MINERAL COMPOSITION OF BOILED, GREEN LEAFY VEGETABLES FOUND IN HAWAIʻI AND IRON BIOAVAILABILITY USING THE IN-VITRO DIGESTION/CACO-2 CELL METHOD

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ABSTRACT

There is an increasing trend toward a more vegetarian diet. The 2010 Dietary Guidelines promote healthier eating patterns by recommending increased consumption of fruits and vegetables. They specifically advocate for the increased consumption of green leafy vegetables (GLV), which are associated with reduced risk of chronic disease, low in calories, and can contribute adequate amounts of folate, magnesium, potassium, dietary fiber, vitamins A, C, and K, nutrients typically under-consumed in the U.S. However, iron deficiency is the most prevalent micronutrient deficiency in the world and plant-based diets low in iron content and low in iron bioavailability are major contributors. Therefore, research is needed to identify plant-based foods with both high iron content and good iron bioavailability. Green leafy vegetables are purportedly good sources of iron. This study objective was to evaluate the mineral content and iron bioavailability of ten green leafy vegetables consumed by many ethnic groups in Hawaii. These included: chrysanthemum, sweet potato leaf, ung choy, watercress, amaranth leaf, bitter melon leaf, edible hibiscus, kale, moringa, and taro leaf. Produce was purchased from local markets or obtained from local growers and prepared by boiling for typical preparation times. Produce was then drained, homogenized using stainless steel equipment, and freeze-dried. Samples were sent to the LSU Agricultural Center and analyzed using inductively coupled plasma-emission spectroscopy. Mineral contents were compared with Daily Values (DV) per 85 g reference amount (RA). Bitter melon leaf met the DV definition of good (10% of DV per RA for calcium, copper, iron, magnesium, and manganese. Four additional vegetables would be ranked as good or excellent (20% of DV per RA) in

calcium (amaranth leaf, edible hibiscus, moringa, and taro leaf). Only bitter melon and amaranth were good in magnesium. Copper and manganese were good in most produce. The four highest iron containing GLV (amaranth, bitter melon, edible chrysanthemum, and moringa) were further screened for iron bioavailability using the in-vitro digestion/Caco-2 cell system. Beef and spinach were used as reference foods. All foods were subjected to in-vitro gastric and intestinal digestions followed by application to monolayers of human intestinal (Caco-2) cells to assess iron bioavailability. In experiment 1, all samples were normalized to 67 μM Fe in the digests to compare absorption efficiency. In experiment 2, equal amounts of sample (0.5 g dry matter) were digested. Results showed that spinach and moringa had the highest efficiency of iron absorption, while amaranth and bitter melon had the lowest efficiency. However, iron bioavailability per recommended 85 g serving amount (RA) was the same among all GLV and spinach. Beef had 50% higher bioavailability per RA compared to all plant foods. We conclude that while there are differences in iron content and efficiency of iron absorption among these selected plant foods, the amount of iron absorbed from a serving of all of these GLV is equal to that from a serving of spinach. Further investigation is warranted to find other local, plant-based foods with greater potential to provide adequate iron. Our results present new information on mineral content and iron bioavailability of Hawaiian-grown boiled produce. This data could be used to improve local and national databases and in turn improve the nutritional quality of the diet.

