferritin/mg cell protein) formation in response to 12-hour treatment with aqueous fractions collected from digested FBFs. Control cells were collected at time zero, to measure baseline ferritin content. Basal salt solution (BSS) containing no iron was used as a negative control. Dry FBF iron concentrations were measured on atomic absorption spectrophotometer (AAS).

Results: Iron content in extruded dry FBFs ranged from 15.6 to 21.1 mg/100g, while it is 8.0 and 31.8 mg/100g in CSB+ and CSB13, respectively. Except two, extruded FBFs contained 2- to 4-fold significantly higher ($p<0.05$) aqueous fraction iron levels than CSB13 and CSB+. Following 12-hours of aqueous fraction treatment to Caco-2 cells, there were no

significant differences in ferritin levels between newly developed extruded FBFs, traditional corn-soy FBFs nor the basal salt solution control.

Conclusions: Results suggest that consumption of newly developed extruded sorghum-cowpea, sorghum-soy and corn-soy FBFs will result in bioavailable iron levels comparable to traditional non-extruded CSB13 and CSB+ FBFs. Thus, an extruded sorghum-cowpea FBF may be a suitable alternative to corn-soybean based FBFs.

Introduction

Iron deficiency anemia (IDA) is the major nutritional problem of public health significance affecting most of the women and children throughout the world. Iron deficiency along with vitamin A, iodine and zinc deficiencies are the four major micronutrient deficiencies worldwide [1]. Globally, more than 2 billion people are at risk of these nutritional deficiencies [2].

Fortified blended foods (FBFs) are porridge mixes composed of cereals and legumes that have been milled, blended, pre-cooked and fortified with vitamins and minerals. These are the major food aid products for young children, women and other vulnerable groups in developing countries. Corn soy blend (CSB) is the most widely distributed FBF in a majority of the food aid receiving countries [3].

An United States Agency for International Development (USAID) food aid quality review (FAQR) recommended to introduce novel FBFs using cereals that are both culturally and nutritionally acceptable in Africa. It recommends sorghum as an alternative to corn or wheat because of its low-cost, drought-tolerance and cultural acceptability in Africa (FAQR recommendation 18) [4]. Cowpea is a legume that may also be beneficial component of FBFs.

Sorghum and cowpea are rich in iron, complement each other by providing the limiting amino acids lysine and cysteine missing from the other commodity, which improves protein quality [5, 6]. Sorghum and cowpea contain the antinutritional factors, phytates and tannins, which interfere with iron bioavailability [7, 8, 9, 10]. However, recent evidence indicates that individuals may adapt to the negative effects of phytates [11]. In support of this finding was an adaptive response that led to higher iron absorption from low bioavailable diet and lower absorption from high bioavailable diet in iron-replete men [12].

Extrusion is a food processing technique that cooks food using high temperature under high pressure for a short period of time in combination with moisture and mechanical shear [13]. The desirable effects of this cost-effective method are that it decreases viscosity, increases palatability, increases digestibility, and partial or total destruction of anti-nutritional factors that leads to improved iron bioavailability [14, 15].

Hence, for the current project we developed extrusion cooked novel sorghum-cowpea blend (SCB) sorghum-soy blend (SSB) and corn-soy blend (CSB) FBFs following the recommendations provided for new FBFs [4] and USDA commodity requirements [16, 17]. Traditional non-extruded FBFs; CSB13 and CSB+ were procured to use as comparisons to the newly developed FBFs. The purpose of this study was to assess the bioavailable iron levels of newly developed extruded SCB, SSB and CSB FBFs compared to traditional non-extruded FBFs; CSB13 and CSB+ in the in-vitro digestion-Caco-2 cell model. The in-vitro digestion-Caco-2 model is a widely used model to study the bioavailability of nutrients from foods and supplements [18-22]. It has been successfully used to screen different varieties of complementary foods [23], lentils [24], and grains like wheat [25]. This model is a cost-effective alternative to in-vivo studies to assess iron bioavailability that is superior to iron solubility and

dialyzability methods [26]. To the best of our knowledge, the bioavailable iron levels of FBFs has not been investigated before.

Materials and Methods

Chemicals

Unless stated otherwise, all the reagents used in the experiment were purchased from Sigma-Aldrich Corp (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA).

FBF formulations

Extruded sorghum-cowpea (n=7), sorghum-soy (n=4) and corn-soy (n=3) were formulated based on FAQR requirements [4] (Table 2-1). Two white (Fontanelle 4575, 738Y) and one red (217X Burgundy) (Delimont et al, manuscript in preparation) sorghum varieties whole or decorticated, were used in making extruded sorghum-cowpea FBFs. Cowpea flour was sourced commercially. For extruded sorghum-soy FBFs; white sorghum variety 1 (Fontanelle 4575), whole or decorticated, with low-fat (1.85%) or medium-fat (6.94%) or full-fat (16.93%) soybeans were used. For extruded corn-soy FBFs; whole or degermed corn with medium- and full-fat soybeans were used. The other ingredients; sugar, oil, whey protein concentrate with 80% protein content (WPC 80) and vitamin-mineral premix were added after extrusion to prevent destruction of micronutrients in the mix. The premix that we used provided lower levels of vitamins A, D and E than the FAQR recommended (Table 2-2). Both the non-extruded FBFs; CSB13 and CSB+ were produced by Bunge Milling (St. Louis, MO) according to the USDA commodity requirements [16, 17]. The major difference between these two is that, CSB+ is a more recent and enhanced form of CSB13 with improved micronutrient formulation [27]. Cerelac (Nestle, NJ), a commercially available fortified infant food, was purchased from a local store and used as the reference control as has been done previously [23]. The fortification form of iron, was different

between extruded and non-extruded FBFs. Ferrous sulfate ($FeSO_4$) and sodium iron EDTA (NaFeEDTA) were added in the extruded FBFs, whereas ferrous fumarate and NaFeEDTA were present in CSB+ and only ferrous fumarate was present in CSB13. All the FBFs were stored at -20°C in zip lock bags covered with aluminum foil.

FBF porridge preparation

To prevent iron contamination, glassware used in the sample preparation, in-vitro digestion and iron analysis was acid washed by soaking in 5% nitric acid solution for no less than 12 hours and rinsing with double-deionized water before use. Twenty grams of dry FBF or Cerelac was slowly added to 80 g of boiling water in a beaker on a hot plate and stirred vigorously for 2 min, removed from hot plate and stirred for another minute. Non-extruded CSB13 and CSB+ were prepared in a similar manner using 11.75 g and 13.79 g dry FBF instead and cooked for 10 min on a hot plate following the preparation instructions for CSB13 and CSB+. FBF porridge was then covered with aluminum foil and kept in water bath at 37°C for 10 min to prevent skin formation. FBF porridge was weighed and water lost during preparation was added back in to bring the final weight to 100 g.

Following cooking, Cerelac and FBF porridges were transferred to 50ml polypropylene tubes, covered in aluminum foil, blanketed with nitrogen, sealed and stored at -80°C. FBFs were cooked in duplicates on two different days. Later, these two sets of porridge were used for different in-vitro digestion/Caco-2 cell experiments.

Dry FBF and aqueous fraction iron determination

Dry FBF and aqueous fraction iron content were analyzed by atomic absorption spectrophotometer (AAS). Dry FBF and Cerelac iron concentration was analyzed (AACC 40-70, 1999) by AIB international analytical services (Manhattan, KS). Briefly, ten grams of the sample

was taken in ashing vessels and dried to ash overnight at 500°C in muffle furnace. Residue was dissolved in 10 ml of concentrated HCl, boiled and evaporated to near dryness on a hot plate. Resulting residue was redissolved in 20 ml of 2N HCl, filtered and diluted to 100 ml with water. Iron concentrations were then measured on atomic absorption spectrophotometer. For aqueous fraction iron concentrations, filtered samples were directly measured on an AAS (Perkin Elmer, AAnalyst 100).

In-vitro digestion

Aliquots of Cerelac, extruded and non-extruded FBF porridges were subjected to in-vitro digestion as described previously [28].Two and half grams of thawed FBF was weighed into a beaker and 10 ml of basal salt solution (120 mmol/L NaCl, 5 mmol/L KCl, 6 mmol/L $CaCl_2$) was added, homogenized with a laboratory homogenizer (Omni GLH, Kennesaw, GA) for 2 min, and then mixed on a magnetic stir plate for 5 min. Ten ml aliquots of homogenized FBFs were subjected to a three phase in-vitro digestion consisting of 10 min oral, 1 hour gastric and 2 hour intestinal digestion.

Oral digestion

Stock saliva solution containing 0.9 mg KCl, 0.89 mg $NaPO_4$, 0.57 mg $NaSO_4$, 0.3 mg NaCl and 1.69 mg $NaHCO_3$ /ml deionized water was prepared and used for all the experiments. Artificial saliva solution was prepared by adding uric acid (0.015 mg/ml), urea (0.2 mg/ml), mucin (0.025 mg/ml) and α–amylase (10.55 mg/ml) to stock saliva solution just before use. Ten ml of homogenized FBF solution was added to a 50 ml conical tube and 8 ml of freshly prepared artificial saliva was added. Tubes were mixed well, blanketed with nitrogen, sealed with parafilm and incubated in shaking water bath at 37°C, 85 rpm for 10 min.

Gastric digestion

After oral digestion, pH was decreased to 2.5 ± 0.1 by slowly adding 1M HCl. Then 2 ml of freshly made pepsin solution (40 mg/ml in100 mM HCl) was added to each tube. The final volume was adjusted to 40 ml with basal salt solution, blanketed with nitrogen, sealed with parafilm and incubated in shaking water bath at 37°C, 85 rpm for 1 hour. The gastric phase was terminated by increasing pH to 6.0 ± 0.1 with 1M $NaHCO_3$ and placing the tubes on ice.

Small intestinal digestion

After terminating gastric digestion, 2 ml of pancreatin (10 mg/ml in 100mM $NaHCO_3$) and lipase (5 mg/ml in 100mM $NaHCO_3$) solution and 3 ml of bile extract (40 mg/ml 100mM $NaHCO_3$) solution were added to each tube and pH was adjusted to 6.5 ± 0.1 with 1 M NaOH. The final volume was adjusted to 50 ml with basal salt solution, blanketed with nitrogen, sealed with parafilm and incubated in shaking water bath at 37°C, 85 rpm for 2 hours.

Isolation of aqueous fraction from digesta

After small intestinal digestion, 10 ml aliquots of digesta were transferred to 15 ml polypropylene tubes and centrifuged at 5000g for 45 min at 5°C. Supernatant aqueous fraction was collected by puncturing the side of the tube with an 18 gauge needle and 10 ml syringe without disturbing the pellet at the bottom of the tube. Aqueous fractions were filtered using 0.22 μm syringe filters (SLGP 033 RS, Millipore, MA), and fresh filtrate was used to treat the Caco-2 cells. Aliquots of filtered aqueous fraction were blanketed with nitrogen and stored in the dark at -80°C for later analysis.

Caco-2 cell cultures

Caco-2 cells (ATCC HTB37) purchased from American Type Culture Collection (Manassas, VA) were used in the experiment at passage 40 and 41. The cells were maintained at

37°C in an incubator with a 5% CO_2/95% humidity, and media was changed every other day. Caco-2 cells were initially cultured in growth-enhanced treated T-75 flasks (TP 90076, Midsci, MO) in the presence of Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Grand Island, NY), supplemented with 15% fetal bovine serum (Atlanta Biologicals, GA), 1% antibiotic/ antimycotic (penicillin/streptomycin) solution, 1% L-glutamine, 1% non-essential amino acid and 0.2% amphotericin B [28]. Confluent cells were subcultured by incubating with 5 ml of 0.25% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) solution for 5 min, which was then inactivated by adding 10 ml of 15% DMEM. Suspended cells were collected into 50 ml conical tubes and centrifuged at 800 rpm for 5 min at room temperature. After resuspending and counting with a hemocytometer, cells were seeded at 50,000 cells/cm^2 in tissue culture treated 6-well plates (Corning Inc, Corning, NY). After being seeded at day 0, cells were switched from 15 to 7.5% fetal bovine serum, to slow growth once confluent, usually 4-5 days after seeding. Cells were used in the iron bioavailability experiments 14 days post-seeding [29, 30].

Aqueous fraction Caco-2 treatment

A day before the experiment, on day 13, Caco-2 monolayers were supplemented with fresh media. On day 14, spent media was removed before treating the cells with 0.25 ml fresh aqueous fraction added to 1.75 ml of DMEM. Samples were randomly assigned to wells; Cerelac was used as a reference control on each plate. In-vitro digestion and Caco-2 cell culture experiments were completed in duplicate on different days using different passage cells.

Harvesting Caco-2 monolayers

After 12 hours of incubation [31, 32], treatments were removed and cells were washed with 2 ml of ice cold 2X PBS followed by 2 ml of ice cold 2 g/L EDTA in PBS. Caco-2 monolayers were scraped with a cell scraper and collected into 10 ml polystyrene tubes using 1

ml ice cold deionized water. This process was repeated twice more using 0.5 ml deionized water and collected into the same tube for a total of 2 ml of deionized water. Cells in this solution were homogenized for 2 min on ice and stored at -20°C for ferritin and protein determination that was completed within 24 hours [33, 22].

Caco-2 cell ferritin and protein determination

Ten µl cell homogenates were used for determining ferritin concentrations (ng/ml) using an enzyme-linked immunosorbent assay (ELISA) (Spectro Ferritin kit, S-22, Ramco Laboratories Inc., Stafford, TX) as done previously [24, 33]. Twenty five µl cell homogenates were used for measuring protein concentrations using Pierce bicinchoninic acid protein assay kits (Rockford, IL). Ferritin content (ng/mg cell protein) was calculated as a ratio of cell ferritin (ng/ml) / cell protein (mg/ml) [33].

Statistical analysis

Data were analyzed using one-way ANOVA with Tukey's test on SAS 9.3 (SAS Institute Inc., Cary, NC, USA), with $p < 0.05$ considered significant. Natural logs were used to transform data that did not meet the model assumptions.

Results

Iron concentration in dry FBF and aqueous fraction

Iron concentration in dry FBFs ranged from 8.0 to 31.8 mg/100g and iron levels in aqueous fractions ranged from 2.1 to 8.5 µg/10 ml (Table 2-3). The mean iron concentrations in extruded dry FBFs was higher than CSB+, but lower than CSB13. Whole white sorghum 2-cowpea FBF had higher dry FBF iron levels compared to all other FBFs except CSB13. All extruded FBFs had significantly higher levels of iron in aqueous fractions compared to CSB13

with the exception of whole corn-soy (medium fat) FBF. Also, extruded FBFs had significantly higher aqueous fraction iron levels compared to CSB+ with the exception of decorticated sorghum-soy (medium-fat) and whole corn-soy (medium fat) FBFs. There was no difference in aqueous fraction iron levels between CSB13 and CSB+.

Ferritin concentration in Caco-2 cells treated with different FBFs

There were no significant differences in ferritin levels between extruded and non-extruded FBFs (Table 2- 4). There also was no significant difference between basal salt solution alone (negative control) and FBF treatments on ferritin concentrations.

Discussion

All fourteen extruded FBFs were supplemented with iron 13mg iron/100g dry mix in the ratio of 11 mg iron from ferrous sulfate ($FeSO_4$) and 2 mg iron from sodium iron EDTA (NaFeEDTA). However, iron analysis showed differences in dry mix iron levels for some of the FBFs. These differences are most likely due to differences in intrinsic grain iron content. All of the whole sorghum-cowpea FBFs, and most of the whole sorghum-soy FBFs had slightly higher iron levels compared to their corresponding decorticated FBFs. As iron is distributed in different regions of the grain including the outer layers, it is not surprising that decortication or dehulling results in lower iron levels [34, 35]. Of the non-extruded FBFs, CSB13 had 2-fold higher iron levels (31.8 mg/100g) and CSB+ had 2-fold lower iron (8.0 mg/100g) levels compared to the extruded FBFs. Both CSB13 and CSB+ were procured from millers that prepared these FBFs following the USDA commodity requirements [16, 17], that require that CSB13 should contain 14.7 to 30.0 mg/100g iron and CSB+ should contain 9.0 to 21.0 mg/100g iron. Iron levels in

CSB13 were slightly higher than the upper limit, whereas CSB+ iron levels were slightly lower than the lower limit set by USDA.

All of the extruded FBFs contained 2- to 4-fold significantly higher aqueous fraction iron levels compared to CSB13 and CSB+ with the exception of whole corn-soy (medium fat) vs CSB13 and decorticated sorghum-soy (medium-fat) and whole corn-soy (medium fat) vs CSB+. One of the reasons for the low aqueous fraction iron concentration in CSB13 and CSB+ may be that extruded FBFs were cooked using 20% solids, whereas, CSB13 and CSB+ porridges were cooked using 11.75% and 13.79% solids. Another possibility is that the extrusion cooking reduced the anti-nutrient content [14, 36], thereby improved extruded FBFs' aqueous fraction iron levels. There was no difference in aqueous fraction iron levels between CSB13 and CSB+ in spite of a 4-fold higher iron in dry CSB13 FBF. CSB+ is an improved formulation of CSB13 with enhanced nutrient profile and with ingredients partially cooked through dry roasting [17]. This heat processing might have contributed to improved aqueous fraction iron levels in CSB+, like in previous studies that observed 16-32% increase in iron availability with roasting and malting [37]. Another reason for the improved iron availability might be that CSB+ is fortified with both ferrous fumarate and NaFeEDTA, the latter iron fortificant chelates with native iron in the diet and protects it from iron binding ANF [38, 39, 40].

Treatment with both extruded and non-extruded FBFs resulted in similar ferritin levels in the in-vitro digestion Caco-2 model. The mean ferritin concentrations ranged between 45.0-58.9 ng/mg. The ferritin values are similar to a previous study that observed 6.58-72.38 ng/mg in different wheat flours [41]. The amount of iron added to Caco-2 cells ranged from 0.05-0.21 µg/well, which was similar range levels (0.05-0.39 µg/well) found in different rice varieties that also did not find significant differences in ferritin levels [21]. Also, no differences were observed

in ferritin concentration between cells treated with basal salt solution and FBF aqueous fractions. Previous studies have also found ferritin levels that were not different from baseline levels [42, 43]. However, in Glahn et al (2002) study, after adding ascorbic acid, iron bioavailability of the rice sample significantly increased beyond the baseline levels [21]. It should be noted that extruded FBFs are fortified with 40 mg/100g ascorbic acid, and CSB13 and CSB+ are fortified with 40 mg/100g and 90 mg/100g ascorbic acid, respectively. However, we did not add exogenous ascorbic acid in our experiments.

The aqueous fraction iron levels used to treat the Caco-2 cells were not correlated to ferritin synthesis as has found previously [33, 22, 21]. One possibility is that the aqueous fraction iron may bind to low molecular weight phytates, making it unavailable for uptake and ultimately ferritin synthesis [44].

Some studies have achieved significant ferritin response by increasing the food sample quantity. Eagling et al (2014), increased the sample quantity from 0.5g to 1g and then to 3g due to low ferritin response with lower amounts (< 3g) of food [25]. For our experiment, we used only 2g porridge, as > 2g resulted in thick sample that did not digest well. It should be noted that their unleavened bread flour samples contained very low iron concentrations (0.67-4.67 mg/100g) compared to our FBF samples (8.0-31.8 mg iron/100g).

We anticipated that bioavailable iron may be higher in decorticated sorghum FBFs compared to corresponding whole FBFs because ANF localized in the outer layers of the grains will be lost during decortication [35]. In tannin-containing Seredo variety of sorghum, decortication by mechanical abrasion reduced the tannin content by 82% [45]. In another study with both tannin and non-tannin sorghum varieties, decortication reduced tannins by 79-92% [36]. However, ferritin concentrations of FBFs were not different between whole and

decorticated sorghum-cowpea blends. On the other hand, we observed a slight increase in ferritin response for decorticated sorghum-soy FBF (52.2 ng/mg) and degermed corn-soy FBF (51.2 ng/mg) compared to their corresponding whole FBFs with ferritin response 47.9 ng/mg and 47.3 ng/mg respectively.

It is difficult to compare in-vitro digestion/Caco-2 model iron bioavailability studies due to methodological differences. Some studies used two chamber arrangement with dialysis membrane [46, 47, 21, 48], while others treated the Caco-2 cells directly with aqueous fraction [33, 22, 49]. Other studies have grown cells on inserts to allow study of the iron transport across the Caco-2 monolayer [50]. Even though the Caco-2 cell density/well (50,000 cells/cm^2) is similar in majority of the studies, the day cells were used for bioavailability experiment varies. Some studies used cells grown on 6-well or 12-well plates 13 days post-seeding [25, 44], others used cells between a range of 14-16 days post-seeding [22]. In addition to the above mentioned differences, presentation of iron bioavailability data greatly varied among studies. Some authors provided iron bioavailability as a percent of control sample or $FeSO_4$ positive control [50, 41, 51, 49], whereas others provided actual ferritin values [23-25, 33].

The major limitation of our study was that we did not observe a greater ferritin response with our samples. However, many of the iron bioavailability studies also did not observe above baseline ferritin response [42, 43]. Our study also lacks specific information about levels of antinutritional factors in FBFs such as phytates and tannins that have direct relevance to iron bioavailability. Study results should be extrapolated with caution, because previous study with red and white beans that observed little ferritin response in Caco-2 cells, found significant improvements in iron status when the same samples were tested in animals [42]. However, a rat study conducted by others in our laboratory, extruded sorghum-cowpea, extruded sorghum-soy

FBFs and CSB+ showed no significant difference in hemoglobin and liver iron levels (Delimont et al, manuscript in preparation), confirming our Caco-2 results of similar iron availability among FBFs. In line with our results, Pynaert et al (2006), found significantly higher soluble iron in processed compared to unprocessed complementary foods (CF). However the iron bioavailability of processed and unprocessed CF was similar due to a proportional decrease in Caco-2 cell iron uptake from processed CF [52]. In a previous clinical study conducted with the same complementary foods, no difference in iron status was observed in infants fed processed and unprocessed CF [53].

In conclusion, our study results suggest that consumption of newly developed extruded sorghum-cowpea, sorghum-soy and corn-soy FBFs will result in iron status comparable to traditional non-extruded CSB13 and CSB+ FBFs. Our results also indicate the need for future studies looking at the reasons for poor in-vitro iron bioavailability of FBFs.

Table 2-1 Composition of extruded FBFs and non-extruded FBFs.

Ingredients (%)	**SCB**	**SSB**	**CSB**	**CSB13**	**CSB+**
Sorghum Flour	24.7	47.6			
Cowpea Flour	38.6				
Corn flour			48.1		
Corn (White or Yellow)					78.5
Cornmeal				69.5	
Whole Soybeans					20.0
Soy Flour		15.7	15.2		
Soy Flour, defatted				21.9	
Soybean oil, Refined				5.5	
Sugar	15.0	15.0	15		
Whey Protein Concentrate (WPC80)	9.5	9.5	9.5		
Vegetable Oil	9.0	9.0	9		
Vitamin & Mineral Premix	3.2	3.2	3.1		
Minerals				3.0	
Vitamin Antioxidant Premix				0.1	
Vitamin/Mineral					0.2
Tri-Calcium Phosphate					1.2
Potassium Chloride					0.2

† For extruded FBFs; SSB (full-fat soy) and CSB (full-fat soy), WPC80 was increased from 9.5% to 13% and vegetable oil was decreased from 9% to 5.5%.

Table 2-2 Macronutrient and micronutrient formulation of FBFs.

Nutrient in 100g	**Unit**	**SCB/SSB/CSB**	**CSB 13**	**CSB+**
Energy	kcal	380	386	380
Protein	g	17	16	14
Fat	g	8	9	6
Vitamin A†	mg	0.120 (0.488)	0.819	1.04
Thiamin (B_1)	mg	0.652	0.61	0.2
Riboflavin (B_2)	mg	0.933	0.481	1.4
Niacin (B_3)	mg	9.07	6.29	8.0
Pantothenic Acid (B_5)	mg	3.646	3.285	1.6
Vitamin B_6	mg	0.752	0.532	1.0
Folic Acid (B_9)	mg	0.087	0.247	0.11
Vitamin B_{12}	mg	0.0015	0.001	0.002
Vitamin D†	mg	0.0006 (0.029)	0.005	0.011
Vitamin E†	mg	10.856 (13.22)	0.98	8.3
Vitamin K	mg	0.033	0.001	0.03
Vitamin C	mg	40.0	40.2	90
Calcium	mg	279.08	650	452
Total Iron (Fe)	mg	13.0	10.6	6.5
Fe ($FeSO_4$)	mg	11.0	0.0	4.0
Fe (NAFeEDTA)	mg	2.0	0.0	2.5
Fe (Ferrous fumarate)	mg	0.0	10.6	4.0
Iodine	mg	0.23	0.0	0.04
Phosphorus	mg	290.97	522.0	290.0
Potassium	mg	163.19	563.0	140.0
Zinc	mg	5.5	5.94	5.0

† For the extruded FBFs, vitamin A, D and E values in the parentheses are the recommendations.

Table 2-3 Iron concentration in dry FBF (mean) and aqueous fraction (mean ± SEM) of extruded and non-extruded FBFs.

Cereal	Cereal type	Legume	Dry FBF (mg/100g)	Aqueous fraction (µg/10ml)
		Sorghum-Cowpea blends		
White Sorghum 1	Whole	Cowpea	16.8	6.8 ± 1.1^{ab}
White Sorghum 1	Decorticated	Cowpea	15.6	6.4 ± 0.2^{ab}
White Sorghum 1	Whole, coarse	Cowpea	16.4	6.8 ± 0.4^{ab}
White Sorghum 2	Whole	Cowpea	21.1	6.7 ± 0.6^{ab}
White Sorghum 2	Decorticated	Cowpea	16.3	6.6 ± 0.4^{ab}
Red Sorghum	Whole	Cowpea	16.8	5.7 ± 0.1^{ab}
Red Sorghum	Decorticated	Cowpea	15.9	7.3 ± 0.4^{ab}
		Sorghum-Soy blends		
White Sorghum 1	Whole	Low Fat soy	19.5	7.2 ± 0.4^{ab}
White Sorghum 1	Whole	Medium Fat Soy	16.1	8.5 ± 0.3^{ac}
White Sorghum 1	Decorticated	Medium Fat Soy	16.7	5.4 ± 0.1^{abe}
White Sorghum 1	Whole	Full Fat soy	18.2	7.3 ± 0.5^{ab}
		Corn-Soy blends		
Corn	Whole	Medium Fat Soy	16.1	5.0 ± 0.2^{bde}
Corn	Degermed, coarse	Medium Fat Soy	16.1	7.0 ± 0.4^{ab}
Corn	Whole	Full Fat soy	16.0	7.4 ± 0.1^{ab}
CSB13	Corn meal	Defatted Soy flour	31.8	2.1 ± 0.1^{d}
CSB Plus	Whole corn	Whole soy	8.0	2.4 ± 0.1^{de}

Within a column, means without a common superscript are significantly different ($p<0.05$).

Table 2-4 Caco-2 cell ferritin levels following aqueous fraction treatment (n=2, mean ± SEM).

Cereal	Cereal type	Legume	Ferritin (ng/mg protein)
Sorghum-Cowpea blends			
White Sorghum 1	Whole	Cowpea	54.8 ± 17.3
White Sorghum 1	Decorticated	Cowpea	49.2 ± 7.9
White Sorghum 1	Whole, Coarse	Cowpea	45.0 ± 12.2
White Sorghum 2	Whole	Cowpea	53.3 ± 9.7
White Sorghum 2	Decorticated	Cowpea	55.4 ± 12.3
Red Sorghum	Whole	Cowpea	58.2 ± 18.8
Red Sorghum	Decorticated	Cowpea	51.5 ± 5.1
Sorghum-Soy blends			
White Sorghum 1	Whole	Low Fat soy	58.9 ± 13.3
White Sorghum 1	Whole	Medium Fat Soy	47.9 ± 4.1
White Sorghum 1	Decorticated	Medium Fat Soy	52.2 ± 12.7
White Sorghum 1	Whole	Full Fat soy	45.2 ± 9.2
Corn-Soy blends			
Corn	Whole	Medium Fat Soy	47.3 ± 10.3
Corn	Degermed, Coarse	Medium Fat Soy	51.2 ± 8.3
Corn	Whole	Full Fat soy	50.4 ± 5.3
CSB13	Corn meal	Defatted Soy flour	48.2 ± 7.4
CSB+	Whole corn	Whole soy	52.6 ± 10.5
Control sample			
Basal salt solution			52.8 ± 6.9

References

1. Ahmed T, Hossain M, Sanin KI. Global burden of maternal and child undernutrition and micronutrient deficiencies. Ann Nutr Metab. 2012;61 Suppl 1: 8-17.

2. Bhutta ZA, Salam RA. Global nutrition epidemiology and trends. Ann Nutr Metab. 2012;61 Suppl 1: 19-27.

3. Fleige LE, Moore WR, Garlick PJ, Murphy SP, Turner EH, Dunn ML, et al. Recommendations for optimization of fortified and blended food aid products from the United States. Nutr Rev. 2010;68: 290-315.

4. Webb P, Rogers B, Rosenberg I, Schlossman N, Wanke C, Bagriansky J, et al. Delivering improved nutrition: recommendations for changes to US food aid products and programs. 2011.

5. Henley E, Rooney L, Dahlberg J, Bean S, Weller C, Turner N, et al. Sorghum: an ancient, healthy and nutritious old world cereal. JPZ Consulting Group: St.Louis, MI, USA 2010.

6. Akibode S, Maredia M. Global and regional trends in production, trade and consumption of food legume crops. 2011: 87.

7. Kayodé AP, Linnemann AR, Hounhouigan JD, Nout MJ, van Boekel MA. Genetic and environmental impact on iron, zinc, and phytate in food sorghum grown in Benin. J Agric Food Chem. 2006;54: 256-262.

8. Punia KP, Darshan. Proximate composition, phytic acid, polyphenols and digestibility (in-vitro) of four brown cowpea varieties. Int J Food Sci Nutr. 2000;51: 189-193.

9. Radhakrishnan MR, Sivaprasad J. Tannin content of sorghum varieties and their role in iron bioavailability. J Agric Food Chem. 1980;28: 55-57.

10. Plahar W, Annan N, Nti C. Cultivar and processing effects on the pasting characteristics, tannin content and protein quality and digestibility of cowpea (Vigna unguiculata). 1997;51: 343-356.

11. Armah SM, Boy E, Chen D, Candal P, Reddy MB. Regular consumption of a high-phytate diet reduces the inhibitory effect of phytate on nonheme-iron absorption in women with suboptimal iron stores. J Nutr. 2015;145: 1735-1739.

12. Hunt JR, Roughead ZK. Adaptation of iron absorption in men consuming diets with high or low iron bioavailability. Am J Clin Nutr. 2000;71: 94-102.

13. Ilo S, Schoenlechner R, Berghofe E. Role of lipids in the extrusion cooking processes. Grasas Y Aceites. 2000;51: 97-110.

14. Alonso R, Rubio L, Muzquiz M, Marzo F. The effect of extrusion cooking on mineral bioavailability in pea and kidney bean seed meals. Anim Feed Sci Technol. 2001;94: 1-13.

15. Tran QD, Hendriks WH, van der Poel, Antonius FB. Effects of extrusion processing on nutrients in dry pet food. J Sci Food Agric. 2008;88: 1487-1493.

16. USDA Commodity Requirements C. Corn-Soy Blend for use in export programs. Available at https://www.fsa.usda.gov/Internet/FSA_File/csb13_110507.pdf. Accessed on September 29, 2015.

17. USDA Commodity Requirements C. Corn-Soy Blend Plus for Use in International Food Aid Programs. Available at https://www.fsa.usda.gov/Internet/FSA_File/csbp2.pdf. Accessed on July 29, 2014.

18. Sandberg A. The use of Caco-2 cells to estimate Fe absorption in humans--A critical appraisal. Int J Vitam Nutr Res. 2010;80: 307-13.

19. Pullakhandam R, Nair KM, Pamini H, Punjal R. Bioavailability of iron and zinc from multiple micronutrient fortified beverage premixes in Caco-2 cell model. J Food Sci. 2011;76: H38-42.

20. Glahn RP, Lee OA, Yeung A, Goldman MI, Miller DD. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in-vitro digestion/Caco-2 cell culture model. J Nutr. 1998;128: 1555-61.

21. Glahn R, Cheng Z, Welch R, Gregorio G. Comparison of iron bioavailability from 15 rice genotypes: studies using an in-vitro digestion/caco-2 cell culture model. J Agric Food Chem. 2002;50: 3586-91.

22. Viadel B. Ferritin synthesis by Caco-2 cells as an indicator of iron bioavailability: Application to milk-based infant formulas. Food Chem. 2007;102: 925-931.

23. Lung'aho MG, Glahn R. In-vitro estimates of iron bioavailability in some Kenyan complementary foods. Food Nutr Bull. 2009;30: 145-52.

24. DellaValle D, Vandenberg A, Glahn R. Seed coat removal improves iron bioavailability in cooked lentils: studies using an in-vitro digestion/Caco-2 cell culture model. J Agric Food Chem. 2013;61: 8084-9.

25. Eagling T, Wawer A, Shewry P, Zhao F, Fairweather Tait S. Iron bioavailability in two commercial cultivars of wheat: comparison between wholegrain and white flour and the effects of nicotianamine and 2'-deoxymugineic acid on iron uptake into Caco-2 cells. J Agric Food Chem. 2014;62: 10320-5.

26. Fairweather-Tait, Wortley, Harvey, Glahn. The use of solubility, dialyzability, and Caco-2 cell methods to predict iron bioavailability. 2007;77: 158-165.

27. General Food Distribution, Module 11. Part2: Technical Notes. 2011. Available at http://www.unscn.org/layout/modules/htp/pdf/M11P2.pdf. Accessed on September,29,2015.

28. Garrett DA, Failla ML, Sarama RJ. Development of an in-vitro digestion method to assess carotenoid bioavailability from meals. J Agric Food Chem. 1999;47: 4301-9.

29. Jin F, Frohman C, Thannhauser T, Welch R, Glahn R. Effects of ascorbic acid, phytic acid and tannic acid on iron bioavailability from reconstituted ferritin measured by an in-vitro digestion-Caco-2 cell model. Br J Nutr. 2009;101: 972-81.

30. Etcheverry P, Miller D, Glahn R. A low-molecular-weight factor in human milk whey promotes iron uptake by Caco-2 cells. J Nutr. 2004;134: 93-8.

31. Liu C, Glahn RP, Liu RH. Assessment of carotenoid bioavailability of whole foods using a Caco-2 cell culture model coupled with an in-vitro digestion. J Agric Food Chem. 2004;52: 4330-4337.

32. Gallardo-Guerrero L, Gandul-Rojas B, Mínguez-Mosquera MI. Digestive stability, micellarization, and uptake by Caco-2 human intestinal cell of chlorophyll derivatives from different preparations of pea (Pisum sativum L.). J Agric Food Chem. 2008;56: 8379-8386.

33. Cilla A, Perales S, Lagarda M, Barbera R, Farre R. Iron bioavailability in fortified fruit beverages using ferritin synthesis by Caco-2 cells. J Agric Food Chem. 2008;56: 8699-703.

34. Ghavidel RA, Prakash J. The impact of germination and dehulling on nutrients, antinutrients, in-vitro iron and calcium bioavailability and in-vitro starch and protein digestibility of some legume seeds. 2007;40: 1292-1299.

35. Lestienne I, Buisson M, Lullien-Pellerin V, Picq C, Trèche S. Losses of nutrients and anti-nutritional factors during abrasive decortication of two pearl millet cultivars (Pennisetum glaucum). Food Chem. 2007;100: 1316-1323.

36. Dlamini NR, Taylor JR, Rooney LW. The effect of sorghum type and processing on the antioxidant properties of African sorghum-based foods. Food Chem. 2007;105: 1412-1419.

37. Gahlawat P, Sehgal S. In-vitro starch and protein digestibility and iron availability in weaning foods as affected by processing methods. 1994;45: 165-173.

38. Chang S, Huang Z, Ma Y, Piao J, Yang X, Zeder C, et al. Mixture of ferric sodium ethylenediaminetetraacetate (NaFeEDTA) and ferrous sulfate: an effective iron fortificant for complementary foods for young Chinese children. 2012;33: 111-116.

39. Gibson RS, Bailey KB, Gibbs M, Ferguson EL. A review of phytate, iron, zinc, and calcium concentrations in plant-based complementary foods used in low-income countries and implications for bioavailability. 2010;31: 134-146.

40. Bothwell, MacPhail. The potential role of NaFeEDTA as an iron fortificant. 2004;74: 421-434.

41. Lei J, Zhang Y, Chen X, Zhang M, Bai L, Huang C, et al. Assessment of iron bioavailability in ten kinds of Chinese wheat flours using an in-vitro digestion/Caco-2 cell model. Biomed Environ Sci. 2012;25: 502-8.

42. Tan SY, Yeung CK, Tako E, Glahn RP, Welch RM, Lei X, et al. Iron bioavailability to piglets from red and white common beans (Phaseolus vulgaris). J Agric Food Chem. 2008;56: 5008-5014.

43. Tako E, Beebe SE, Reed S, Hart JJ, Glahn RP. Polyphenolic compounds appear to limit the nutritional benefit of biofortified higher iron black bean (Phaseolus vulgaris L.). Nutr J. 2014;13: 28-2891-13-28.

44. Glahn R, Wortley G, South P, Miller D. Inhibition of iron uptake by phytic acid, tannic acid, and ZnCl2: studies using an in-vitro digestion/Caco-2 cell model. J Agric Food Chem. 2002;50: 390-5.

45. Yetneberk S, Rooney LW, Taylor J. Improving the quality of sorghum injera by decortication and compositing with tef. J Sci Food Agric. 2005;85: 1252-1258.

46. Bejjani S, Pullakhandam R, Punjal R, Nair KM. Gastric digestion of pea ferritin and modulation of its iron bioavailability by ascorbic and phytic acids in caco-2 cells. World J Gastroenterol. 2007;13: 2083-8.

47. DellaValle D, Glahn R. Differences in relative iron bioavailability in traditional Bangladeshi meal plans. Food Nutr Bull. 2014;35: 431-9.

48. Tako E, Blair M, Glahn R. Biofortified red mottled beans (*Phaseolus vulgaris L.*) in a maize and bean diet provide more bioavailable iron than standard red mottled beans: studies in poultry (*Gallus gallus*) and an in-vitro digestion/Caco-2 model. Nutr J. 2011;10: 113. doi: 10.1186/1475-2891-10-113.

49. Bodnar A, Proulx A, Scott MP, Beavers A, Reddy M. Iron bioavailability of maize hemoglobin in a Caco-2 cell culture model. J Agric Food Chem. 2013;61: 7349-56.