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
ANOVA	Analysis of Variance
AMDR	Acceptable Macronutrient Distribution Ranges
AP-2	Assembly peptide-2
Caco-2	Human colonic adenocarcinoma cells
CO_2	Carbon dioxide
DW	Dry weight
DMEM	Dulbecco's modified eagle medium
DMT1	Divalent metal transporter-1
DNA	Deoxyribonucleic acid
DRI	Dietary reference intakes
DV	Daily Value
EDTA	Ethylenediaminetetraacetic acid
EP	Edible portion
FBS	Fetal bovine serum
FDA	Food and Drug Administration
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
$FeSO_4$	Ferrous sulfate
FW	Fresh weight
GLV	Green leafy vegetables
GSE	Grape seed extract
ECGC	Epigallocatechin gallate
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HBBS	Hank's balanced salt solution
HCl	Hydrochloric acid
HCP-1	Heme carrier protein-1
HO	Heme oxygenase-1
HNO_3	Nitric acid
HRE	Hemoglobin regeneration efficiency
ICP-ES	Inductively coupled plasma-emission spectroscopy
KCl	Potassium chloride
MEM	Minimal essential media
mRNA	Messenger ribonucleic acid
MW	Molecular weight
$NaHCO_3$	Sodium biocarbonate
NaCl	Sodium chloride
NFT	Nutrient film technique
NLEA	Nutrition Labeling and Education Act

PPM	Parts per million
RACC	Recommended Amount commonly consumed per eating occasion
RDA	Recommended Dietary Allowance
UNICEF	The United Nations Children's Fund
USDA	United States Department of Agriculture
WHO	World Health Organization

CHAPTER 1: LITERATURE REVIEW

1.1 IRON DEFICIENCY: THE NEED TO IDENTIFY PLANT SOURCES OF IRON

1.1.1. Iron deficiency

Iron deficiency is the most common and widespread nutritional deficiency in the world. It has been estimated that 30-40% of the world's population are iron deficient (WHO 2001). In the United States, nine percent of toddlers and nine to eleven percent of adolescents and women of child-bearing age are iron deficient (Looker, Dallman et al. 1997).

Iron deficiency is defined as a progressive loss of iron in the body resulting from low dietary intake, inadequate absorption, or iron losses (Haas and Brownlie IV 2001). Common symptoms of iron deficiency include reduced work capacity, impaired cognitive development, and increased rates of preterm delivery and low birth weight (Kretchmer, Beard et al. 1996; Allen 2000; Haas and Brownlie IV 2001).

The reaction to the global scale of iron deficiency has been for large-scale public health agencies to mandate research and practices aimed toward addressing iron deficiency. The three principal strategies that have been used to combat iron deficiency include iron supplementation, food fortification, and the consumption of foods high in iron content and bioavailability. Many of these programs are currently implemented around the world, but evidence has shown that the efficacy of such intervention strategies as a whole have proven to be unsuccessful (Hurrell 1997; Stoltzfus 2003). Therefore,

further research is required to bridge the link between theoretical and real world, applicable, and sustainable approaches. Food-based approaches are advised as the first priority in strategies intended to meet iron requirements. Iron supplementation and food fortification in theory can be effective strategies to combat iron deficiency but the high cost, palatability, low compliance, and adverse health effects associated with supplementation and fortification have made their efforts prohibitive (Allen 2008). Furthermore, while iron supplementation in clinical trials has proven effective, its translation to public health practices has not. The limitations of iron supplementation and food fortification place importance on the food-based approach. Food-based approaches have been suggested as the best method to meet iron requirements (Walker 1998).

1.1.2. Iron bioavailability

Iron bioavailability is defined as the measure of the proportion of the total iron in a food or diet that is digested, absorbed, and metabolized by normal pathways (Fairweather-Tait 1987). The predominant factors affecting iron bioavailability include the amounts of non-heme and heme iron in the diet, dietary factors that influence iron bioavailability, and the iron status of the individual (Hallberg 1981).

Dietary Factors affecting non-heme iron absorption

Enhancers	Inhibitors
Ascorbic Acid	Calcium
Alcohol	Phytate
Meat/poultry/fish	Polyphenols
	Soy protein
	Egg
	Zinc
	Caffeine

SOURCE: (Hallberg and Hulthen 2000)

Vegetarian diets can theoretically meet or exceed dietary iron requirements but research indicates that vegetarians generally have lower iron stores than non-vegetarians (Alexander, Ball et al. 1994). One major reason for this discrepancy is due to the difference in iron bioavailability between vegetables and animal/meat sources. In vegetarian diets, the form of iron and inhibitory components found in vegetables limit the amount of iron absorbed. Meat provides iron in a highly bioavailable form and is unaffected by these inhibitory components. The Recommended Dietary Allowance (RDA) for iron has been set at 8 milligrams (mg) per day for men, 18 mg per day for women, and 27 mg per day for pregnant mothers. These values are based on a person consuming 10% of dietary iron from heme sources. The bioavailability of iron from a vegetarian diet is approximately 10% and the bioavailability of iron from a mixed-Western diet is 18%. To account for the difference in absorption, the DRI's for vegetarians are set at 1.8 times the amount of the original DRI's, or 14 mg per day for adult men and postmenopausal women, 33 mg per day for postmenopausal women, and 26 mg per day for adolescent girls (Food and Nutrition Board 2001). The increase in iron requirements for vegetarians makes it difficult for vegetarians to meet iron demands. In particular, vegetarian women of reproductive age are at high risk for iron deficiency (Hunt 2003).

Foods can be expressed as good and excellent sources of nutrients. Through the Nutrition Labeling and Education Act (NLEA), the FDA requires nutritional labeling on all packaged products. Raw vegetables are usually exempted from nutrition labeling, however if a nutrient claim is made, such as a good or excellent source, nutrition labeling is required. A nutrient content claim for a 'good' source is a food item that contains 10-

19% of the Daily Value (DV) per Reference amounts customarily consumed per eating occasion (RACC). A claim for 'excellent' is 20% or more of the DV per RACC. The DV for each nutrient is a unified term that encompasses both the Dietary Reference Values (DRV) and Recommended Dietary Intakes (RDI). The aim of the DV is to be an easy, and simple nutritional label for consumers. The value represents the daily intake of a nutrient required to meet the needs for 97-98% of the population. The RACC determines the appropriate serving sizes for specific foods. This paper will use 'reference amount' synonymously with RACC. The reference amounts for vegetables are listed as 85 grams per serving. For example, the DV for iron is 18 milligrams per day. Using these definitions and criteria, for a selected green leafy vegetable to be considered a good source of iron, it would need to meet 1.8 to 3.6 milligrams of iron per 85 gram serving. A green leafy vegetable excellent in iron would contain greater than 3.6 mg iron per RACC. The labeling of green leafy vegetables as good or excellent sources of nutrients helps to disseminate important information to consumers looking to meet their nutrient needs.

1.1.3. Green leafy vegetables as potential sources of bioavailable iron

The inclusion of lesser known, underutilized vegetarian foods with good to excellent iron content and good iron bioavailability may provide an important dietary strategy in preventing iron deficiency. Green leafy vegetables can be good sources of some minerals and may provide adequate intakes of iron in vegetarian diets (Gupta, Jyothi Lakshmi et al. 2005). Green leafy vegetables, like all vegetables, contain inhibitors that limit its potential as an iron source, but there is evidence to suggest that

green leafy vegetables may constitute a bioavailable source of iron, especially for vegetarians. Green leafy vegetables have been demonstrated to be better sources of bioavailable iron than cereals and legumes. The isocaloric substitution of a portion of cereals and legumes with green leafy vegetables can increase the iron density and iron absorption of meals (Agte, Tarwadi et al. 2000). Moreover, the bioavailable iron density of meals can be increased by including a green leafy vegetable salad sprinkled with added lemon juice (Chiplonkar, Tarwadi et al. 1999).

1.2. SPINACH: IRON CONTENT AND BIOAVAILABILITY

1.2.1. Spinach (*Spinacea oleracea*)

Spinach is a green leafy vegetable with a reputation as a good source of vitamins and minerals, specifically Vitamins A and C, iron, folic acid, and magnesium. Spinach was initially cultivated in China in the 7th century and over time has spread throughout the world. It propagates well in the cold season and is able to withstand frost-like conditions (Yamaguchi 1983). Spinach is widely popular consumed green leafy vegetable. From 1990-92 to 2000-02, the consumption of spinach has risen more than 66%. It is typically eaten either raw as a salad or cooked as a plate vegetable (Yamaguchi 1983).