50. He W, Feng Y, Wei Y, Yang X, Shi C, He Z, et al. Differential iron-bioavailability with relation to nutrient compositions in polished rice among selected Chinese genotypes using Caco-2 cell culture model. Int J Food Sci Nutr. 2013;64: 822-9.

51. Proulx AK, Reddy MB. Iron bioavailability of hemoglobin from soy root nodules using a Caco-2 cell culture model. J Agric Food Chem. 2006;54: 1518-1522.

52. Pynaert I, Armah C, Fairweather-Tait S, Kolsteren P, Van Camp J, Henauw SD. Iron solubility compared with in-vitro digestion–Caco-2 cell culture method for the assessment of iron bioavailability in a processed and unprocessed complementary food for Tanzanian infants (6–12 months). Br J Nutr. 2006;95: 721-726.

53. Mamiro PS, Kolsteren PW, van Camp JH, Roberfroid DA, Tatala S, Opsomer AS. Processed complementary food does not improve growth or hemoglobin status of rural tanzanian infants from 6-12 months of age in Kilosa district, Tanzania. J Nutr. 2004;134: 1084-1090.

Chapter 3 - Determination of Bioavailable Iron and Vitamin A in Sorghum, Cowpea, Corn and Soybean Fortified Blended Foods - Cell Lysis Buffer Method

Abstract

Background: Fortified blended foods (FBFs), particularly corn-soybean blend (CSB), are food aid products distributed in developing countries. In order to improve the FBFs, a United States Agency for International Development (USAID) Food Aid Quality Review report recommended developing novel FBFs using local alternative commodities such as sorghum and improving the nutritional quality of FBFs by using extrusion processing. Given the high prevalence of iron and vitamin A deficiency in Africa, and that the continent receives almost 70% of global food aid, it is important to determine the bioavailable levels of these two micronutrients in FBFs.

Objective: The objective of the study was to determine bioavailable iron and vitamin A levels, from these novel, extruded sorghum-cowpea, sorghum-soy and corn-soy FBFs compared to the traditional food aid FBFs; corn-soy blend 13 (CSB13) and corn-soy blend plus (CSB+).

Methods: Eleven extruded FBFs; sorghum-cowpea (n=7), sorghum-soy (n=3) and corn-soy (n=1), two non-extruded FBFs; CSB13 and CSB+, and Cerelac, a commercial infant cereal, were prepared as porridges, and bioavailable iron and vitamin A levels were assessed using the in-vitro digestion/Caco-2 cell model by measuring Caco-2 cell ferritin (ng ferritin/mg cell protein) and vitamin A (µg /g cells) levels in response to 12-hour and 4-hour treatments, respectively, with aqueous fractions from digested FBFs. Cerelac was used as a control on each plate. Control cells collected at time zero, 0.1 and 0.2 µg iron ferrous sulfate ($FeSO_4$) solutions, and basal salt

solution (BSS), were used as baseline, positive, and negative controls, respectively. Dry FBF iron concentrations were measured using a atomic absorption spectrophotometer (AAS). Vitamin A was extracted from dry FBFs, aqueous fractions and Caco-2 cell pellets and analyzed using HPLC.

Results: Ion and vitamin A content in Cerelac and dry FBFs ranged from 8.0 to 31.8 mg/100g and 0.3 to 1.67 mg/100g, respectively. All of the extruded FBFs contained 4- to 7-fold significantly higher ($p<0.05$) aqueous fraction iron concentrations compared to CSB13 and CSB+. There were no significant differences in aqueous fraction vitamin A content. There were no significant differences in Caco-2 cell ferritin and vitamin A levels between extruded FBFs, non-extruded FBFs, nor the basal salt solution control. Iron sulfate ($FeSO_4$) positive control treatments significantly increased ($p<0.05$) ferritin levels compared to all FBFs except CSB+ and CSB14.

Conclusions: The significant differences in aqueous fraction iron in extruded compared to non-extruded FBFs did not result in similar responses in Caco-2 cells after treatment. Our results support that consumption of newly developed extruded sorghum-cowpea, sorghum-soy and corn-soy FBFs will result in bioavailable iron and vitamin A levels comparable to traditional non-extruded CSB13 and CSB+ FBFs. Thus, an extruded sorghum-cowpea FBF may be a suitable alternative to corn-soybean based FBFs.

Introduction

In food aid recipients in Africa, iron deficiency anemia (IDA) and vitamin A deficiency are the most prevalent micronutrient deficiencies [1]. Both IDA and vitamin A deficiency are the nutritional problems of public health significance affecting most of the women and children throughout the world [2]. Globally, more than 2 billion people are at risk for vitamin A, iodine

and iron deficiencies with higher prevalence in Southeast Asia and sub-Saharan Africa. More than one micronutrient deficiency exists in most of the settings, indicating the need to address multiple micronutrient deficiencies [3].

Fortified blended foods (FBFs) are porridge mixes composed of cereals and legumes that have been milled, blended and fortified with vitamins and minerals, that are the major food aid products for young children, women and other vulnerable groups in developing countries. Corn-soy blend (CSB) is the most widely distributed FBF in a majority of the food aid receiving countries [4]. A United States Agency for International Development (USAID) food aid quality review (FAQR) recommended developing novel FBFs using cereals that are both culturally and nutritionally acceptable in Africa. It recommends sorghum as an alternative to corn or wheat (FAQR recommendation 18) [5]. Cowpea is a legume that may also be beneficial component of FBFs. Both sorghum and cowpea are drought-tolerant, sustainable and cost-effective.

Sorghum and cowpea are rich in iron, complement each other by providing the limiting amino acids lysine and cysteine missing from the other commodity, which improves protein quality [6, 7]. Sorghum and cowpea contain the antinutritional factors, phytates and tannins, which interfere with iron bioavailability [8-11]. However, recent evidence indicates that individuals may adapt to the negative effects of phytates [12]. In support of this finding was an adaptive response that led to higher iron absorption from low bioavailable diet and lower absorption from high bioavailable diet in iron-replete men [13].

Extrusion is a food processing technique that cooks food using high temperature under high pressure for a short period of time in combination with moisture and mechanical shear [14]. The desirable effects of this cost-effective method are that it decreases viscosity, increases

palatability, increases digestibility, and partial or total destruction of anti-nutritional factors that leads to improved iron bioavailability [15, 16].

For the current project, we developed extruded novel sorghum-cowpea blend (SCB) sorghum-soy blend (SSB) and corn-soy blend (CSB) FBFs based on the new FBF recommendation [5] and USDA commodity requirements [17, 18]. Traditional non-extruded FBFs; CSB13 and CSB+ were procured to use as comparisons for the newly developed FBFs. The purpose of this study was to assess the in-vitro bioavailable iron and vitamin A levels of newly developed extruded SCB, SSB and CSB FBFs compared to traditional non-extruded FBFs; CSB13 and CSB+ in the in-vitro digestion/Caco-2 cell model. The in-vitro digestion Caco-2 model is a widely used, simple and inexpensive model to study the bioavailability of nutrients from foods and supplements [19-23]. It has been successfully used to screen different varieties of complementary foods [24], lentils [25], wheat [26], cassava [27] and supplemented food stuffs [28]. This is the first study to develop novel sorghum based FBFs, and to the best of our knowledge, both iron and vitamin A in-vitro bioavailability of FBFs has not been assessed previously.

Materials and Methods

Chemicals

Unless stated otherwise, all the reagents used in the experiment were purchased from Sigma-Aldrich Corp (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA). Water used for porridge preparation, in-vitro digestion, reagent preparation and vitamin A extraction was double deionized. To prevent iron contamination, glassware used in the sample preparation, in-vitro digestion and iron analysis were acid washed by soaking in 5% nitric acid solution for no

less than 12 hours and rinsing with double deionized water before use. HPLC grade acetonitrile, methanol, chloroform, hexane and ethanol were used.

FBF formulations

Extruded sorghum-cowpea (n=7), sorghum-soy (n=3) and corn-soy (n=1) were formulated based on FAQR requirements [5] (Table 3-1). Two white (Fontanelle 4575, 738Y) and one red (217X Burgundy) sorghum varieties, whole or decorticated, were used in making extruded sorghum-cowpea FBFs (Delimont et al, manuscript in preparation). Cowpea flour was sourced commercially. For extruded sorghum-soy FBFs; white sorghum variety 1 (Fontanelle 4575), whole or decorticated, with low-fat (1.85%) medium-fat (6.94%) or full-fat (16.93%) soybeans were used. For extruded corn-soy FBFs; whole or degermed corn with medium- and full-fat soybeans were used. The other ingredients; sugar, oil, whey protein concentrate with 80% protein content (WPC 80) and vitamin-mineral premix were added after extrusion to prevent destruction of micronutrients in the mix (Table 3-2). Both the non-extruded FBFs; CSB13 and CSB+ were produced by Bunge Milling (St. Louis, MO) according to the USDA commodity requirements [17, 18]. The major difference between these two is that, CSB+ is a more recent and processed form of CSB13 with improved micronutrient formulation [29]. Cerelac (Nestle, NJ), a commercially available fortified infant food, was purchased from a local store and used as the reference control as has been done previously [24]. The fortification form of iron and vitamins A were different between Cerelac, extruded and non-extruded FBFs. Retinyl palmitate was added in extruded and non-extruded FBFs, whereas retinyl acetate was added in the Cerelac. For iron, ferrous sulfate ($FeSO_4$) and sodium iron EDTA (NaFeEDTA) were added in the extruded FBFs, whereas ferrous fumarate and NaFeEDTA was added in CSB+ and only ferrous

fumarate was added in CSB13 and Cerelac. All the FBFs were stored at -20°C in zip lock bags covered with aluminum foil.

FBF porridge preparation

Twenty grams of dry FBF or Cerelac was slowly added to 80 g of boiling water in a beaker on a hot plate and stirred vigorously for 2 min, removed from hot plate and stirred for another minute. Non-extruded CSB13 and CSB+ were prepared in a similar manner but 11.75 g and 13.79 g dry FBF, respectively, was used and they were cooked for 10 min on a hot plate with 11.75 g and 13.79 g dry FBF, respectively. These changes were made following the preparation instructions for CSB13 and CSB+ [17, 18]. FBF porridge was then covered with aluminum foil and kept in water bath at 37°C for 10 min to prevent skin formation. FBF porridge was weighed and water lost during preparation was added back in to bring the final weight to 100 g.

Following cooking, Cerelac and FBF porridges were transferred to 50 ml polypropylene tubes, covered in aluminum foil, blanketed with nitrogen, sealed and stored at -80°C until analysis. FBFs were cooked in duplicates on two different days. Later, these two sets of porridge were used for in-vitro digestion/Caco-2 cell experiments.

In-vitro digestion

Aliquots of Cerelac, extruded and non-extruded FBF porridges were subjected to in-vitro digestion as described previously [30].Two and half grams of thawed FBF was weighed into a beaker and 10 ml of basal salt solution (120 mmol/L NaCl, 5 mmol/L KCl, 6 mmol/L $CaCl_2$) was added, homogenized with a laboratory homogenizer (Omni GLH, Kennesaw, GA) for 2 min, and then mixed on a magnetic stir plate for 5 min. Ten ml aliquots of homogenized FBFs were subjected to a three phase in-vitro digestion consisting of 10 min oral, 1 hour gastric and 2 hour intestinal digestion.

Oral digestion

Stock saliva solution containing 0.9 mg KCl, 0.89 mg $NaPO_4$, 0.57 mg $NaSO_4$, 0.3 mg NaCl and 1.69 mg $NaHCO_3$ /ml deionized water was prepared and used for all the experiments. Artificial saliva solution was prepared by adding uric acid (0.015 mg/ml), urea (0.2 mg/ml), mucin (0.025 mg/ml) and α–amylase (10.55 mg/ml) to stock saliva solution just before use. Ten ml of homogenized FBF solution was added to a 50 ml conical tube and 8 ml of freshly prepared artificial saliva was added. Tubes were mixed well, blanketed with nitrogen, sealed with parafilm and incubated in shaking water bath at 37°C, 85 rpm for 10 min.

Gastric digestion

After oral digestion, pH was decreased to 2.5 ± 0.1 by slowly adding 1M HCl. Then 2 ml of freshly made pepsin solution (40 mg/ml in100mM HCl) was added to each tube. The final volume was adjusted to 40 ml with basal salt solution, blanketed with nitrogen, sealed with parafilm and incubated in a shaking water bath at 37°C, 85 rpm for 1 hour. The gastric phase was terminated by increasing pH to 6.0 ± 0.1 with 1M $NaHCO_3$ and placing the tubes on ice.

Small intestinal digestion

After terminating gastric digestion, 2 ml of pancreatin (10 mg/ml in 100mM $NaHCO_3$) and lipase (5 mg/ml in 100mM $NaHCO_3$) solution and 3 ml of bile extract (40 mg/ml 100mM $NaHCO_3$) solution were added to each tube and pH was adjusted to 6.5 ± 0.1 with 1 M NaOH. The final volume was adjusted to 50 ml with basal salt solution, blanketed with nitrogen, sealed with parafilm and incubated in shaking water bath at 37°C, 85 rpm for 2 hours.

Isolation of aqueous fraction from digesta

After small intestinal digestion, 10 ml aliquots of digesta were transferred to 15 ml polypropylene tubes and centrifuged at 5000g for 45 min at 5°C. Supernatant aqueous fraction was collected by puncturing the side of the tube with an 18 gauge needle and 10 ml syringe without disturbing the pellet at the bottom of the tube. Aqueous fractions were filtered using 0.22 μm syringe filters (SLGP 033 RS, Millipore, MA), and the fresh filtrate was used to treat the Caco-2 cells. Aliquots of filtered aqueous fraction were blanketed with nitrogen and stored in the dark at -80°C for later analysis.

Dry FBF and aqueous fraction iron determination

Dry FBF and aqueous fraction iron content was analyzed by atomic absorption spectrophotometer (AAS). Dry FBF and Cerelac iron levels was analyzed (AACC 40-70, 1999) by AIB International Analytical Services (Manhattan, KS). Briefly, ten grams of sample was taken in ashing vessels and dried to ash overnight at 500°C in a muffle furnace. Residue was dissolved in 10 ml of concentrated HCl, boiled and evaporated to near dryness on a hot plate. Resulting residue was redissolved in 20 ml of 2N HCl, filtered and diluted to 100 ml with water. Iron concentrations were then measured on an atomic absorption spectrophotometer. Aqueous fraction iron concentrations, filtered samples were directly measured on an AAS (Perkin Elmer, AAnalyst 100).

Caco-2 cell cultures

Caco-2 cells (ATCC® HTB37™) purchased from American Type Culture Collection (Manassas, VA) were used in the experiment at passage 32 and 33. The cells were maintained at 37 °C in an incubator with a 5% CO_2/95% humidity, and media was changed every other day. Caco-2 cells were initially cultured in growth-enhanced treated T-75 flasks (TP 90076, Midsci,

MO) in the presence of Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Grand Island, NY), supplemented with 15% fetal bovine serum (Atlanta Biologicals, GA), 1% antibiotic/ antimycotic (penicillin/streptomycin) solution, 1% L-glutamine, 1% non-essential amino acid and 0.2% amphotericin B [30]. Confluent cells were subcultured by incubating with 5 ml of 0.25% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) solution for 5 min, which was then inactivated by adding 10 ml of 15% DMEM. Suspended cells were collected into 50 ml conical tubes and centrifuged at 800 rpm for 5 min at room temperature. After resuspending and counting with a hemocytometer, cells were seeded at 50,000 cells/cm^2 in tissue culture treated 6-well plates (Corning Inc, Corning, NY). After being seeded at day 0, cells were switched from 15 to 7.5% fetal bovine serum, to slow growth once confluent, usually 4-5 days after seeding. Cells were used in the iron and vitamin A bioavailability experiments 14 days post-seeding [31, 32].

Aqueous fraction Caco-2 treatment for bioavailable iron experiment

A day before the experiment, on day 13, Caco-2 monolayers were supplemented with fresh media. On day 14, spent media was removed before treating the cells with 0.25 ml fresh aqueous fraction added to 1.75 ml of DMEM. Samples were randomly assigned to wells; Cerelac was used as a reference control on each plate. A negative control with 0.25 ml of basal salt solution containing no iron and 1.75 ml of DMEM was used to treat Caco-2 cells. A positive control was prepared with 100 mg of $FeSO_4.7H_2O$ in 100ml of basal salt solution. This was serial diluted to provide 100 µg/ml. Cells were treated with 5 µl or 10 µl of the diluted $FeSO_4$ solution to provide 0.1 µg iron/well or 0.2 µg iron/well. In-vitro digestion and Caco-2 cell culture experiments were completed in duplicate on different days using different passage cells.

Harvesting Caco-2 monolayers for ferritin determination

After 12 hours of incubation [33, 34], treatments were removed and cells were washed with 2 ml of ice cold PBS. Caco-2 monolayers were lysed by adding 350 µl/well of mammalian protein extraction reagent (M-PER, Thermo Fisher Scientific, Rockford, IL) [35] and incubated in 6-well plates for 10 minutes on a plate shaker at 120 rpm. Caco-2 monolayers were scraped with a cell scraper (Fisher Scientific, Pittsburgh, PA), collected into microcentrifuge tubes, sonicated for 3 minutes and centrifuged at 14,000g for 10 minutes. Cell lysate supernatants were transferred to other tubes and stored at -20°C for ferritin and protein determination that was completed within 24 hours [35, 36, 23].

Caco-2 cell ferritin and protein determination

Ten µl cell lysate solutions were used for determining ferritin concentrations (ng/ml) using enzyme-linked immunosorbent assay (ELISA) (Spectro Ferritin kit, S-22, Ramco Laboratories Inc., Stafford, TX) as done previously [25, 36]. Twenty five µl cell lysate solutions were used for measuring protein concentrations using Pierce bicinchoninic acid protein assay kits (Rockford, IL). Ferritin content (ng/mg cell protein) was calculated as a ratio of cell ferritin (ng/ml)/cell protein (mg/ml) [36].

Aqueous fraction Caco-2 treatment for bioavailable vitamin A experiment

A day before the experiment, Caco-2 monolayers were supplemented with fresh media. On the day of the experiment, spent media was removed before treating the cells with 0.5 ml fresh aqueous fraction added to 1.5 ml of DMEM. Samples were randomly assigned to wells; Cerelac was used as a reference control on each plate. In-vitro digestion and Caco-2 cell culture experiments were completed in duplicate on different days using different passage cells. Control cells were collected at time zero without any food treatment.

Harvesting Caco-2 monolayers for vitamin A assessment

After 4 hours of incubation [37, 28, 27] treatments were removed and cells were washed with 2 ml of ice cold 2X PBS followed by 2 ml of ice cold 2 g/L albumin in PBS. Caco-2 monolayers were scraped with a cell scraper (Fisher Scientific, Pittsburgh, PA), and collected into amber colored microcentrifuge tubes using 1 ml ice cold PBS. This process was repeated twice more using 0.5 ml ice cold PBS and collected into the same microcentrifuge tube and centrifuged at 2000 rpm for 45 minutes at 5°C. PBS was discarded by carefully inverting the microcentrifuge tubes and leaving the cell pellet at the bottom. Microcentrifuge tubes were placed upside down for 20 seconds to remove excess moisture. Tubes were then blanketed with nitrogen, and stored at -80°C for vitamin A analysis.