1.2.2. Iron content of spinach, effects of soil and season.

Spinach has been recognized as a good source of iron content. The iron content of spinach is one of the highest among all vegetables (Van Campen and Welch 1980). In early studies that evaluated the iron bioavailability of spinach using rat models, it was necessary for the iron content of spinach to be determined. In one study, spinach contained 19.8 mg Fe per kg fresh weight (FW) and 213.5 mg Fe per kg dry weight (DW) (Zhang, Hendricks et al. 1989). In another study, freeze-dried spinach contained 167 μg Fe per g DM (Gordon and Chao 1984). The current USDA National Nutrient Database for Standard Reference (Release 24) value for iron is 2.71 ± 0.522 mg per 100 g in raw spinach (n=10) and 3.57 ± 0.996 mg per 100 g in cooked, boiled spinach (n=12).

The iron content in spinach has shown to vary depending on the variety, cultivar, season, and soil content. Winter Bloomsdale and Tuftegards varieties were grown in

nutrient solutions containing 0.5 (parts per million) ppm iron and 1.5 ppm iron. The Bloomsdale variety contained 79 and 92 μg per g Fe freeze-dried spinach and the Tuftegards contained 71 and 87 μg per g Fe (Van Campen and Welch 1980). The values suggest that the iron content of spinach varieties positively correlates with the iron content in the nutrient solutions. In a CV Whitney cultivar, spinach grown in soilless potting media containing 2 mg/liter Fe-DTPA contained 71 μg per g Fe DW (Rutzke, Glahn et al. 2004). Another study added graded levels of iron and zinc directly to the soil. Iron added to the soil (0, 15, 30, 45 ppm) dose dependently increased the iron content from 11.4 to 16.9 mg per 100 grams DW (Reddy, Sondge et al. 1993). In a study investigating the effects of season and various ratios of fertilizers (chicken manure, farmyard manure, and blood meal) on the iron content of spinach, the iron content ranged from 11-634 mg per kg DW in the autumn and 500-1255 mg per kg DW in the early winter, indicating that the iron content can range widely in plants depending on the fertilizer used and the season of growth (Citak and Sonmez 2009).

Variation in the Iron Content of Spinach

Effect	Fe Content, uncooked (μg Fe per g DW)	Reference
Variety	Bloomsdale: 79 and 92 μg Fe per g DW; Tuftegards: 71 and 87 μg Fe per g DW	Van Campen and Welch, 1980
Cultivar	CV Whitney: 71 μg Fe per g DW	Rutzke et al 2004
Season	Autumn: 11-634 μg Fe per g DW, Winter: 500-1255 μg Fe per g DW	Citak and Sonmez, 2009
Soil	0-45 ppm iron: 114 - 169 μg Fe per g DW	Reddy et al 1993

1.2.3. Cooking effects on the iron content of spinach

The effect of cooking on the iron content of spinach has also been investigated. Depending on the cooking method, different values would be expected. Investigators found that the amount of iron in spinach decreased from 21.69 to 13.89 mg Fe per 100 g DW after 15 minutes of blanching (Yadav and Sehgal 2002). Another study did not find a difference in the total iron content of spinach when cooked, which may have resulted from their cooking methods. Researchers in this particular study boiled spinach using a one to one ratio of water volume to spinach volume until the water became completely evaporated. They found that the iron content of fresh spinach was 67.86 mg Fe per 100 g and boiled spinach, 67.49 mg Fe per 100 g (Sotelo, Gonzalez-Osnaya et al. 2010). Cooking has also shown to increase the iron content of spinach. Kuti and Kuti (1999) steamed spinach in the microwave. Raw spinach contained 5.7 mg Fe per 100 g and steamed spinach, 6.3 mg Fe per 100 g. They hypothesize that the increase of iron due to steaming may be caused by the decrease in moisture content relative to increase in solids. In summary, the effects of cooking have led to variability in the iron content of spinach and may be based on cooking method used. Cooking was shown to increase, decrease, and cause no effect on iron content, presumably due to different cooking methodologies.