Extraction of vitamin A in dry FBF, Aqueous fraction and Caco-2 cells

Vitamin A was extracted from dry FBF, aqueous fraction and Caco-2 cell pellets as described previously [38] with modifications. For dry FBF, approximately 1.0 g of dry FBF was taken in 50 ml screw cap glass tubes and homogenized in 4 ml of deionized water before adding 10 ml of ethanol with 0.1% butylated hydroxyltoluene (BHT) and 4 mL of super saturated potassium hydroxide (KOH). Samples were vortexed and incubated in a water bath at 70°C for 30 minutes. The tubes were kept on ice and 6 ml of DI water was added. The samples were initially extracted with 10 ml of hexane and repeated twice with 5 ml of hexane. Tubes were vortexed briefly each time hexane was added and left on ice to allow the layers separate. Top hexane layers were collected into a clean glass tube with pasteur pipette, completely dried in vacufuge (Model No, 5301, Eppendorf North America, Hauppauge, NY, USA), reconstituted in 400 µl of mobile phase and analyzed on HPLC on the same day.

For aqueous fractions, 8 ml of the thawed aqueous fraction was extracted with 10 ml of ethanol with 0.1% BHT and 4 mL of super saturated KOH in the manner similar to dry FBFs. For cell pellets, 2 ml of ethanol with 0.1% BHT and 1 mL of super saturated KOH was added to thawed cell pellet and incubated at 70°C for 30 minutes. The tubes were kept on ice and 1 ml of DI water was added. Samples were extracted with 5 ml of hexane three times and dried the hexane layers in the manner similar to dry FBFs. Reconstituted (in 400 µl mobile phase) aqueous fractions and cell pellet samples were syringe filtered using whatman PVDF filter with pore size 0.45µm (Cat No. 6779-1304, GE Healthcare Biosciences, Pittsburgh, PA, USA) before placing in HPLC vials. The values were expressed as mg/100g for dry FBF and ng/ml for aqueous fraction. All the Caco-2 cell pellets were weighed before extraction and average pellet weight was used to calculate vitamin A (µg) per cell pellet weight (g).

HPLC analysis

A Shimadzu HPLC system (Kyoto, Japan) consisting of a DGU-20A3 built in degasser, a LC-20AB solvent delivery pump, a SIL-20ACHT auto-sampler, a CTO-20AC column holding oven, a CBM-20A communicator module, and a SPD-M20A Photodiode Array Detector was used to analyze samples for retinol. A Waters (Milford, MA) C18 reversed phase column was used. The column temperature was maintained at 25°C Data was analyzed using LC Solution software (Kyoto, Japan). A mobile phase of methanol/ acetonitrile/chloroform (47:47:6, v/v/v) at a flow rate of 1.0 mL/min was used, detection was at 325 nm [39] and data was analyzed using LC solution software. Vitamin A peaks were identified and quantified. An external retinyl acetate (catalog # 1716002, USP, Rockville, MD, USA) standard curve was used for quantification, whose concentration was determined using a spectrophotometer (Jenway 6305, Bibby Scientific US, NJ, USA) on the day of analysis. Retinyl acetate stock solution

concentration was determined on spectrophotomer (Jenway 6305, Bibby Scientific US, NJ, USA) on the day of the experiment. Statistical analysis

Data were analyzed using one-way ANOVA with Tukey's test on SAS 9.3 (SAS Institute Inc., Cary, NC, USA), with $p < 0.05$ considered significant. Natural logs were used to transform data that did not meet the model assumptions.

Results

Iron concentration in dry FBF and aqueous fraction

Iron concentration in dry FBFs ranged from 8.0 to 31.8 mg/100g (Table 3-3). Extruded dry FBF iron concentrations were higher than CSB+, but lower than CSB13. Among the sorghum-cowpea FBFs, iron levels are higher in all the whole sorghum FBFs compared to the decorticated varieties. Even in sorghum-soy FBFs, both the whole sorghum varieties had higher iron concentrations compared to the decorticated variety. Extruded, degermed corn-soy FBF had iron levels that are similar to the decorticated sorghum FBFs. Cerelac iron concentrations are lower than all the FBFs except CSB+.

Iron levels in aqueous fractions ranged from 1.4 to 10.3 µg/10ml (Table 3-3). All extruded FBFs had significantly higher levels of iron in aqueous fractions compared to CSB13 and CSB+. There were no significant differences in aqueous fraction iron levels between extruded FBFs. Also, there was no significant difference in aqueous fraction iron levels between CSB13, CSB+ and Cerelac. Aqueous fraction iron levels in Cerelac (4.2 µg/10ml) were intermediate between extruded and non-extruded FBFs, and were significantly lower compared to whole white sorhum1-cowpea, decorticated white sorhum1-cowpea, whole sorghum-soy (low fat) and whole sorghum-soy (Full fat) extruded FBFS.

Ferritin concentration in Caco-2 cells treated with aqueous fractions

There were no significant differences in ferritin levels following treatment with the different aqueous fractions. (Table 3-4). However, $FeSO_4$ positive control (0.2 µg iron/well) showed significantly higher ferritin response compared to FBFs, Cerelac and negative control. There was no significant difference between basal salt solution alone (negative control) and FBF treatments on ferritin concentrations. Among the extruded FBFs, corn-soy (medium fat) had highest iron uptake (6.78 ng/mg) and whole sorghum-soy (full fat) had lowest iron uptake (4.10 ng/mg). There was a non-significant dose dependent increase in ferritin response with $FeSO_4$ positive controls with 0.1 µg and 0.2 µg iron/well.

Vitamin A concentration in dry FBF and aqueous fraction

In dry FBFs, vitamin A concentration ranged from 0.54-1.67 mg/100g, with highest concentration in non-extruded FBFs (Table 3-5). CSB+ had the highest vitamin A concentration of 1.67 mg/100g, while Cerelac contained the lowest concentration of 0.3 mg vitamin A/100g among the studied samples. Whole sorghum-cowpea and whole sorghum-soy FBFs contain vitamin A concentrations that are higher than Cerelac. FBFs with whole sorghum had slightly higher vitamin A levels compared to their corresponding decorticated sorghum FBFs.

In aqueous fractions, vitamin A concentrations in all the FBFs and Cerelac are similar. However sorghum-cowpea FBFs contain higher levels (50.8-80.1 ng/ml) compared to sorghum-soy and corn-soy FBFs (33.0-49.1 ng/ml).

Vitamin A concentration in Caco-2 cells treated with aqueous fraction

Vitamin A concentration in Caco-2 cell pellets was not significantly different among the samples (Table 3-6). The interesting trend was that red sorghum levels trended lower than white sorghum. The white sorghum cowpea blend trended to be higher than the other extruded blends

as well. The vitamin A levels in all the FBFs and Cerelac are higher than the negative control levels of 2.03 µg/g, with the exception of red sorghum FBFs and CSB13. Control cells collected at time zero without any treatment showed even lower levels of 1.82 µg/g.

Discussion

All eleven extruded FBFs were supplemented with iron 13 mg/100g dry mix in the ratio of 11 mg iron from ferrous sulfate ($FeSO_4$) and 2 mg iron from sodium iron EDTA (NaFeEDTA) (Table 3-2). However, iron analysis showed differences in dry mix iron levels between whole and decorticated FBFs (Table 3-3). All of the whole sorghum-cowpea FBFs, and whole sorghum-soy FBFs had higher iron levels compared to their corresponding decorticated FBFs. As iron is distributed in different regions of the grain, including the outer layers, it is not surprising that decortication or dehulling results in lower iron levels [40, 41]. Of the non-extruded FBFs, CSB13 had approximately 2-fold higher iron levels (31.8 mg/100g) and CSB+ had approximately 2-fold lower iron (8.0 mg/100g) levels compared to extruded FBFs average iron concentration of 18.0 mg/100g. Both CSB13 and CSB+ were procured from millers that prepared these FBFs following the USDA commodity requirements [17, 18], that require that CSB13 should contain 14.7 to 30.0 mg/100g iron and CSB+ should contain 9.0 to 21.0 mg/100g iron. Iron levels in CSB13 were slightly higher than the upper limit, whereas CSB+ iron levels were slightly lower than the lower limit set by USDA.

All of the extruded FBFs contained 4- to 7-fold significantly higher aqueous fraction iron concentrations compared to CSB13 and CSB+ (Table 3-3). One of the reasons for the low aqueous fraction iron concentration in CSB13 and CSB+ may be that extruded FBFs were cooked using 20% solids, whereas, CSB13 and CSB+ porridges were cooked using 11.75% and

13.79% solids. Another possibility is that the extrusion cooking reduced the antrinutritional factor concentration [15, 42], thereby improving extruded FBFs' aqueous fraction iron levels. There was no difference in aqueous fraction iron levels between CSB13 and CSB+ in spite of a 4-fold higher iron in CSB13 dry FBF. CSB+ is an improved formulation of CSB13 with enhanced nutrient profile and with ingredients partially cooked through dry roasting [18]. This heat processing might have contributed to improved aqueous fraction iron levels in CSB+, like in previous studies that observed 16-32% increase in iron availability with roasting and malting [43]. Another reason for the improved iron availability may have been that CSB+ is fortified with both ferrous fumarate and NaFeEDTA. The latter iron fortificant chelates with native iron in the diet and protects it from binding to ANF [44, 45, 46].

Treatment with both extruded and non-extruded FBFs resulted in similar ferritin levels in the in-vitro digestion-Caco-2 model. However, compared to $FeSO_4$ positive control (0.2 µg iron/well), with the exception of CSB+ and corn-soy (medium fat), all other FBFs, Cerelac and negative control showed significantly lower ferritin levels. The mean ferritin concentrations ranged between 4.1-7.4 ng/mg. Positive controls prepared with $FeSO_4$ solution providing 0.1 µg iron/well and 0.2 µg iron/well showed a non-significant dose dependent increase in ferritin synthesis of 15.87 ng/mg and 29.65 ng/mg respectively. Proulx & Reddy (2006), also treated the cells with $FeSO_4$ solution providing 25 µg iron/well, which is > 100 times higher than our study concentrations [47]. However, comparison of our positive control ferritin response with the previous study was not possible as they presented results in % relative bioavailability considering $FeSO_4$ values as 100%. Also, our $FeSO_4$ control samples were not subjected to in-vitro digestion, unlike in the previous study. The baseline ferritin, observed with basal salt solution treatment was 4.75 ng/mg. In a previous study conducted with red and white beans, the

baseline ferritin response was 5.56 ng/mg and all of the sample ferritin responses were at or significantly below the baseline level [48]. Seven of the extruded FBFs and one non-extruded FBF had ferritin response higher than baseline levels. However none of the FBFs showed significantly different ferritin levels.

The amount of iron added to Caco-2 cells in each well ranged from 0.11-0.25 µg/well, which was about similar range levels (0.05- 0.39 µg/well) found in different rice varieties that also did not find significant differences in ferritin levels [22]. Also, no differences were observed in ferritin concentration of cells treated with basal salt solution containing no iron and with digested FBFs. Previous studies have also found ferritin levels that were not different from baseline levels [48, 49]. However, in Glahn et al (2002) study, after adding ascorbic acid, iron bioavailability of the rice sample significantly increased beyond the baseline levels [22]. It should be noted that extruded FBFs are fortified with 40 mg/100g ascorbic acid, and CSB13 and CSB+ are fortified with 40 mg/100g and 90 mg/100g ascorbic acid, respectively. However, we did not add exogenous ascorbic acid in our experiments.

The aqueous fraction iron levels used to treat the Caco-2 cells were not correlated to ferritin synthesis as has found previously [22, 23, 36]. One possibility is that the aqueous fraction iron may bind to low molecular weight phytates, making it unavailable for uptake and ultimately ferritin synthesis [50].

Some studies have achieved significant ferritin response by increasing the food sample quantity. Eagling et al (2014), increased the sample quantity from 0.5 g to 1 g and then to 3 g due to low ferritin response with lower amounts (< 3 g) of food [26]. For our experiment, we used only 2 g porridge, as > 2 g resulted in thick sample that did not digest well. However, their

unleavened bread flour samples contained very low iron concentrations (0.67-4.67 mg/100g) compared to our FBF samples (8.0-31.8 mg iron/100g).

We anticipated that bioavailable iron may be higher in decorticated sorghum FBFs compared to corresponding whole FBFs because ANF localized in the outer layers of the grains would be lost during decortication [41]. In tannin-containing Seredo variety of sorghum, decortication by mechanical abrasion reduced the tannin content by 82% [51]. In another study with both tannin and non-tannin sorghum varieties, decortication reduced tannins by 79-92% [42]. However, ferritin concentrations after treatments with aqueous fractions from whole and decorticated sorghum-cowpea FBFs were not significantly different. On the other hand, we observed a slight decrease in ferritin response for all the decorticated sorghum-cowpea and sorghum-soy FBFs compared to their corresponding whole FBFs.

With respect to vitamin A, data on the in-vitro bioavailability of vitamin A is very limited. While there are many studies on the carotenoid bioavailability [27, 37], there is so far only one study looked at the vitamin A bioavailability using the Caco-2 cell model [28]. Others studied vitamin A bioaccessibility using only in-vitro digestion [52] or bioavailability in clinical studies [53]. Vitamin A content in the current study ranged from 0.3 mg/100g in Cerelac to 1.67 mg/100g in CSB+ (Table 3-5). Vitamin A content in Cerelac matched its labeled value, however CSB+ had 61% and CSB13 had 41% higher vitamin A levels than the fortified amount (Table 3-2). The decorticated sorghum FBFs and degermed corn FBF contain lower vitamin A levels compared to their respective whole FBFs. Sorghum-cowpea and sorghum-soy FBFs contained 10-58% higher vitamin A than the fortified levels of 0.488 mg/100g. It is not clear what caused these differences.

Vitamin A concentration in aqueous fraction was similar in all the samples. However, non-extruded CSB13 and CSB+ that contain highest vitamin A levels in dry FBF, showed lowest concentration in aqueous fractions compared to extruded FBFs and Cerelac. Cooking during porridge preparation and in-vitro digestion may have affected the vitamin A concentration [28] of non-extruded FBFs most than extruded ones. Another possibility for the lower aqueous fraction vitamin A levels in CSB+ may relate to its lower fat levels. CSB13 is a little harder to understand in this regard. Also, sorghum-soy and corn-soy FBFs had lower vitamin A compared to sorghum-cowpea FBFs in aqueous fractions.

Bioavailable vitamin A levels of FBFs measured by its concentration in Caco-2 cells after 4 hour FBF treatment was not different between treatments. Red sorghum FBFs trended lower bioavailable vitamin A compared to white sorghum-cowpea FBFs. Even though the aqueous fraction vitamin A levels are lowest for CSB13 and CSB+, Caco-2 cell vitamin A concentrations were on par with white sorghum FBFs. This improvement might be caused by the cellular conversion of provitamin A carotenoids to vitamin A [54] for traditional corn-soy FBFs (Table 3-7). The similar increase in bioavailable vitamin A was not observed for extruded corn-soy FBF, may be related to only 48% corn compared to 70% corn in CSB13 and 79% corn in CSB+ (Table 3-1).

It is difficult to compare in-vitro digestion/Caco-2 model iron bioavailability studies due to methodological differences. Some studies used a two chamber arrangement with dialysis membrane [55, 56, 22, 57], while others treated the Caco-2 cells directly with aqueous fraction [36, 23, 58]. Other studies have grown cells on inserts to study iron transport across the Caco-2 monolayer [59]. Even though the Caco-2 cell density/well (50,000 cells/cm^2) is similar in a majority of the studies, the day cells were used for bioavailability experiment varies. Some

studies used cells grown on 6 or 12 well plates 13 days post- seeding [26, 50], others used cells between a range of 14-16 days post-seeding [23]. In addition to the above mentioned differences, presentation of ferritin data greatly varied among studies. Some authors provided iron bioavailability as a percent of control or $FeSO_4$ positive control [59, 60, 47, 58], whereas others provided actual ferritin values [26, 25, 24, 36].

The major limitation of our study was that we did not observe a greater ferritin response with our samples. However multiple other studies also did not observe increases above baseline ferritin response [48, 49]. Some have found benefits from adding ascorbic acid externally, but the FBFs were fortified with ascorbic acid, which is probably not true of other foods assessed in this model [26, 61]. Also our study FBFs were fortified with ascorbic acid, which is probably not true of most of the other foods that were being used in other studies. Our study also lacks specific information about levels of antinutritional factors in FBFs such as phytates and tannins that have direct relevance to iron bioavailability. Study results should be extrapolated with caution, because a previous study that observed at or below baseline ferritin response in Caco-2 cells, found significant improvements in iron status in animals when fed with the same samples [48]. However, in a rat study conducted with, by others in our laboratory, extruded sorghum-cowpea, extruded sorghum-soy FBFs and CSB+ showed no significant difference in hemoglobin and liver iron levels (Delimont et al, manuscript in preparation), confirming our Caco-2 results of similar iron availability among FBFs. In line with our results, Pynaert et al (2006), found significantly higher soluble iron in processed compared to unprocessed complementary foods (CF). However the iron bioavailability of processed and unprocessed CF was similar due to a proportional decrease in Caco-2 cell iron uptake from processed CF [62]. In a previous clinical

study conducted with the same complementary foods, no difference in iron status was observed in infants fed processed and unprocessed CF [63].

To the best of our knowledge, this is the first study looked at both bioavailable iron and vitamin A in FBFs. This research is important as major food aid receiving regions have high prevalence of anemia and vitamin A deficiency [64, 65]. In conclusion, our study results suggest that consumption of newly developed extruded sorghum-cowpea, sorghum-soy and corn-soy FBFs will result in iron and vitamin A status comparable to traditional non-extruded CSB13 and CSB+ FBFs. As the antinutrient phytate content influenced iron bioavailability more than the iron content in the food [26], future studies should give emphasis to analyzing the antinutrient levels in iron bioavailability studies. Also these novel sorghum-FBFs should be tested in animal and clinical trials to confirm the current study results.

Table 3-1 Composition of Cerelac, extruded FBFs and non-extruded FBFs.

Extruded FBFs				**Traditional FBFs**			**Cerelac**
Ingredient (%)	**SCB**	**SSB†**	**CSB†**	**Ingredient (%)**	**CSB13**	**CSB+**	**Ingredient**
Sorghum Flour	24.7	47.6	-	Cornmeal	69.5	-	Wheat Flour
Cowpea Flour	38.6	-	-	Soy Flour, Defatted	21.9	-	Fat Free Milk
Corn Flour	-	-	48.1	Soybean Oil, Refined	5.5	-	Sugar
Soy Flour	-	15.7	15.2	Minerals	3.0	-	Milk Fat
Sugar	15.0	15.0	15.0	Vitamin Antioxidant Premix	0.1	-	Corn Oil
Whey Protein Concentrate	9.5	9.5	9.5	Corn (White or Yellow)	-	78.5	Palm Oil
Vegetable Oil	9.0	9.0	9.0	Whole Soybeans	-	20.0	Calcium Carbonate
Vitamin & Mineral Premix	3.2	3.2	3.1	Vitamin/Mineral	-	0.2	Sodium Phosphate
				Tri-Calcium Phosphate	-	1.2	Bifidus Cultures
				Potassium Chloride	-	0.2	Vitamin & Mineral Mix

† For extruded SSB (full-fat soy) & extruded CSB (full-fat soy), WPC80 was increased from 9.5% to 13% and vegetable oil was decreased from 9% to 5.5%. For Cerelac, ingredient % was not available, ingredients were listed in the order on the label.

Table 3-2 Macronutrient and micronutrient formulation of FBFs and Cerelac.

Nutrient in 100g	**Unit**	**SCB/SSB /CSB**	**CSB 13**	**CSB+**	**Cerelac**
Energy	kcal	380	386	380	400
Protein	g	17	16	14	13
Fat	G	8	9	6	10
Vitamin A	Mg	0.488	0.819	1.04	0.344
Thiamin (B_1)	Mg	0.652	0.61	0.20	0.28
Riboflavin (B_2)	Mg	0.933	0.481	1.40	0.80
Niacin (B_3)	Mg	9.07	6.29	8.0	2.40
Pantothenic Acid (B_5)	Mg	3.646	3.285	1.60	1.33
Vitamin B_6	Mg	0.752	0.532	1.0	0.280
Folic Acid (B_9)	Mg	0.087	0.247	0.11	0.053
Vitamin B_{12}	Mg	0.0015	0.001	0.002	0.001
Vitamin D	Mg	0.029	0.005	0.011	0.004
Vitamin E	Mg	13.22	0.98	8.3	3.0
Vitamin K	Mg	0.033	0.001	0.03	0.03
Vitamin C	Mg	40.0	40.2	90	16.0
Calcium	Mg	279.08	650	452	426.67
Total Iron (Fe)	Mg	13.0	10.6	6.5	5.33
Fe ($FeSO_4$)	Mg	11.0	0.0	4.0	0.0
Fe (NAFeEDTA)	Mg	2.0	0.0	2.5	0.0
Fe (Ferrous fumarate)	Mg	0.0	10.6	4.0	5.33
Iodine	Mg	0.23	0.0	0.04	0.03
Phosphorus	Mg	290.97	522.0	290.0	426.67
Potassium	mg	163.19	563.0	140.0	NA
Zinc	mg	5.5	5.94	5.0	2.13

For Cerelac, macronutrient and micronutrient values were calculated based on the label information for % Daily Value (% DV), and daily values for children under 4 years provided by US Food and Drug Administration, Guidance for Industry: A Food Labeling Guide (15. Appendix G: Daily Values for Infants, Children Less Than 4 Years of Age, and Pregnant and Lactating Women). NA-values not available.

Table 3-3 Iron concentrations in dry FBFs, Cerelac and aqueous fractions (mean ± SEM).

Cereal	Cereal type	Legume	Dry FBF (mg/100g)	Aqueous fraction* (µg/10ml)
Sorghum-Cowpea blends				
White Sorghum 1	Whole	Cowpea	19.5	9.3 ± 0.2^{a}
White Sorghum 1	Decorticated	Cowpea	18.6	10.3 ± 0.6^{a}
White Sorghum 1	Decorticated, commercial	Cowpea	15.1	6.6 ± 1.1^{ac}
White Sorghum 2	Whole	Cowpea	19.5	6.8 ± 1.7^{ac}
White Sorghum 2	Decorticated	Cowpea	15.9	8.2 ± 0.3^{ac}
Red Sorghum	Whole	Cowpea	19.1	8.0 ± 0.8^{ac}
Red Sorghum	Decorticated	Cowpea	15.3	6.2 ± 1.8^{ac}
Sorghum-Soy blends				
White Sorghum 1	Whole	Low Fat soy	23.4	9.5 ± 0.6^{a}
White Sorghum 1	Decorticated	Medium Fat Soy	15.6	8.5 ± 0.5^{ac}
White Sorghum 1	Whole	Full Fat soy	20.7	9.2 ± 0.3^{a}
Corn-Soy blends				
Corn	Degermed, coarse, commercial	Medium Fat Soy	15.6	7.5 ± 0.9^{ac}
CSB13	Cornmeal	Defatted Soy flour	31.8	1.7 ± 0.3^{b}
CSB+	Whole Corn	Whole Soy	8.0	1.4 ± 0.3^{b}
Cerelac	Wheat		11.4	4.2 ± 0.1^{bc}

*Aqueous fractions (n=2) from two different in-vitro digestion experiments were included for statistical analysis. Within a column, means without a common superscript are significantly different ($p<0.05$).

Table 3-4 Caco-2 cell ferritin levels following aqueous fraction treatment (n=2, mean ± SEM).

Cereal	Cereal type	Legume	Ferritin (ng/mg)
Sorghum-Cowpea blends			
White Sorghum 1	Whole	Cowpea	6.21 ± 1.68 [a]
White Sorghum 1	Decorticated	Cowpea	4.66 ± 0.05 [a]
White Sorghum 1	Decorticated, commercial	Cowpea	4.74 ± 0.05 [a]
White Sorghum 2	Whole	Cowpea	6.52 ± 1.83 [a]
White Sorghum 2	Decorticated	Cowpea	5.47 ± 2.05 [a]
Red Sorghum	Whole	Cowpea	5.24 ± 1.35 [a]
Red Sorghum	Decorticated	Cowpea	4.13 ± 1.01 [a]
Sorghum-Soy blends			
White Sorghum 1	Whole	Low Fat soy	6.51 ± 2.17 [a]
White Sorghum 1	Decorticated	Medium Fat Soy	5.97 ± 3.06 [a]
White Sorghum 1	Whole	Full Fat soy	4.10 ± 1.22 [a]
Corn-Soy blends			
Corn	Degermed, coarse, commercial	Medium Fat Soy	6.78 ± 0.64 [ab]
CSB13	Cornmeal	Defatted Soy flour	4.72 ± 0.73 [a]
CSB+	Whole Corn	Whole Soy	7.39 ± 1.76 [ab]
Controls			
Cerelac	Wheat		4.89± 0.53 [a]
Basal salt solution (negative control)			4.75 ± 1.04 [a]
$FeSO_4$ (0.1 µg Fe/well) (positive control)			15.87 ± 5.73 [ab]
$FeSO_4$ (0.2 µg Fe/well) (positive control)			29.65 ± 3.26 [b]

Within a column, means without a common superscript are significantly different ($p<0.05$).

Table 3-5 Vitamin A content in dry FBFs, Cerelac and aqueous fractions (mean ± SEM).

Cereal	Cereal type	Legume	Dry FBF (mg/100g)	Aqueous fraction (ng/ml)
		Sorghum-Cowpea blends		
White Sorghum 1	Whole	Cowpea	0.75	60.8 ± 10.0
White Sorghum 1	Decorticated	Cowpea	0.70	69.6 ± 15.9
White Sorghum 1	Decorticated, commercial	Cowpea	0.54	50.8 ± 5.0
White Sorghum 2	Whole	Cowpea	0.71	52.7 ± 7.7
White Sorghum 2	Decorticated	Cowpea	0.56	80.1 ± 15.4
Red Sorghum	Whole	Cowpea	0.76	77.6 ± 2.8
Red Sorghum	Decorticated	Cowpea	0.54	67.4 ± 27.7
		Sorghum-Soy blends		
White Sorghum 1	Whole	Low Fat soy	0.72	48.0 ± 4.0
White Sorghum 1	Decorticated	Medium Fat Soy	0.59	44.8 ± 10.9
White Sorghum 1	Whole	Full Fat soy	0.75	49.1 ± 0.8
		Corn-Soy blends		
Corn	Degermed, coarse, commercial	Medium Fat Soy	0.56	45.9 ± 12.8
CSB13	Cornmeal	Defatted Soy flour	1.16	33.0 ± 0.4
CSB Plus	Whole Corn	Whole Soy	1.67	41.5 ± 28.2
		Cerelac		
Cerelac	Wheat		0.30	55.6 ± 25.6

Table 3-6 Caco-2 cell vitamin A levels following aqueous fraction treatment (n=2, mean ± SEM).

Cereal	Cereal type	Legume	Vitamin A (µg/g cells)
	Sorghum-Cowpea blends		
White Sorghum 1	Whole	Cowpea	2.59 ± 0.35
White Sorghum 1	Decorticated	Cowpea	2.52 ± 0.13
White Sorghum 1	Decorticated, commercial	Cowpea	2.34 ± 0.15
White Sorghum 2	Whole	Cowpea	2.23 ± 0.12
White Sorghum 2	Decorticated	Cowpea	2.29 ± 0.15
Red Sorghum	Whole	Cowpea	1.89 ± 0.52
Red Sorghum	Decorticated	Cowpea	1.99 ± 0.14
	Sorghum-Soy blends		
White Sorghum 1	Whole	Low Fat Soy	1.96 ± 0.11
White Sorghum 1	Decorticated	Medium Fat Soy	2.13 ± 0.01
White Sorghum 1	Whole	Full Fat Soy	2.09 ± 0.69
	Corn-Soy blends		
Corn	Degermed, coarse, commercial	Medium Fat Soy	1.96 ± 0.01
CSB13†	Cornmeal	Defatted Soy Flour	2.03
CSB Plus†	Whole Corn	Whole Soy	2.43
	Controls		
Cerelac	Wheat		2.24 ± 0.10
Basal salt solution			2.09 ± 0.21
Control cells†			1.82

† For CSB13, CSB+ and control cells single analysis data was included due to sample loss.

References

1. Drorbaugh N, Neumann CG. Micronutrient deficiencies in food aid beneficiaries: A review of seven African countries. 2009;9.

2. Ahmed T, Hossain M, Sanin KI. Global burden of maternal and child undernutrition and micronutrient deficiencies. Ann Nutr Metab. 2012;61 Suppl 1: 8-17.

3. Bhutta ZA, Salam RA. Global nutrition epidemiology and trends. Ann Nutr Metab. 2012;61 Suppl 1: 19-27.

4. Fleige LE, Moore WR, Garlick PJ, Murphy SP, Turner EH, Dunn ML, et al. Recommendations for optimization of fortified and blended food aid products from the United States. Nutr Rev. 2010;68: 290-315.

5. Webb P, Rogers B, Rosenberg I, Schlossman N, Wanke C, Bagriansky J, et al. Delivering improved nutrition: recommendations for changes to US food aid products and programs. 2011.

6. Henley E, Rooney L, Dahlberg J, Bean S, Weller C, Turner N, et al. Sorghum: an ancient, healthy and nutritious old world cereal. 2010.

7. Akibode S, Maredia M. Global and regional trends in production, trade and consumption of food legume crops. 2011: 87.

8. Kayodé AP, Linnemann AR, Hounhouigan JD, Nout MJ, van Boekel MA. Genetic and environmental impact on iron, zinc, and phytate in food sorghum grown in Benin. J Agric Food Chem. 2006;54: 256-262.

9. Punia KP, Darshan. Proximate composition, phytic acid, polyphenols and digestibility (in-vitro) of four brown cowpea varieties. Int J Food Sci Nutr. 2000;51: 189-193.

10. Radhakrishnan MR, Sivaprasad J. Tannin content of sorghum varieties and their role in iron bioavailability. J Agric Food Chem. 1980;28: 55-57.

11. Plahar W, Annan N, Nti C. Cultivar and processing effects on the pasting characteristics, tannin content and protein quality and digestibility of cowpea (Vigna unguiculata). 1997;51: 343-356.

12. Armah SM, Boy E, Chen D, Candal P, Reddy MB. Regular Consumption of a High-Phytate Diet Reduces the Inhibitory Effect of Phytate on Nonheme-Iron Absorption in Women with Suboptimal Iron Stores. J Nutr. 2015;145: 1735-1739.

13. Hunt JR, Roughead ZK. Adaptation of iron absorption in men consuming diets with high or low iron bioavailability. Am J Clin Nutr. 2000;71: 94-102.

14. Ilo S, Schoenlechner R, Berghofe E. Role of lipids in the extrusion cooking processes. Grasas Y Aceites. 2000;51: 97-110.

15. Alonso R, Rubio L, Muzquiz M, Marzo F. The effect of extrusion cooking on mineral bioavailability in pea and kidney bean seed meals. Anim Feed Sci Technol. 2001;94: 1-13.

16. Tran QD, Hendriks WH, van der Poel, Antonius FB. Effects of extrusion processing on nutrients in dry pet food. J Sci Food Agric. 2008;88: 1487-1493.

17. USDA Commodity Requirements. Corn-Soy Blend for use in export programs. Available at https://www.fsa.usda.gov/Internet/FSA_File/csb13_110507.pdf. Accessed on September 29, 2015.

18. USDA Commodity Requirements. Corn-Soy Blend Plus for Use in International Food Aid Programs. Available at https://www.fsa.usda.gov/Internet/FSA_File/csbp2.pdf. Accessed on July 29, 2014.

19. Sandberg A. The use of Caco-2 cells to estimate Fe absorption in humans--A critical appraisal. Int J Vitam Nutr Res. 2010;80: 307-13.

20. Pullakhandam R, Nair KM, Pamini H, Punjal R. Bioavailability of iron and zinc from multiple micronutrient fortified beverage premixes in Caco-2 cell model. J Food Sci. 2011;76: H38-42.

21. Glahn RP, Lee OA, Yeung A, Goldman MI, Miller DD. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in-vitro digestion/Caco-2 cell culture model. J Nutr. 1998;128: 1555-61.

22. Glahn R, Cheng Z, Welch R, Gregorio G. Comparison of iron bioavailability from 15 rice genotypes: studies using an in-vitro digestion/caco-2 cell culture model. J Agric Food Chem. 2002;50: 3586-91.

23. Viadel B. Ferritin synthesis by Caco-2 cells as an indicator of iron bioavailability: Application to milk-based infant formulas. Food Chem. 2007;102: 925-931.

24. Lung'aho MG, Glahn R. In-vitro estimates of iron bioavailability in some Kenyan complementary foods. Food Nutr Bull. 2009;30: 145-52.

25. DellaValle D, Vandenberg A, Glahn R. Seed coat removal improves iron bioavailability in cooked lentils: studies using an in-vitro digestion/Caco-2 cell culture model. J Agric Food Chem. 2013;61: 8084-9.

26. Eagling T, Wawer A, Shewry P, Zhao F, Fairweather Tait S. Iron bioavailability in two commercial cultivars of wheat: comparison between wholegrain and white flour and the effects of nicotianamine and 2'-deoxymugineic acid on iron uptake into Caco-2 cells. J Agric Food Chem. 2014;62: 10320-5.

27. Thakkar SK, Maziya-Dixon B, Dixon AG, Failla ML. Beta-carotene micellarization during in-vitro digestion and uptake by Caco-2 cells is directly proportional to beta-carotene content in different genotypes of cassava. J Nutr. 2007;137: 2229-2233.

28. O'Callaghan Y, O'Brien N. Bioaccessibility, cellular uptake and transepithelial transport of α-tocopherol and retinol from a range of supplemented foodstuffs assessed using the caco-2 cell model. Int J Food Sci Tech. 2010;45: 1436-1442.

29. General Food Distribution, Module 11. Part2: Technical Notes. 2011. Available at http://www.unscn.org/layout/modules/htp/pdf/M11P2.pdf. Accessed on September,29,2015.

30. Garrett DA, Failla ML, Sarama RJ. Development of an in-vitro digestion method to assess carotenoid bioavailability from meals. J Agric Food Chem. 1999;47: 4301-9.

31. Jin F, Frohman C, Thannhauser T, Welch R, Glahn R. Effects of ascorbic acid, phytic acid and tannic acid on iron bioavailability from reconstituted ferritin measured by an in-vitro digestion-Caco-2 cell model. Br J Nutr. 2009;101: 972-81.

32. Etcheverry P, Miller D, Glahn R. A low-molecular-weight factor in human milk whey promotes iron uptake by Caco-2 cells. J Nutr. 2004;134: 93-8.

33. Liu C, Glahn RP, Liu RH. Assessment of carotenoid bioavailability of whole foods using a Caco-2 cell culture model coupled with an in-vitro digestion. J Agric Food Chem. 2004;52: 4330-4337.

34. Gallardo-Guerrero L, Gandul-Rojas B, Mínguez-Mosquera MI. Digestive stability, micellarization, and uptake by Caco-2 human intestinal cell of chlorophyll derivatives from different preparations of pea (Pisum sativum L.). J Agric Food Chem. 2008;56: 8379-8386.

35. Latunde-Dada GO, Li X, Parodi A, Edwards C, Ellis P, Sharp PA. Micromilling enhances iron bioaccessibility from wholegrain wheat. J Agric Food Chem. 2014;62: 11222-11227.

36. Cilla A, Perales S, Lagarda M, Barbera R, Farre R. Iron bioavailability in fortified fruit beverages using ferritin synthesis by Caco-2 cells. J Agric Food Chem. 2008;56: 8699-703.

37. Failla ML, Chitchumronchokchai C, Ferruzzi MG, Goltz SR, Campbell WW. Unsaturated fatty acids promote bioaccessibility and basolateral secretion of carotenoids and α-tocopherol by Caco-2 cells. 2014;5: 1101-1112.

38. Lindshield BL, King JL, Wyss A, Goralczyk R, Lu CH, Ford NA, et al. Lycopene biodistribution is altered in 15,15'-carotenoid monooxygenase knockout mice. J Nutr. 2008;138: 2367-2371.

39. Deming DM, Teixeira SR, Erdman JW,Jr. All-trans beta-carotene appears to be more bioavailable than 9-cis or 13-cis beta-carotene in gerbils given single oral doses of each isomer. J Nutr. 2002;132: 2700-2708.

40. Ghavidel RA, Prakash J. The impact of germination and dehulling on nutrients, antinutrients, in-vitro iron and calcium bioavailability and in-vitro starch and protein digestibility of some legume seeds. 2007;40: 1292-1299.

41. Lestienne I, Buisson M, Lullien-Pellerin V, Picq C, Trèche S. Losses of nutrients and anti-nutritional factors during abrasive decortication of two pearl millet cultivars (Pennisetum glaucum). Food Chem. 2007;100: 1316-1323.

42. Dlamini NR, Taylor JR, Rooney LW. The effect of sorghum type and processing on the antioxidant properties of African sorghum-based foods. Food Chem. 2007;105: 1412-1419.

43. Gahlawat P, Sehgal S. In-vitro starch and protein digestibility and iron availability in weaning foods as affected by processing methods. 1994;45: 165-173.

44. Chang S, Huang Z, Ma Y, Piao J, Yang X, Zeder C, et al. Mixture of ferric sodium ethylenediaminetetraacetate (NaFeEDTA) and ferrous sulfate: an effective iron fortificant for complementary foods for young Chinese children. 2012;33: 111-116.

45. Gibson RS, Bailey KB, Gibbs M, Ferguson EL. A review of phytate, iron, zinc, and calcium concentrations in plant-based complementary foods used in low-income countries and implications for bioavailability. 2010;31: 134-146.

46. Bothwell, MacPhail. The potential role of NaFeEDTA as an iron fortificant. 2004;74: 421-434.

47. Proulx AK, Reddy MB. Iron bioavailability of hemoglobin from soy root nodules using a Caco-2 cell culture model. J Agric Food Chem. 2006;54: 1518-1522.

48. Tan SY, Yeung CK, Tako E, Glahn RP, Welch RM, Lei X, et al. Iron bioavailability to piglets from red and white common beans (Phaseolus vulgaris). J Agric Food Chem. 2008;56: 5008-5014.

49. Tako E, Beebe SE, Reed S, Hart JJ, Glahn RP. Polyphenolic compounds appear to limit the nutritional benefit of biofortified higher iron black bean (Phaseolus vulgaris L.). Nutr J. 2014;13: 28-2891-13-28.

50. Glahn R, Wortley G, South P, Miller D. Inhibition of iron uptake by phytic acid, tannic acid, and ZnCl2: studies using an in-vitro digestion/Caco-2 cell model. J Agric Food Chem. 2002;50: 390-5.

51. Yetneberk S, Rooney LW, Taylor J. Improving the quality of sorghum injera by decortication and compositing with tef. J Sci Food Agric. 2005;85: 1252-1258.