1.2.4. Iron bioavailability of spinach in humans

Spinach may be a good iron content source (2.3 mg Fe per 85 g RACC) but its value to contribute in reducing iron deficiency is limited due to its inherent low iron bioavailability (Scrimshaw 1991). All iron contained in spinach is in the non-heme form. Non-heme iron has been widely demonstrated to be not as well absorbed as heme iron.

Non-heme iron is found primarily in plant products while heme iron is found primarily in hemoglobin and myoglobin proteins in the tissues of animal/meat. For example, iron in ground beef is predominantly in the heme form and the iron bioavailability of ground beef is estimated to be 20% (Scrimshaw 1991). According to the USDA National Nutrient Database for Standard Reference (Release 24), ground beef, 90% lean/10% fat, raw, contains 2.24 mg Fe per 100 g (n=36) and ground beef ground beef, 90% lean/10% fat, patty, cooked, broiled contains 2.71 mg Fe per 100 g (n=36). In comparison, iron bioavailability from spinach is relatively low, with estimates as low as 1.4% of the iron actually absorbed (Scrimshaw 1991). Radioactive iron isotopes have been used to measure the iron bioavailability of various foods, including spinach, in humans. A 2 mg dose of extrinsically, radioactive labeled ^{59}Fe-spinach administered to 9 subjects resulted in iron absorption between 1.9 to 2.1% (Layrisse, Cook et al. 1969).

Although there are no known clinical studies that have used intrinsically labeled iron in food, one study provided evidence that there is no difference in absorption between iron labeled intrinsically or iron labeled extrinsically. Miller, Schricker et al. (1981) compared the iron bioavailability in meals between intrinsic food iron and added extrinsic iron and found no difference in iron bioavailability.

1.2.5. Oxalic acid and its effects on iron bioavailability in spinach

Researchers have attempted to provide an explanation for the poor iron bioavailability found in spinach. Food components inherent in spinach, such as oxalates, were thought to be responsible for inhibiting iron bioavailability. Spinach is an oxalate accumulator in addition to its high iron content. The oxalate ion is a bi-dentate ligand

capable of forming coordinate covalent bonds with ferrous and ferric iron (Kotz and Pence 1996). Ferrous and ferric oxalates form stable complexes and are soluble in water (Van Campen and Welch 1980). Therefore, the oxalate content in spinach has been proposed to the reason for the low amount of iron actually absorbed. Experimental data has demonstrated that oxalates in spinach are primarily responsible for the reduction of calcium bioavailability (Heaney, Weaver et al. 1988). The oxalate content in spinach has shown to reduce calcium bioavailability by binding calcium and forming non-soluble calcium oxalates which are unavailable for absorption (Noonan and Savage 1999). Iron and calcium are minerals with similar cationic charges. Oxalate inhibition of iron bioavailability in spinach was thought to use a similar mechanism as calcium inhibition.

Oxalic acid is a common constituent of green leafy vegetables (Zarembski and Hodgkinson 1962; Chai and Liebman 2005). The largest amounts of oxalates are found in spinach, mangold (chard), and purslane (Chai and Liebman 2005). Although spinach contains ample amounts of oxalic acid and iron, oxalic acid may not be an inhibitor of iron bioavailability.

Rat models were used to investigate the influence of oxalates on the iron bioavailability in spinach. Researchers measured iron bioavailability by using whole body counting techniques (counts radioactive labeled iron) and hemoglobin regeneration efficiency (HRE). The iron bioavailability in iron-depleted rats was similar between spinach, $^{59}FeCl_3$ and ^{59}Fe-oxalate (Van Campen and Welch 1980). Interestingly, they also found that the addition of dietary oxalate (0.75%) to ^{59}Fe labeled spinach marginally enhanced iron utilization by rats as measured by whole body counting techniques and HRE. Another researcher found that spinach contained both high quantity and high

utilization of iron in rats. Anemic and non-anemic rats fed on meat, meat-spinach, and spinach diets gained similar amounts of hemoglobin-Fe. Surprisingly, the mildly deficient rats in the study had increased HRE and iron absorption compared to severely anemic rats (Zhang, Hendricks et al. 1989).