52. Herrero-Barbudo M, Granado-Lorencio F, Blanco-Navarro I, Pérez-Sacristán B, Olmedilla-Alonso B. Applicability of an in-vitro model to assess the bioaccessibility of vitamins A and E from fortified commercial milk. Int Dairy J. 2009;19: 64-67.

53. Herrero-Barbudo C, Olmedilla-Alonso B, Granado-Lorencio F, Blanco-Navarro I. Bioavailability of vitamins A and E from whole and vitamin-fortified milks in control subjects. Eur J Nutr. 2006;45: 391-398.

54. Reboul E. Absorption of vitamin A and carotenoids by the enterocyte: focus on transport proteins. 2013;5: 3563-3581.

55. Bejjani S, Pullakhandam R, Punjal R, Nair KM. Gastric digestion of pea ferritin and modulation of its iron bioavailability by ascorbic and phytic acids in caco-2 cells. World J Gastroenterol. 2007;13: 2083-8.

56. DellaValle D, Glahn R. Differences in relative iron bioavailability in traditional Bangladeshi meal plans. Food Nutr Bull. 2014;35: 431-9.

57. Tako E, Blair M, Glahn R. Biofortified red mottled beans (*Phaseolus vulgaris L.*) in a maize and bean diet provide more bioavailable iron than standard red mottled beans: studies in poultry (*Gallus gallus*) and an in-vitro digestion/Caco-2 model. Nutr J. 2011;10: 113.

58. Bodnar A, Proulx A, Scott MP, Beavers A, Reddy M. Iron bioavailability of maize hemoglobin in a Caco-2 cell culture model. J Agric Food Chem. 2013;61: 7349-56.

59. He W, Feng Y, Wei Y, Yang X, Shi C, He Z, et al. Differential iron-bioavailability with relation to nutrient compositions in polished rice among selected Chinese genotypes using Caco-2 cell culture model. Int J Food Sci Nutr. 2013;64: 822-9.

60. Lei J, Zhang Y, Chen X, Zhang M, Bai L, Huang C, et al. Assessment of iron bioavailability in ten kinds of Chinese wheat flours using an in-vitro digestion/Caco-2 cell model. Biomed Environ Sci. 2012;25: 502-8.

61. Ariza-Nieto M, Blair MW, Welch RM, Glahn RP. Screening of iron bioavailability patterns in eight bean (Phaseolus vulgaris L.) genotypes using the Caco-2 cell in-vitro model. 2007;55: 7950-7956.

62. Pynaert I, Armah C, Fairweather-Tait S, Kolsteren P, Van Camp J, Henauw SD. Iron solubility compared with in-vitro digestion–Caco-2 cell culture method for the assessment of iron bioavailability in a processed and unprocessed complementary food for Tanzanian infants (6–12 months). Br J Nutr. 2006;95: 721-726.

63. Mamiro PS, Kolsteren PW, van Camp JH, Roberfroid DA, Tatala S, Opsomer AS. Processed complementary food does not improve growth or hemoglobin status of rural tanzanian infants from 6-12 months of age in Kilosa district, Tanzania. J Nutr. 2004;134: 1084-1090.

64. World Health Organization. Worldwide prevalence of anaemia 1993-2005: WHO global database on anaemia. 2008.

65. World Health Organization. Global prevalence of vitamin A deficiency in populations at risk 1995-2005: WHO global database on vitamin A deficiency. 2009.

Chapter 4 - Fatty Acid and Phytosterol Content of Commercial Saw Palmetto Supplements

Abstract

Saw palmetto supplements are one of the most commonly consumed supplements by men with prostate cancer and/or benign prostatic hyperplasia (BPH). Some studies have found significant improvements in BPH and lower urinary tract symptoms (LUTS) with saw palmetto supplementation, whereas others found no benefits. The variation in the efficacy in these trials may be a result of differences in the putative active components, fatty acids and phytosterols, of the saw palmetto supplements. To this end, we quantified the major fatty acids (laurate, myristate, palmitate, stearate, oleate, linoleate) and phytosterols (campesterol, stigmasterol, β-sitosterol) in 20 commercially available saw palmetto supplements using GC-FID and GC-MS, respectively. Samples were classified into liquids, powders, dried berries, and tinctures. Liquid saw palmetto supplements contained significantly higher ($p<0.05$) concentrations of total fatty acids (908.5 mg/g), individual fatty acids, total phytosterols (2.04 mg/g), and individual phytosterols, than the other supplement categories. Powders contained significantly higher ($p<0.05$) concentrations of total fatty acids than tinctures, which contain negligible amounts of fatty acids (46.3 mg/g) and phytosterols (0.10 mg/g). Our findings suggest that liquid saw palmetto supplements may be the best choice for individuals who want to take a saw palmetto supplement with the highest concentrations of both fatty acids and phytosterols.

Introduction

Saw palmetto (*Serenoa repens*) herbal supplements are commonly used by men to combat benign prostatic hyperplasia (BPH), a nonmalignant enlargement of the prostate. In addition to BPH, these supplements are commonly consumed by men diagnosed with prostate cancer. In 2007, 5.1% (~1.7 million people) of Americans 18 years of age or older reported using saw palmetto in the past 30 days [1]. In 2011, over $18 million in saw palmetto was sold in the United States, ranking third among herbal dietary supplements [2]. A systematic literature review of 11 studies identified saw palmetto supplements as one of five commonly used complementary or alternative medicine modalities by men with prostate cancer; use was 1.9–24.9% [3]. Another study found that 13.8% of unaffected men whose brothers had been diagnosed with prostate cancer had taken saw palmetto at some point in their lives [4].

Saw palmetto's putative mechanism of action is inhibition of 5α-reductase, the enzyme that converts testosterone into the more potent androgen dihydrotestoterone [5-10]. Studies that have found saw palmetto administration or treatment reduced androgen action support this belief [11-15]; however, not all studies have found that saw palmetto inhibits 5α-reductase or has anti-androgen action [16-18]. The believed bioactive components of saw palmetto are fatty acids and phytosterols. Saw palmetto extracts predominantly consist of fatty acids (~90%) and are unique compared with other extracts and vegetable and nut oils [19] in that they are a rich source of the saturated, medium-chain fatty acids laurate (12:0) and myristate (14:0) [20].

Several studies suggest that the fatty acids in saw palmetto extracts are responsible for its ability to inhibit 5α-reductase [21-26], but which fatty acid(s) is/are responsible for the inhibition varies. Some research suggests that saw palmetto phytosterols, (β-sitosterol, campesterol, stigmasterol) inhibit 5α-reductase, prostate cancer cell/tumor growth, and/or BPH symptoms [27-

31]; however, these phytosterols are not unique to saw palmetto extracts [32]. Nevertheless, a combination of fatty acids, phytosterols, and other bioactive components may be responsible for beneficial effects reported from saw palmetto supplements.

Saw palmetto extract decreased testosterone-induced prostate hyperplasia [15] in rats and prostate cancer progression in TRAMP mice [14]. A number of studies have found that saw palmetto supplements improve lower urinary tract symptoms (LUTS) in men suffering from BPH. The two largest, highest-quality BPH clinical trials [33, 34], however, failed to find a benefit of supplementation, leading a systematic literature review to conclude that saw palmetto supplementation provides no benefit [35].

Although methodological issues or distinctions may be responsible for these conflicting findings, it is also possible that differences in the nutrient profiles of the saw palmetto supplements used in the studies influenced the results. In support of this possibility, different saw palmetto supplements exhibited varied effectiveness in inhibiting 5α-reductase activity and prostate cancer cell proliferation [36, 37]. In addition, Wolsko et al. found that only 6/26 (26%) of published saw palmetto randomized-controlled trials reported performing quantitative analysis on the extract used [38]. This is important because the fatty acid content of saw palmetto supplements has been found to be -97% to +140% of stated dosages [39], and a separate study found that the supplements mean free fatty acid percentages ranged from 40.7% to 80.7% [40].

Others have also measured fatty acid [15, 20, 41-43] or phytosterol content [42, 44-45] of saw palmetto, but despite the reported differences in saw palmetto supplement contents, to the best of our knowledge, no one has characterized both the fatty acid and phytosterol contents of commercially available supplements or compared different supplement categories. Thus, we set

out to characterize these saw palmetto supplement components, hypothesizing that we would find large differences in their quantities and composition.

Materials and Methods

Sample procurement

Twenty commercially available saw palmetto supplements were procured from online and local sources. Standard reference material (SRM) 3251, a *Serenoa repens* extract, was purchased from the National Institute of Standards and Technology (NIST; Gaithersurg, MD) to ensure the accuracy of the fatty acid and phytosterol analysis [20]. Supplements were classified into the following categories based on their physical properties: 1) liquids, 2) powders, 3) dried berries, and 4) tinctures (Table 1).

Sample preparation

Representative samples from each category were prepared as follows. For liquids, 4–5 gel capsules were emptied into a microcentrifuge tube and gently vortexed. For powders, 4–5 powder capsules were emptied into a weigh boat and the contents were mixed thoroughly with a spatula to break up any lumps; tablets were ground to smooth powder using a mortar and pestle. Dried berries were ground using a coffee grinder until they reached a coarse powder texture. Tincture bottles were vortexed before taking a sample. SRM ampoules were vortexed and the contents were transferred to microcentifuge tubes. Samples, and SRM, were extracted and analyzed in duplicate. Care was taken to select the duplicates from the same lot number. All materials were from Fisher Scientific (Pittsburgh, PA) unless noted.

Fatty acid extraction and transesterification

Fatty acids were prepared for analysis using the one-step extraction-transesterification method [46]. Approximately 40 mg of SRM, 40 mg of liquid supplements, 150 mg of powder

supplements, 150 mg of dried berry powder, or 250 mg of tinctures were placed into 15-ml tubes with Teflon-lined screw caps. These amounts were selected because they were estimated to contain ~10–50 mg of total fatty acids based on label information. Two milliliters of benzene containing 2 mg of the internal standard methyl tridecanoate (Sigma-Aldrich, St. Louis, MO) and 3 ml of freshly prepared methanolic-HCl were added. The tubes were capped, vortexed, and incubated at 70^{o}C for 2 h in a water bath. After allowing them to cool to room temperature, 5 ml of 6% potassium carbonate and 2 ml of benzene were added, and the tubes were vortexed and centrifuged at 500x g for 5 min. The supernatant was carefully transferred to vials for analysis.

Fatty acid methyl esters (FAMEs) gas chromatography-flame ionization detector (GC-FID) analysis

Sample extracts were analyzed for fatty acid methyl esters using a Hewlett-Packard model 5890 GC (Hewlett-Packard, Palo Alto, CA, USA) with a SP-2560 capillary column (100m x 0.25mm x d_f 0.2µm, Supelco, Inc., Bellefonte, PA, USA). Injection port and detector temperatures were maintained at 250^{o}C. Helium was used as the carrier gas at a flow rate of 1 ml/min. One microliter of sample was injected at a split ratio of 100:1. A temperature gradient was used with an initial oven temperature of 140°C that increased to 200°C at 2°C/min, then to 245°C at 4°C/min, and the sample held at this temperature for 17 min. The total run time was 66 min. Fatty acid methyl esters (FAMEs) were identified by their retention times using the FAME standard mix (Supelco, Bellefonte, PA, USA) and quantified using Agilent ChemStation software [47]. Thirty-seven fatty acids were analyzed and quantified and contributed to the total fatty acid results reported, but only the six major fatty acids, laurate (C12:0), myristate (C14:0), palmitate (C16:0), stearate (C18:0), oleate (C18:1), and linoleate (C18:2), are reported

individually. The mean percent coefficient of variance between duplicates for total fatty acids was 0.8%.

Phytosterol extraction

Phytosterols were extracted using a modficiation of a method described previously [48]. Approximately 100 mg of SRM, liquid, powder, and dried berry supplements and 1000 mg of tincture supplements were weighed into separate 250-ml Erlenmeyer flasks. Forty milliliters of 0.3M potassium hydroxide in methanol and 10 µl (1 mg/ml chloroform) of cholestanol (Steraloids, Inc. Newport, RI, USA) were added to each flask. The extracts were distilled at 80ºC using a condenser for 1 h with continuous stirring. After cooling the flasks to room temperature, 40 ml of double-distilled water was added. Twenty milliliters of hexane was then added, and the contents were transferred to a separatory funnel. The separatory funnel was carefully inverted, gently rotated, then allowed to stand for 5–10 min. The bottom white turbid aqueous layer was discarded. The contents of the separatory funnel were then extracted by adding 20 ml of hexane twice. The clear hexane layers were collected and filtered through a glass funnel containing glass wool and sodium sulfate. Filtered hexane extracts were concentrated using a Brinkmann Buchi Rotavapor R110 (Buchi, Switzerland) at 50ºC, transferred to test tubes, then evaporated to dryness under nitrogen. The dried contents were redissolved in 1 ml of chloroform and stored at -80ºC for derivatization.

Phytosterol derivatization

Phytosterols were derivitaized and analyzed by gas chromatography-mass spectrometry as described previously with modifications [49]. Two hundred microliters of extracted sample was used for derivatization. A smaller volume was used for samples that contained high levels of phytosterols. Samples were placed in 1.5-ml screw-cap GC vials (Agilent, Santa Clara, CA,

USA), dried completely under nitrogen, and redissolved in 70 μL pyridine (99.5% extra dry with AcroSeal, Acros Organics, Geel, Belgium) for derivatization. The samples in pyridine were derivatized by adding 30 μL of N-trimethylsilyl-N-methyltrifluoroacetamide with 1% trimethylchlorosilane (MSTA +1% TMCS Thermo Scientific, Bellefonte, PA, USA) and incubated at 50°C for 60 min on a hot plate. Derivatized samples were completely dried down under nitrogen, redissolved in 100 μL of chloroform, and analyzed within 24 h of derivatization.

Phytosterol gas chromatography-mass spectrometry (GC-MS)

GC-MS was performed on an Agilent 6890N GC coupled to an Agilent 5975N quadruple mass selective detector. The GC was fitted with a DB-5MS capillary column (60m x 0.25mm, x d_f 0.25 μm, Agilent Technologies, Santa Clara, CA, USA) with a 5% phenyl methyl siloxane stationary phase. Helium was used as the carrier gas at a column flow rate of 1 mL/min. The front inlet was operating at a pressure of 22.33 psi and 280°C. An Agilent 7683 autosampler was used to inject 1 μL of the sample in the splitless mode. The GC temperature program was: initial temperature of 80°C, increased 25°C/min to 300°C, then increased 3°C/min to a final temperature of 325°C, where it was held for 7 min. The total run time was 24.5 min. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV ionization energy. The MS quad temperature was at 150°C, and the MS source temperature was at 230°C. The data were processed with Agilent Chemstation. The three major phytosterols campesterol, stigmasterol, β-sitosterol, were quantified and summed to calculate total phytosterol content. The mean percent coefficient of variance between replicates for total phytosterol was 10.8%.

Statistical analysis

Data were analyzed using SAS 9.3 (SAS Institute Inc., Cary, NC, USA), with $p<0.05$ considered significant. Natural logs were used to transform data that did not meet the

assumptions of normality and/or homogeneity of variance. Differences in individual fatty acid and phytosterol quantities and percentages, total fatty acid and phytosterol quantities, and percentages among the four supplement categories were analyzed using one-way ANOVA with Tukey's test. The variation between duplicates was assessed by dividing the standard deviation with duplicate mean and multiplying with 100 to calculate the mean % coefficient of variation.

Results

Fatty acid quantities and percentages between supplement categories

Fatty acid quantities and percentages of the SRM, liquid, powder, dried berry, and tincture saw palmetto supplements are shown in Tables 2 and 3. Nature's Answer was not included in the tincture means and was excluded from statistical analysis due to its negligible fatty acid content (0.1 mg/g). Oleate and laurate were the predominant fatty acids across the different supplement categories. Liquid supplements contained significantly higher quantities of total (908.5 mg/g) and individual fatty acids than powder, dried berry, and tincture supplements. Liquid supplements contained significantly higher percentages of oleate and total fatty acids and significantly lower percentages of laurate and myristate than the other supplement categories. The total fatty acid content of powder supplements (179.6 mg/g) was similar to dried berry (126.4 mg/g) but significantly higher than tincture supplements (46.3 mg/g). Powder supplements contained significantly higher quantities of palmitate and stearate than dried berries and tinctures and significantly higher quantities of linoleate and total fatty acids than dried berries. Powder supplements contained significantly higher palmitate and stearate percentages than the other supplement categories, and tinctures contained significantly higher percentages of laurate than liquid and powder supplements.

Fatty acid quantities and percentages within supplement categories

Within supplement categories, fatty acid quantities and percentages across the three dried berry supplements were fairly consistent (Tables 2 and 3). Tinctures, on the other hand, varied widely, with total fatty acids ranging from 0.1–97.3 mg/g. There was less variability in total fatty acids in liquids (710.3–941.3 mg/g) and powders (68.6–327.3 mg/g); however, there were some notable trends. Among liquid supplements, Doctor's Best and Saw Palmetto Harvesting Company contained higher laurate and myristate and lower oleate quantities. Jarrow Formulas contained much higher quantities of linoleate than the other liquid supplements. Among powder supplements, Biochem contained lower quantities of laurate and myristate levels but higher quantities of stearate and oleate.

Phytosterol quantities and percentages within supplement categories

Liquid and powder supplements had similar quantities of individual and total phytosterols, with the exception of Jarrow Formulas and Biochem, which contained much higher phytosterol quantities (Tables 4 and 5). Dried berry supplements had similar quantities and percentages of individual and total phytosterols. All tincture supplements contained low phytosterols quantities.

Difference between stated and measured total fatty acid content

Stated total fatty acid content from supplement labels was only available for liquid supplements. These stated quantities are compared to measured content in Table 6. Measured total fatty acid content in five out of six liquid supplements was greater than or equal to the quantities stated on their labels. It is worth noting that one supplement whose measured content (833 mg/g) was below its stated amount, Jarrow Formulas, was only 7% lower than its stated content (850-950 mg/g).

Discussion

We found great variability in total and individual fatty acid and phytosterol quantities and percentages in 20 commercial saw palmetto supplements. There was also a great deal of variability in the total and individual fatty acid and phytosterol quantities and percentages between the four different saw palmetto supplement categories. We believe we are the first to collect and analyze samples from these different supplement categories. Overall, we found that liquid supplements contained the highest fatty acid and phytosterol quantities, followed by powder, dried berry, and tincture supplements.

Fatty acid and phytosterol contents have been reported previously for SRM 3251 [20]. Because total fatty acid quantities were not reported, we multiplied the reported free fatty acid composition by the triglyceride to free fatty acid ratio. Our measured total fatty acid and total phytosterol contents (908.5 mg/g, 2.0 mg/g) are comparable with the calculated SRM 3251 values (983.6 mg/g, 2.4 mg/g). These results support that our extraction and analysis procedures were accurate in measuring these components. The total fatty acid percentages of liquid samples (83.3–94.1%) are consistent with the values reported for hexane (98.7–99.7%) [41], supercritical CO_2 (96.1–97.4%) [42], CO_2 (92.2%) [43], ethanol (88,7%) [43] and lipid (92.5%) [15] saw palmetto extracts. The total fatty acid percentages are also consistent with the sum of the free fatty acids, esters, and glycerides (87.0–95.3%) in 14 European saw palmetto extracts [40]. However, the total fatty acid percentages are higher than those reported for a saw palmetto ethanol extract (68.7%) [43].