Researchers determined iron bioavailability using oxalate and cellulose isolates (another previously thought inhibitor of iron bioavailability) against ferrous sulfate in rats. The addition of 2.1% oxalate (the equivalent found in spinach) to 35 μg Fe per g in ferrous sulfate increased the relative biological value (measured by hemoglobin stores) of rats from 4.6 to 8.8 g/dl while spinach alone decreased hemoglobin stores from 4.7 to 1.7. This study suggests that oxalates increase the iron bioavailability of ferrous sulfate, but these results may be misleading. In this particular study, rats consumed significantly less of the oxalate-ferrous sulfate treatment compared to the spinach treatment, most likely due to the palatability of the oxalate-ferrous sulfate treatment (Gordon and Chao 1984). Estimates of the iron bioavailability of spinach using animal models may be invalid because the data may not be suitable for extrapolation to humans. Some of the reasons for the difficulty in extrapolation include: rats absorb ferrous and ferric iron to an equal extent, the bioavailability of heme and nonheme iron is approximately similar, rats synthesize ascorbic acid, and rats possess intestinal phytase activity, all of which are very different absorption mechanisms relative to humans (Wienk, Marx et al. 1999).

A human study recently conducted is the latest evidence to date suggesting that oxalic acid does not inhibit iron absorption in spinach (Storcksdieck genannt Bonsmann, Walczyk et al. 2007). Researchers provided spinach and kale meals (using bread rolls as a vehicle) extrinsically labeled with iron isotopes and measured iron absorption using a

double stable-isotope method in order to determine erythrocyte incorporation of ^{57}Fe and ^{58}Fe. The spinach meal consisted of inherent high oxalate and the kale meal consisted of inherent low oxalate. The weight of each vegetable was kept equal and unlabeled ferrous sulfate was added to kale to normalize the iron content since the kale meal consisted of less iron than spinach. Using equal iron content, the spinach meal had 24% less iron absorption compared to the kale meal. In a follow-up experiment to determine if the oxalate content was responsible for the decreased iron absorption of spinach relative to kale, the researchers normalized the oxalate content of kale to spinach by adding a potassium oxalate drink to the kale meal (equivalent to the amount of oxalate found in spinach). They found no difference between the iron absorption of kale and the iron absorption of kale with oxalate drink. Furthermore, the ratio of iron absorption between spinach and kale meal, and spinach and kale meal with oxalate drink were equal, clearly demonstrating that iron bioavailability is unaffected by oxalate content. The authors suggested that solubility and the complex stability constants of oxalate salts were the reasons why iron bioavailability was unaffected by oxalate content. They hypothesized that because spinach contains an equal mixture of soluble ferrous oxalates and insoluble ferric oxalates, the net effect in absorption of these ferrous and ferric oxalates would have no effect on iron absorption. Researchers found the calcium and polyphenol content of spinach to be ten and two fold higher than kale, therefore they speculated that polyphenol and calcium content found in spinach are responsible for the low iron bioavailability in spinach.

1.2.6. Polyphenolic effects on iron bioavailability

Fiber, oxalates, polyphenols, and phytates are all classes of compounds found in different concentrations within foods that are known to inhibit iron absorption. They all have high binding affinities for iron that lead to the formation of insoluble complexes. Insoluble iron complexes cannot be absorbed into the small intestine, therefore these compounds inhibit iron absorption (Sotelo, Gonzalez-Osnaya et al. 2010). Among the possible classes of inhibitors in iron absorption, it is likely that polyphenols are predominantly responsible for the low iron bioavailability in spinach.

Polyphenols are secondary metabolites found in plants that provide defense mechanisms against UV light and pathogens. Their general structure consists of aromatic rings with attached hydroxyl groups. Classes of polyphenols are defined by the amount and position of aromatic rings. Subclasses consist of the arrangement of hydroxyl groups and further attachment of carbohydrates and organic acids contribute to the existence of thousands of polyphenols found in foods. It has previously been shown that higher levels of polyphenols found in beverages such as tea and coffee are potent inhibitors of iron absorption in humans (Hurrell, Reddy et al. 1999). Certain polyphenols are more potent inhibitors of iron absorption than others. In a comparison of three small phenolic compounds, gallic acid, catechin, and chlorogenic acid, gallic acid was found to be the greatest inhibitor of iron absorption. Investigators specifically found that the amount of galloyl groups constituted within total polyphenols decreased iron bioavailabiliity (Brune, Rossander et al. 1989). Elucidating mechanisms of binding efficiencies between iron and different polyphenolic compounds, researchers found that the highest binding efficiency (measured as catechin equivalents) between iron and polyphenols were flavonoids

attached to catechols, consisting of 3', 4' dihydroxy groups (Khokhar and Owusu Apenten 2003). Specific phenolic compounds may not be entirely responsible for inhibiting iron bioavailability. Another investigator has shown that total polyphenols, not only specific polyphenols, including flavonoids, inhibit iron bioavailability as well (Hurrell, Reddy et al. 1999).

Several studies have suggested that polyphenols are responsible for the inhibition of iron absorption in spinach. In a clinical study, fasting female subjects consumed 100 g of various individual pureed vegetables, including spinach, containing the addition of 3 mg of extrinsically labeled ferrous sulfate. After 14 days, researchers measured several hematological indices of iron: hemoglobin, transferrin saturation, and ferritin. Iron absorption was quantified by measuring ^{55}Fe and ^{59}Fe. Researchers found that total polyphenol content was inversely correlated to iron absorption. In addition, they found no relationship between oxalate content and iron absorption (Gillooly, Bothwell et al. 1983). Another study investigated the iron bioavailability of a leafy green vegetable, Yod Kratin (*Leucaena glauca*, Lead tree) (Tuntawiroon, Sritongkul et al. 1991). Yod Kratin is widely eaten in Thailand and contains significant amounts of polyphenols (1 gram equivalent to 29.2 tannic acid equivalents). Using three grams of radio iron isotope labeled ferrous sulfate, investigators demonstrated that three grams of Yod Kratin decreased iron absorption by fifty percent in male volunteers. Furthermore, increasing the amount of Yod Kratin consumed decreased iron absorption in a dose dependent manner, concluding with 20 grams of Yod Kratin inhibiting iron absorption by 90%. The study demonstrated the inverse relationship between tannic acid equivalents and iron

absorption. Researchers suggested that the high content of galloyl groups found in Yod Kratin was responsible for the inhibitory effects on iron absorption.

1.2.7. In-vitro techniques in determining iron bioavailability of spinach

Several researchers have investigated iron bioavailability of spinach using in-vitro techniques. One technique frequently used is the measurement of ionizable (free, unbound Fe^{2+} and Fe^{3+}) iron after an in-vitro digestion (Rao and Prabhavathi 1978). A study conducted found that although step-wise addition of 5 ppm iron applied to baseline soil with 'marginal' levels of zinc and 'adequate levels of iron' increased the iron content of spinach, it did not reflect an increase in iron bioavailability. They also found that while the addition of zinc to the soil at 5 ppm decreased iron bioavailability, at 10 and 15 ppm there was an increase in iron bioavailability. They concluded that the requirements for optimal iron bioavailability in spinach are 30 ppm iron and 15 ppm zinc added to soil. Their data indicates that high iron content alone does not accurately reflect iron bioavailability and other components within plants, such as zinc, have effects on iron bioavailability irrespective of iron content (Reddy, Sondge et al. 1993). Using a Caco-2 cell model to investigate iron bioavailability in-vitro, researchers found that 0.8% of the total iron present in spinach was bioavailable. The addition of vitamin C to the spinach digest resulted in an increase to 1.8% of the iron absorbed (Rutzke, Glahn et al. 2004). In a study examining the release of iron from spinach using in-vitro conditions, researchers found only free, soluble non-organic $Fe(aq)^{2+}$ to be available for absorption. They concluded that the low iron bioavailability of spinach was due to the inability of the