Dried berry supplements contained 11.6–13.4% fatty acids, which is similar to the levels reported in SRM 3250 (~15.8%), a saw palmetto berry, when calculated as described above [20]. Permixon, one of the powder supplements, has been analyzed previously and reported to contain

91.4% free fatty acids, esters, and glycerides [40]. This was far higher than the 32.7% fatty acid content that we measured. Our method may not extract fatty acids efficiently from powder samples and could be underestimating the fatty acid content. To the best of our knowledge, we are the first to measure tincture saw palmetto supplements. The levels of the major phytosterols (campesterol, stigamasterol, and β-sitosterol) in the saw palmetto supplements is consistent with quantities found in a variety of previously analyzed saw palmetto supplements [44] and is similar to the β-sitosterol content in a supercritical CO_2 saw palmetto extract [42].

Ingredients added to saw palmetto supplements (Table 1) help explain the differences we found in fatty acid and phytosterol quantities and percentages. Most liquid, and a few powder, supplements contain added ingredients. For instance, among liquid supplements, only Doctor's Best and Saw Palmetto Harvesting Company had no or a limited number of extra ingredients added to them. This probably explains why they have much higher laurate and myristate quantities. Jarrow Formulas, on the other hand, has a pumpkin seed oil base that is enriched in phytosterols, which explains its high linoleate and phytosterol quantities. The remaining four liquid supplements contained either olive oil or extra virgin olive oil (GNC Herbal Plus, Spring Valley Natural Foods, Now Foods, Solaray), which explains their higher oleate percentages compared to other liquid supplements. The powder supplements Nature's Way and Biochem contain magnesium stearate, which explains their high quantities of stearate. Biochem also contains pygeum root extract, which is probably the reason why the supplement contains very high quantities of phytosterols [50].

Industrial preparative extraction method may also help explain the differences in fatty acid and phytosterol quantities in the supplements. Saw palmetto supplements are usually extracted using solvents like hexane, ethyl alcohol, or supercritical CO_2. All the tinctures were

alcohol-extracted, but among liquid and powder supplements, only Doctor's Best and Jarrow Formulas, which were extracted using supercritical CO_2, stated how they were extracted. Thus, the unknowne methods used to produce the supplements prevent us from understanding how extraction affects supplement fatty acid and phytosterol content. Other factors that might also contribute to the variation in the seen are batch and plant growing conditions.

We found that 83% of the liquid supplements contained greater than or equal to their stated fatty acid content. This is higher than the 33% of the measured saw palmetto supplements analyzed in a previous study [39]. Supplement companies that state their fatty acid content may be employing better manufacturing procedures and analyzing their products to ensure they contain the stated values.

Conclusions

We believe we are the first to characterize both fatty acid and phytosterol content of commercial saw palmetto supplements. We plan to determine the anti-androgen action of saw palmetto supplements with different nutrient profiles hoping that the understanding gained will explain the varied response to saw palmetto supplements seen in previous studies.

Implications

Our findings suggest that liquid saw palmetto supplements are the best choice for individuals who want to take a saw palmetto supplement because they contain higher concentrations of both fatty acids and phytosterols than other types of saw palmetto supplements. However, further research is needed to determine whether these supplements are indeed more efficacious.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

This is contribution number 13-371-J from the Kansas Agricultural Experiment Station.

Table 4-1 Saw palmetto supplements' names, manufacturing locations, and other added ingredients.

Saw palmetto supplement	Other label ingredients	Lot Number
Liquids		
Doctor's Best (San Clemente, CA)	Gelatin, glycerin, water	9I1215
GNC Herbal Plus (Pittsburgh, PA)	Olive oil, gelatin, glycerin, caramel color, titanium dioxide	NI
Spring Valley Natural Foods (Springfield, MO)	Olive oil, gelatin, glycerin, water	36994690904
Now Foods (Bloomingdale, IL)	Extra virgin olive oil, gelatin, glycerin	11676001159
Solaray (Park City, UT)	Extra virgin olive oil, gelatin, glycerin	132812
Saw Palmetto Harvesting Company (Lengby, MN)	NI	NI
Jarrow Formulas (Los Angeles, CA)	Pumpkin seed oil, vitamin E, gelatin, glycerin, water	43044J9
Powders		
Permixon (Pierre Fabre Medicament, Boulogne Cedex, France)	NI	G06167
Biochem (Country Life, Hauppauge, NY)	Pygeum extract, magnesium stearate, calcium silicate, magnesium silicate, silica, cellulose	09G802C
Natures' Way Products, Inc. (Springville, UT)	Magnesium stearate, gelatin, glycerin	585612
Solaray (Park City, UT)	Vegetable cellulose capsule	140401
GNC Saw Palmetto Formula (Pittsburgh, PA)	Pumpkin seed meal powder, pygeum bark powder, lycopene, zinc, cellulose, dicalcium phosphate, povidone	5795KJ3613
Dried Berries		
Mountain Rose Herbs (Dried Berries, Eugene, OR)	None	9498
Mountain Rose Herbs (Powdered Berries, Eugene, OR)	None	9646
More than alive (Powdered Berries, Lobelville, TN)	None	NI
Tinctures		
Oregon's Wild Harvest (Sandy, OR)	Organic alcohol, water	SB12159E01
GAIA Herbs, Inc. (Brevard, NC)	Grain alcohol	6212054600
LA Naturals (Michigan City, IN)	Grain alcohol 70–80%, deionized water	7R44ALL
Teeter Creek Herbs (Ava, MO)	Grain alcohol 40–50%, distilled water	NI
Nature's Answer (Hauppauge, NY)	Vegetable glycerin, purified United States Pharmacopeia (USP) water	092469

NI–no information on the label

Table 4-2 Fatty acid quantities (mg/g) and composition (% of total fatty acids) in SRM, liquid, and powder saw palmetto supplements.

Supplement	Laurate (C12:0)		Myristate (C14:0)		Palmitate (C16:0)		Stearate (C18:0)		Oleate (C18:1)		Linoleate (C18:2)		Total fatty acid	
	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%*
Liquid extracts *(n = 7)*														
Doctor's Best	276.6	29.4	105.4	11.2	86.9	9.2	17.5	1.9	313.9	33.4	43.6	4.6	941.3	94.1
GNC Herbal Plus	82.9	8.9	33.8	3.6	94.0	10.1	25.4	2.7	582.3	62.4	59.1	6.3	933.8	93.4
Spring Valley Natural Foods	170.3	18.5	67.6	7.3	85.8	9.3	24.1	2.6	448.5	48.6	53.9	5.8	923.4	92.3
Now Foods	141.6	15.5	51.6	5.7	107.0	11.7	21.1	2.3	438.1	47.9	81.0	8.9	914.7	91.5
Solaray	141.9	15.6	57.1	6.3	90.9	10.0	21.2	2.3	476.0	52.1	55.6	6.1	913.0	91.3
Saw Palmetto Harvesting Company	254.9	28.3	98.5	11.0	84.4	9.4	16.2	1.8	312.5	34.7	34.1	3.8	900.0	90.0
Jarrow Formulas	127.2	15.3	47.5	5.7	93.1	11.2	32.5	3.9	230.2	27.6	248.2	29.8	833.4	83.3
Mean	**170.8^{a}**	**18.8^{a}**	**65.9^{a}**	**7.2^{a}**	**91.7^{a}**	**10.1^{a}**	**22.6^{a}**	**2.5^{a}**	**400.2^{a}**	**43.8^{a}**	**82.2^{a}**	**9.3^{a}**	**908.5^{a}**	**90.9^{a}**
SEM	**18.0**	**1.9**	**6.9**	**0.7**	**2.0**	**0.2**	**1.4**	**0.2**	**30.9**	**3.2**	**19.2**	**2.4**	**9.6**	**1.0**
Powders *(n = 5)*														
Permixon	96.7	29.5	38.5	11.8	30.5	9.3	5.6	1.7	111.7	34.1	11.5	3.5	327.3	32.7
Biochem	40.7	17.1	16.2	6.8	58.2	24.4	47.0	19.7	36.4	15.3	19.1	8.0	238.3	23.8
Natures' Way	38.0	26.5	14.3	10.0	18.2	12.7	14.4	10.0	40.1	27.9	6.5	4.5	143.6	14.4
Solaray	40.5	33.7	14.8	12.3	11.9	9.9	2.5	2.1	34.2	28.5	6.1	5.1	120.1	12.0
GNC Saw Palmetto Formula	14.6	21.3	5.6	8.2	15.2	22.1	9.6	13.9	14.6	21.2	4.6	6.7	68.6	6.9
Mean	**46.1^{b}**	**25.6^{b}**	**17.9^{b}**	**9.8^{b}**	**26.8^{b}**	**15.7^{b}**	**15.8^{b}**	**9.5^{b}**	**47.4^{b}**	**25.4^{b}**	**9.6^{b}**	**5.6^{a}**	**179.6^{b}**	**18.0^{b}**
SEM	**9.0**	**2.0**	**3.7**	**0.7**	**5.7**	**2.1**	**5.4**	**2.3**	**11.1**	**2.2**	**1.8**	**0.5**	**30.7**	**3.1**
Standard reference material														
SRM 3251	259.9	26.3	10.6	10.6	86.6	8.5	16.9	1.7	331.1	34.6	50.2	6.0	983.6	98.4

* % of dry mass (weight/weight), different letters indicate significant differences ($p<0.05$) between the four categories. Samples, and SRM from the same lot, were extracted and analyzed in duplicate.

Table 4-3 Fatty acid quantities (mg/g) and composition (% of total fatty acids) in dried berry and tincture saw palmetto supplements.

Supplement	Laurate (C12:0)		Myristate (C14:0)		Palmitate (C16:0)		Stearate (C18:0)		Oleate (C18:1)		Linoleate (C18:2)		Total fatty acid	
	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%*
Dried berries *(n =3)*														
Mountain Rose Herbs (dried berries)	41.7	31.1	15.1	11.3	12.4	9.3	2.6	1.9	41.2	30.8	6.6	4.9	134.0	13.4
Mountain Rose Herbs (powdered berries)	37.1	28.6	15.6	12.0	12.6	9.7	2.1	1.7	43.9	33.9	4.8	3.7	129.7	13.0
More than Alive (powdered berries)	37.5	32.5	14.1	12.2	11.5	9.9	2.2	1.9	34.6	30.0	5.6	4.9	115.7	11.6
Mean	**38.8^{b}**	**30.7^{b,c}**	**14.9^{b}**	**11.8^{b}**	**12.2^{c}**	**9.6^{a}**	**2.3^{c}**	**1.8^{a}**	**39.9^{b}**	**31.5^{b}**	**5.7^{b}**	**4.5^{a}**	**126.4^{b,c}**	**12.6^{b,c}**
SEM	**0.9**	**0.7**	**0.3**	**0.2**	**0.2**	**0.1**	**0.1**	**0.1**	**1.7**	**0.8**	**0.3**	**0.3**	**3.5**	**0.3**
Tinctures *(n = 5)*														
Oregon's Wild Harvest	28.4	29.2	10.6	10.9	8.7	9.0	1.7	1.8	32.0	32.9	4.5	4.6	97.3	9.7
GAIA Herbs	22.8	35.2	9.3	14.3	5.5	8.4	0.6	1.0	20.1	31.0	1.2	1.9	64.8	6.5
LA Naturals	5.1	40.2	1.7	13.4	1.1	8.6	0.2	1.4	3.0	23.1	0.7	5.6	12.8	1.3
Teeter Creek	3.7	36.5	1.2	11.6	0.7	7.4	0.1	1.3	2.4	23.6	0.3	2.7	10.1	1.0
Nature's Answer**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	19.3	0.0	0.0	0.0	0.0	0.1	0.0
Mean	**15.0^{b}**	**35.3^{c}**	**5.7^{b}**	**12.5^{b}**	**4.0^{c}**	**8.3^{a}**	**0.7^{c}**	**1.4^{a}**	**14.3^{b}**	**27.6^{b}**	**1.7^{c}**	**3.7^{a}**	**46.3^{c}**	**4.6^{c}**
SEM	**4.1**	**1.5**	**1.6**	**0.5**	**1.2**	**0.2**	**0.2**	**0.1**	**4.7**	**1.6**	**0.6**	**0.6**	**13.9**	**1.4**

* % of dry mass (weight/weight), ** Nature's Answer was excluded from statistical analysis due to its negligible fatty acid content compared with other products. Different letters indicate significant differences ($p<0.05$) between the four categories Samples, and SRM from the same lot, were extracted and analyzed in duplicate.

Table 4-4 Phytosterol quantities (mg/g) and composition (% of total phytosterols) in SRM, liquid and powder saw palmetto supplements.

Supplement	**Campesterol**		**Stigmasterol**		**β-sitosterol**		**Total phytosterols**	
	mg/g	**%**	**mg/g**	**%**	**mg/g**	**%**	**mg/g**	**%***
Liquids								
Jarrow Formulas**	8.33	29.09	4.01	14.03	16.29	56.88	28.63	2.86
Doctor's Best	0.54	19.21	0.24	8.53	2.04	72.26	2.83	0.28
Now Foods	0.29	12.56	0.13	5.66	1.92	81.78	2.35	0.23
Saw Palmetto Harvesting Company	0.34	21.05	0.16	10.09	1.12	68.86	1.62	0.16
Solaray	0.23	11.10	0.11	5.19	1.73	83.71	2.07	0.21
Spring Valley Natural Foods	0.15	7.20	0.11	5.52	1.80	87.28	2.06	0.21
GNC Herbal Plus	0.15	11.61	0.07	5.40	1.08	82.99	1.31	0.13
Mean	**0.28[a]**	**13.79[a]**	**0.14[a]**	**6.73[a]**	**1.62[a]**	**79.48[a]**	**2.04[a]**	**0.20[a]**
SEM	**0.04**	**1.45**	**0.02**	**0.57**	**0.12**	**1.99**	**0.15**	**0.02**
Powders								
Biochem**	22.80	30.30	19.75	26.55	32.55	43.15	75.11	7.51
Permixon	0.18	21.06	0.06	7.68	0.60	71.27	0.84	0.08
Nature's Way	0.10	24.65	0.04	11.11	0.25	64.24	0.39	0.04
Solaray	0.07	23.89	0.03	9.30	0.20	66.80	0.30	0.03
GNC Saw Palmetto Formula	0.03	19.83	0.01	9.19	0.10	70.98	0.15	0.01
Mean	**0.09[b]**	**22.36[b]**	**0.04[b,c]**	**9.32[a]**	**0.29[b]**	**68.32[b]**	**0.42[b]**	**0.04[b]**
SEM	**0.02**	**0.90**	**0.01**	**0.53**	**0.07**	**1.33**	**0.10**	**0.01**
Standard reference material								
SRM 3251	0.41	19.69	0.18	8.57	1.48	71.74	2.06	0.21

*% dry mass (weight/weight), ** Jarrow Formulas and Biochem were excluded from statistical analysis due to their very high phytosterol content compared with other supplements. Different letters indicate significant differences ($p<0.05$) between the four categories. Samples, and SRM from the same lot, were extracted and analyzed in duplicate.

Table 4-5 Phytosterol quantities (mg/g) and composition (% of total phytosterols) in dried berry and tincture saw palmetto supplements.

Supplement	Campesterol		Stigmasterol		β-sitosterol		Total sterols	
	mg/g	%	mg/g	%	mg/g	%	mg/g	%*
Dried berries								
Mountain Rose Herbs (dried berries)	0.16	41.45	0.09	21.91	0.13	36.64	0.38	0.04
Mountain Rose Herbs (powdered berries)	0.09	27.93	0.06	17.41	0.17	54.66	0.32	0.03
More Than Alive (powdered berries)	0.08	27.28	0.07	22.06	0.15	50.66	0.30	0.03
Mean	**0.11^{b}**	**32.22^{c}**	**0.07^{b}**	**20.46^{b}**	**0.15^{b}**	**47.32^{c}**	**0.33^{b,c}**	**0.03^{b}**
SEM	**0.02**	**3.30**	**0.01**	**2.86**	**0.01**	**5.16**	**0.03**	**0.00**
Tinctures								
Oregon's Wild Harvest	0.04	16.40	0.02	9.16	0.18	74.45	0.24	0.02
GAIA Herbs	0.03	17.95	0.02	11.65	0.11	70.40	0.16	0.02
LA Naturals Saw Palmetto drops	0.01	16.73	0.01	13.72	0.04	69.55	0.06	0.01
Nature's Answer	0.00	21.12	0.00	25.23	0.01	53.65	0.02	0.00
Teeter Creek	0.01	22.27	0.00	14.71	0.02	63.02	0.02	0.00
Mean	**0.02^{b}**	**18.89^{a,b}**	**0.01^{c}**	**14.89^{b}**	**0.07^{b}**	**66.21^{b}**	**0.10^{c}**	**0.01^{b}**
SEM	**0.00**	**0.83**	**0.00**	**1.86**	**0.02**	**2.47**	**0.03**	**0.00**

* % of dry mass (weight/weight), different letters indicate significant differences ($p<0.05$) between the four categories. Samples, and SRM from the same lot, were extracted and analyzed in duplicate.

Table 4-6 Stated, measured, and percentage difference in total fatty acid content in liquid saw palmetto supplements.

Product name	Stated content (mg/g)	Measured content (mg/g)	% difference
Doctor's Best	900	941	+5
GNC Herbal Plus	850	934	+10
Spring Valley Natural Foods	850-950	923	+3
Now Foods	850-950	915	+2
Solaray	850	913	+7
Jarrow Formulas	850-950	833	-7

References

1. Barnes, P.M.; Bloom, B.; Nahin, R.L. CDC National Health Statistics Report #12. Complementary and Alternative Medicine Use Among Adults and Children: United States, 2007. CDC National Health Statistics Report #12 2008, 1-23.

2. Blumenthal, M.; Lindstrom, A.; Ooyen, C.; Lynch, M.E. Herb Supplement Sales Increase 4.5% in 2011. HerbalGram 2012, 60-64.

3. Bishop, F.L.; Rea, A.; Lewith, H.; Chan, Y.K.; Saville, J.; Prescott, P.; von Elm, E.; Lewith, G.T. Complementary medicine use by men with prostate cancer: a systematic review of prevalence studies. Prostate Cancer Prostatic Dis. 2011, 14, 1-13.

4. Bauer, C.; Ishak, M.; Johnson, E.; Beebe Dimmer, J.; Cooney, K. Prevalence and correlates of vitamin and supplement usage among men with a family history of prostate cancer. Integr. Cancer. Ther. 2012, 11, 83-89.

5. Iehlé, C.; Délos, S.; Guirou, O.; Tate, R.; Raynaud, J.P.; Martin, P.M. Human prostatic steroid 5 alpha-reductase isoforms--a comparative study of selective inhibitors. J. Steroid Biochem Mol Biol. 1995, 54, 273.

6. Bayne, C.W.; Donnelly, F.; Ross, M.; Habib, F.K. Serenoa repens (Permixon): a 5alpha-reductase types I and II inhibitor-new evidence in a coculture model of BPH. Prostate 1999, 40, 232-241.

7. Bayne, C.W.; Ross, M.; Donnelly, F.; Habib, F.K. The selectivity and specificity of the actions of the lipido-sterolic extract of Serenoa repens (Permixon) on the prostate. J. Urol. 2000, 164, 876-881.

8. Habib, F.K.; Ross, M.; Ho, C.K.H.; Lyons, V.; Chapman, K. Serenoa repens (Permixon) inhibits the 5alpha-reductase activity of human prostate cancer cell lines without interfering with PSA expression. Int. J. Cancer 2005, 114, 190.

9. Anderson, M.L. A preliminary investigation of the enzymatic inhibition of 5alpha-reduction and growth of prostatic carcinoma cell line LNCap-FGC by natural astaxanthin and Saw Palmetto lipid extract in-vitro. J. Herbal Pharmacother. 2005, 5, 17-26.

10. Pais, P. Potency of a novel saw palmetto ethanol extract, SPET-085, for inhibition of 5alpha-reductase II. Adv. Ther. 2010, 27, 555-563.

11. Van Coppenolle, F.; Le Bourhis, X.; Carpentier, F.; Delaby, G.; Cousse, H.; Raynaud, J.P.; Dupouy, J.P.; Prevarskaya, N. Pharmacological effects of the lipidosterolic extract of Serenoa repens (Permixon) on rat prostate hyperplasia induced by hyperprolactinemia: comparison with finasteride. Prostate 2000, 43, 49-58.

12. Talpur, N.; Echard, B.; Bagchi, D.; Bagchi, M.; Preuss, H.G. Comparison of Saw Palmetto (extract and whole berry) and Cernitin on prostate growth in rats. Mol. Cell. Biochem. 2003, 250, 21-26.

13. Carbajal, D.; Molina, V.; Mas, R.; Arruzazabala, M.L. Therapeutic effect of D-004, a lipid extract from Roystonea regia fruits, on prostate hyperplasia induced in rats. Drugs Exp. Clin. Res. 2005, 31, 193-7.

14. Wadsworth, T.L.; Worstell, T.R.; Greenberg, N.M.; Roselli, C.E. Effects of dietary saw palmetto on the prostate of transgenic adenocarcinoma of the mouse prostate model (TRAMP). Prostate 2007, 67, 661-673.

15. Arruzazabala, M.L.; Molina, V.; Mas, R.; Carbajal, D.; Marrero, D.; González, V.; Más, R.; Rodríguez, E. Effects of coconut oil on testosterone-induced prostatic hyperplasia in Sprague-Dawley rats. J. Pharm. Pharmacol. 2007, 59, 995-999.

16. Rhodes, L.; Primka, R.L.; Berman, C.; Vergult, G.; Gabriel, M.; Pierre-Malice, M.; Gibelin, B. Comparison of finasteride (Proscar), a 5 alpha reductase inhibitor, and various commercial plant extracts in in-vitro and in vivo 5 alpha reductase inhibition. Prostate 1993, 22, 43-51.

17. Hill, B.; Kyprianou, N. Effect of permixon on human prostate cell growth: lack of apoptotic action. Prostate 2004, 61, 73-80.

18. Strauch, G.; Perles, P.; Vergult, G.; Gabriel, M.; Gibelin, B.; Cummings, S.; Malbecq, W.; Malice, M.P. Comparison of finasteride (Proscar) and Serenoa repens (Permixon) in the inhibition of 5-alpha reductase in healthy male volunteers. Eur. Urol. 1994, 26, 247-52.

19. U.S. Department of Agriculture, Agricultural Research Service USDA National Nutrient Database for Standard Reference, Release 25. http://ndb.nal.usda.gov/ (accessed May/28, 2013).

20. Schantz, M.M.; Bedner, M.; Long, S.E.; Molloy, J.L.; Murphy, K.E.; Porter, B.J.; Putzbach, K.; Rimmer, C.A.; Sander, L.C.; Sharpless, K.E.; Thomas, J.B.; Wise, S.A.; Wood, L.J.; Yen, J.H.; Yarita, T.; NguyenPho, A.; Sorenson, W.R.; Betz, J.M. Development of saw palmetto (Serenoa repens) fruit and extract standard reference materials. Anal. Bioanal. Chem. 2008, 392, 427-438.

21. Liang, T.; Liao, S. Growth suppression of hamster flank organs by topical application of gamma-linolenic and other fatty acid inhibitors of 5alpha-reductase. J. Invest. Dermatol. 1997, 109, 152-157.

22. Weisser, H.; Tunn, S.; Behnke, B.; Krieg, M. Effects of the sabal serrulata extract IDS 89 and its subfractions on 5 alpha-reductase activity in human benign prostatic hyperplasia. Prostate 1996, 28, 300-6.

23. Niederprum, H.J.; Schweikert, H.U.; Thuroff, J.W.; Zanker, K.S. Inhibition of steroid 5 alpha-reductase activity by aliphatic fatty acids. Candidates for chemoprevention of prostate cancer. Ann. N. Y. Acad. Sci. 1995, 768, 227-30.

24. Raynaud, J.; Cousse, H.; Martin, P. Inhibition of type 1 and type 2 5alpha-reductase activity by free fatty acids, active ingredients of Permixon. J. Steroid Biochem. Mol. Biol. 2002, 82, 233-9.

25. Abe, M.; Ito, Y.; Suzuki, A.; Onoue, S.; Noguchi, H.; Yamada, S. Isolation and pharmacological characterization of fatty acids from saw palmetto extract. Anal. Sci. 2009, 25, 553-7.

26. Abe, M.; Ito, Y.; Yunzul, L.; Oki Fujino, T.; Yamada, S.; Oyunzul, L. Pharmacologically relevant receptor binding characteristics and 5alpha-reductase inhibitory activity of free Fatty acids contained in saw palmetto extract. Biol. Pharm. Bull. 2009, 32, 646-50.

27. Berges, R.R.; Windeler, J.; Senge, T.; Aeikens, B.; Trampisch, H.J. Randomised, placebo-controlled, double-blind clinical trial of beta-sitosterol in patients with benign prostatic hyperplasia. Beta-sitosterol Study Group. Lancet 1995, 345, 1529-32.

28. von Holtz, R.L.; Fink, C.S.; Awad, A.B. Beta-sitosterol activates the sphingomyelin cycle and induces apoptosis in LNCaP human prostate cancer cells. Nutr. Cancer 1998, 32, 8-12.

29. Awad, A.B.; Williams, H.; Kim, U.; Fink, C.S. In-vitro and in vivo (SCID mice) effects of phytosterols on the growth and dissemination of human prostate cancer PC-3 cells. Eur. J. Cancer Prev. 2001, 10, 507-13.

30. Cabeza, M.; Bratoeff, E.; Heuze, I.; Ramirez, E.; Sanchez, M.; Rami-rez, E.; Sanchez, M.; Flores, E. Effect of beta-sitosterol as inhibitor of 5 alpha-reductase in hamster prostate. Proc. West. Pharmacol. Soc. 2003, 46, 153-5.

31. Scholtysek, C.; Krukiewicz, A.; Alonso, J.; Sharma, K.; Sharma, P.; Goldmann, W. Characterizing components of the Saw Palmetto Berry Extract (SPBE) on prostate cancer cell growth and traction. Biochem. Biophys. Res. Commun. 2009, 379, 795-798.

32. Weihrauch, J.L.; Gardner, J.M. Sterol content of foods of plant origin. J. Am. Diet. Assoc. 1978, 73, 39-47.

33. Bent, S.; Kane, C.; Shinohara, K.; Neuhaus, J.; Hudes, E.; Goldberg, H.; Avins, A. Saw palmetto for benign prostatic hyperplasia. N. Engl. J. Med. 2006, 354, 557-566.

34. Barry, M.; Meleth, S.; Lee, J.; Kreder, K.; Avins, A.; Nickel, J.C.; Roehrborn, C.; Crawford, E.D.; Foster, H.; Kaplan, S.; McCullough, A.; Andriole, G.; Naslund, M.; Williams, O.D.; Kusek, J.; Meyers, C.; Betz, J.; Cantor, A.; McVary, K. Effect of increasing doses of saw palmetto extract on lower urinary tract symptoms: a randomized trial. JAMA-J.Am. Med. Assoc. 2011, 306, 1344-1351.

35. MacDonald, R.; Tacklind, J.; Rutks, I.; Wilt, T. Serenoa repens monotherapy for benign prostatic hyperplasia (BPH): an updated Cochrane systematic review. BJU Int. 2012, 109, 1756-1761.

36. Scaglione, F.; Lucini, V.; Pannacci, M.; Caronno, A.; Leone, C. Comparison of the potency of different brands of Serenoa repens extract on 5alpha-reductase types I and II in prostatic co-cultured epithelial and fibroblast cells. Pharmacology 2008, 82, 270-275.

37. Scaglione, F.; Lucini, V.; Pannacci, M.; Dugnani, S.; Leone, C. Comparison of the potency of 10 different brands of Serenoa repens extracts. Eur. Rev. Med. Pharmacol. Sci. 2012, 16, 569-574.

38. Wolsko, P.M.; Solondz, D.K.; Phillips, R.S.; Schachter, S.C.; Eisenberg, D.M. Lack of herbal supplement characterization in published randomized controlled trials. Am. J. Med. 2005, 118, 1087-1093.

39. Feifer, A.H.; Fleshner, N.E.; Klotz, L. Analytical accuracy and reliability of commonly used nutritional supplements in prostate disease. J. Urol. 2002, 168, 150-154.

40. Habib, F.K.; Wyllie, M.G. Not all brands are created equal: a comparison of selected components of different brands of Serenoa repens extract. Prostate Cancer Prostatic Dis. 2004, 7, 195-200.

41. Priestap, H.; Houle, P.; Bennett, B. Fatty acid composition of fruits of two forms of Serenoa repens. Chem. Nat. Compd. 2011, 47, 511-514.

42. Catchpole, O.J.; Perry, N.B.; da Silva, B.M.T.; Grey, J.B.; Smallfield, B.M. Supercritical extraction of herbs I: Saw Palmetto, St John's Wort, Kava Root, and Echinacea. J. Supercrit. Fluids 2002, 22, 129-138.

43. Ganzera, M.; Croom, E.M.; Khan, I.A. Determination of the Fatty Acid content of pumpkin seed, pygeum, and saw palmetto. J. Med. Food 1999, 2, 21-27.

44. Sorenson, W.R.; Sullivan, D. Determination of campesterol, stigmasterol, and beta-sitosterol in saw palmetto raw materials and dietary supplements by gas chromatography: collaborative study. J AOAC Int. 2007, 90, 670-678.

45. Sorenson, W.R.; Sullivan, D. Determination of campesterol, stigmasterol, and beta-sitosterol in saw palmetto raw materials and dietary supplements by gas chromatography: single-laboratory validation. J. AOAC Int. 2006, 89, 22.

46. Sukhija, P.; Palmquist, D.L. Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. J. Agric. Food Chem. 1988, 36, 1202-1206.

47. Hawkins, A.; Yuan, K.; Armendariz, C.K.; Highland, G.; Bello, N.M.; Winowiski, T.; Drouillard, J.S.; Titgemeyer, E.C.; Bradford, B.J. Effects of urea formaldehyde condensation polymer treatment of flaxseed on ruminal digestion and lactation in dairy cows. J. Dairy Sci. 2013, 96, 3907-15.

48. Zhang, X.; Julien-David, D.; Miesch, M.; Geoffroy, P.; Raul, F.; Roussi, S.; Aoudé-Werner, D.; Marchioni, E. Identification and quantitative analysis of beta-sitosterol oxides in vegetable oils by capillary gas chromatography-mass spectrometry. Steroids 2005, 70, 896-906.

49. Merath, K.M.; Chang, B.; Dubielzig, R.; Jeannotte, R.; Sidjanin, D.J.A. spontaneous mutation in Srebf2 leads to cataracts and persistent skin wounds in the lens opacity 13 (lop13) mouse. Mamm. Genome 2011, 22, 661-673.

50. Kadu, C.A.C.; Parich, A.; Schueler, S.; Konrad, H.; Muluvi, G.; Eyog-Matig, O.; Muchugi, A.; Williams, V.; Ramamonjisoa, L.; Kapinga, C.; Foahom, B.; Katsvanga, C.; Hafashimana, D.; Obama, C.; Vinceti, B.; Schumacher, R.; Geburek, T. Bioactive constituents in Prunus africana: geographical variation throughout Africa and associations with environmental and genetic parameters. Phytochemistry 2012, 83, 70-8.

Chapter 5 - Summary & Future Directions

The current research was carried out to screen different varieties of sorghum-based novel fortified blended foods and compare them with the traditional corn-soy FBFs. Products were assessed for bioavailable iron and vitamin A using the in-vitro digestion/Caco-2 cell model. In preliminary research (Chapter 2) with FBFs low in vitamins A, D and E; most extruded FBFs showed 2- to 4-fold significantly higher levels of iron in aqueous fractions than traditional FBFs. However similar differences were not observed in ferritin response. Extruded sorghum-cowpea, sorghum-soy and corn-soy showed iron bioavailability that was similar to non-extruded CSB13 and CSB+.

In the second part of our research (Chapter 3), all of the extruded FBFs had 4- to 7-fold significantly higher aqueous fraction iron concentrations compared to non-extruded CSB13 and CSB+. However, the Caco-2 cell ferritin response was similar between extruded and non-extruded FBFs. Also, vitamin A levels in aqueous fractions and Caco-2 cells were not different between extruded and non-extruded FBFs.

The in vitro digestion-Caco-2 cell model needs further refinement to optimize the culture conditions and to standardize the technique to measure the bioavailability of multiple micronutrients. Inter-laboratory methodological differences such as growing cells on inserts compared to plastic surface, passage number, harvesting time and heat treatment of aqueous fraction may cause variation in the study outcomes. Even though both the studies (Chapter 2 and Chapter 3) were conducted on similar FBFs, the methodology for Chapter-3 was modified to use cell lysis buffer as mechanical lysis did not work any longer. Ferritin response was also different between the two studies, and is approximately 3-times lower in Chapter-3. As this is the first study looking at both bioavailable iron and vitamin A levels, we used a method that did not grow cells on inserts, a common technique used in most iron

bioavailability studies. This modification allowed us to treat the Caco-2 cells directly with filtered aqueous fractions. Caution should be warranted for future studies using the in-vitro digestion/Caco-2 cell model as there were studies that did not find ferritin response above baseline levels [1, 2]. Future studies should also consider assessing the cell differentiation by measuring the brush border enzyme activity [3]. If the Caco-2 cells are not fully differentiated they express less number of transporters and cause lower nutrient uptake by cells. This information on cell differentiation time should be used in determining the day Caco-2 cells can be used for iron and vitamin A bioavailability experiment.

The major difficulty in direct comparison of our results is that the extruded and traditional FBFs vary from each other in their composition, cooking, and iron fortificants used. Another difference was that the porridges were prepared at different solid concentrations. For example, extruded FBFs were cooked at 20% solids, whereas traditional FBFs were cooked at 12-14% solids. Also, unlike in other Caco-2 iron bioavailability studies [4, 5], iron concentration among the porridge aliquots used for in-vitro digestion were not same. However, the advantage of our porridges was that they were prepared similar to the porridges that are made at household level in food aid receiving countries such as Tanzania. This uniformity in porridge cooking methods would help in translation of current research findings to future human trials.

Although, two sets of FBFs showed similar results with regards to bioavailable iron, this study could have been improved by including a non-extruded sorghum-cowpea or sorghum-soy FBF. This would have allowed us to determine the effect of extrusion on micronutrient bioavailability in sorghum FBFs. Even though extrusion is widely used in different food and feed applications [6], and has been shown to reduce antinutritional factors and improve iron bioavailability [7, 8], this processing technique was used for the first time on sorghum FBFs. As the study FBFs were newly developed, there were no other extruded

sorghum FBF studies available to compare our results. Hence, the study findings on extruded sorghum based FBFs may help in designing the food aid products, particularly in sorghum-consuming regions like Africa and Asia.

The amount of dietary fat and fiber were shown to have increasing and decreasing effect on vitamin A micellarization and absorption in animal [9, 10]. However, we did not observe differences in bioavailable vitamin A levels among FBFs with different fat levels. More Caco-2 cell model studies should determine the effect of fat and fiber on bioavailable vitamin A levels in FBFs.

Another future direction is determining the antinutrient concentrations in extruded and non-extruded FBFs. In the present study, the increase in aqueous fraction iron concentrations might have partly contributed by phytate and tannin degradation to lower forms caused by extrusion. Quantification of total phytate, tannin and their degradation products in FBFs may help further understand the results.

Finally, animal studies and field trials are needed to confirm the equal nutritional benefits of extruded sorghum based FBFs compared to traditional corn-soy FBFs. The broiler chicken model may be a suitable in-vivo model, as this model was shown to respond to iron deficiency and has advantages over rodent and pig models [11].

Liquid saw palmetto supplements contained significantly higher concentrations of total fatty acids, individual fatty acids, total phytosterols, and individual phytosterols than other saw palmetto categories such as powders, tinctures and dried berries. Studies suggest that the fatty acids in saw palmetto extracts are responsible for its ability to inhibit 5α-reductase [12, 13], an enzyme that converts testosterone to dihydrotestoterone leading to prostate hyperplasia, but which fatty acids are responsible for the inhibition varies. Hence future studies should determine the anti-androgen action of liquid saw palmetto supplements with different nutrient profiles in LNCap cells and prostate cancer animal models.

References

1. Tan SY, Yeung CK, Tako E, Glahn RP, Welch RM, Lei X, et al. Iron bioavailability to piglets from red and white common beans (Phaseolus vulgaris). J Agric Food Chem. 2008;56: 5008-5014.

2. Tako E, Beebe SE, Reed S, Hart JJ, Glahn RP. Polyphenolic compounds appear to limit the nutritional benefit of biofortified higher iron black bean (Phaseolus vulgaris L). Nutr J. 2014;13: 28-2891-13-28.

3. Jovaní M, Barberá R, Farré R, Martín de Aguilera E. Calcium, iron, and zinc uptake from digests of infant formulas by Caco-2 cells. J Agric Food Chem. 2001;49: 3480-5.

4. Latunde-Dada GO, Li X, Parodi A, Edwards C, Ellis P, Sharp PA. Micromilling enhances iron bioaccessibility from wholegrain wheat. J Agric Food Chem. 2014;62: 11222-11227.

5. Lei J, Zhang Y, Chen X, Zhang M, Bai L, Huang C, et al. Assessment of iron bioavailability in ten kinds of Chinese wheat flours using an in-vitro digestion/Caco-2 cell model. Biomed Environ Sci. 2012;25: 502-8.

6. Ilo S, Schoenlechner R, Berghofe E. Role of lipids in the extrusion cooking processes. Grasas Aceites. 2000;51: 97-110.

7. Alonso R, Rubio L, Muzquiz M, Marzo F. The effect of extrusion cooking on mineral bioavailability in pea and kidney bean seed meals. Anim Feed Sci Technol. 2001;94: 1-13.

8. Tran QD, Hendriks WH, van der Poel, Antonius FB. Effects of extrusion processing on nutrients in dry pet food. J Sci Food Agric. 2008;88: 1487-1493.

9. Reboul E. Absorption of vitamin A and carotenoids by the enterocyte: focus on transport proteins. 2013;5: 3563-3581.

10. Goda T, Yasutake H, Takase S. Dietary fat regulates cellular retinol-binding protein II gene expression in rat jejunum. 1994;1200: 34-40.

11. Tako E, Rutzke MA, Glahn RP. Using the domestic chicken (Gallus gallus) as an in vivo model for iron bioavailability. Poult Sci. 2010;89: 514-521.

12. Liang, T.; Liao, S. Growth suppression of hamster flank organs by topical application of gamma-linolenic and other fatty acid inhibitors of 5alpha-reductase. J. Invest. Dermatol. 1997, 109, 152-157.

13. Abe, M.; Ito, Y.; Yunzul, L.; Oki Fujino, T.; Yamada, S.; Oyunzul, L. Pharmacologically relevant receptor binding characteristics and 5alpha-reductase inhibitory activity of free Fatty acids contained in saw palmetto extract. Biol. Pharm. Bull. 2009, 32, 646-50.