digestive process to release non-heme iron from their protein -bound clusters (Crispin and Varey 2002).

1.2.8. Cooking effects on the iron bioavailability of spinach

Cooking decreases the amount of oxalates, phytates, and tannins in spinach (Park, Jang et al. 1994; Mosha, Gaga et al. 1995) and therefore theoretically increases iron bioavailability in spinach by minimizing the inhibitory effects. In-vitro techniques have been utilized in understanding the effect of cooking on spinach iron bioavailability and have lead to different conclusions. In one study, HCl extractability of iron increased from 64.75 to 69.38% concomitantly with increase in cooking time. Longer blanching times increased ionizable iron and available (soluble) iron from 6.47 to 7.74% and 3.53 to 4.13% between 5 to 15 minutes. The authors attribute the increase in iron bioavailability to the reduction of oxalic acid, phytic acid, and polyphenolics by the blanching process (Yadav and Sehgal 2002). Using another in-vitro iron bioavailability measurement (Miller, Schricker et al. 1981), researchers found bioavailable iron from raw and boiled spinach to be 4.59 mg Fe per 100 g and 4.45 mg Fe per 100 g, respectively, from an initial iron content of 67.86 and 67.49 Fe per 100 g, leading to an approximate bioavailability of seven percent. They found no difference in iron bioavailability between raw and boiled spinach even though boiling decreased the oxalate and tannin content of spinach (Sotelo, Gonzalez-Osnaya et al. 2010).

A proposed mechanism has been suggested that describes how cooking increases iron bioavailability in spinach and other vegetables. In the process of digestion, iron is

released from ferritin by proteases, heat denaturing, or low acidic conditions. The free iron released becomes bound to polyphenols formed by polyphenol oxidases in the foods thus creating iron-polyphenol complexes that are unavailable for absorption. Cooking denatures polyphenol oxidases, but leaves sufficient amount of ascorbic acid intact. Ascorbic acid chelates iron and keeps the iron in the soluble form, rendering it available for absorption (Yang and Tsou 2006).

1.2.9. Summary

The iron content and bioavailability of spinach has been extensively studied. The iron content of spinach can vary widely depending on various factors, including cooking. Human studies have consistently shown that iron bioavailability of spinach is low. The low iron bioavailability of spinach has also been corroborated in multiple studies using rat models and in-vitro techniques. Researchers have attempted to identify food constituents responsible for the low iron bioavailability using different models. It was previously thought that oxalic acid in spinach inhibited iron absorption, but studies investigating the inhibitory effects of oxalates on iron have not been substantiated. Polyphenols are currently thought to be the primary reason for the low iron bioavailability in spinach.

1.3. IRON CONTENT AND BIOAVAILABILITY OF SELECTED HAWAIIAN AND ASIAN GREEN LEAFY VEGETABLES

1.3.1. Amaranth leaf (*Amaranthus tricolor*)

Amaranth leaf has been suggested as a good source of calcium, iron, beta-carotene and protein (Singh, Punia et al. 2009). It is consumed in many parts of the world, serving a vital component in the diet of Central America, Asia, and Africa (Rangarajan and Kelly 1998). Amaranth leaf has been shown to be a promising food source for populations combating iron deficiency due to its abundant growth and ease of cultivation (Gupta, Jyothi Lakshmi et al. 2005). Amaranth leaf is one of the few greens that can survive in hot summer climates. For this reason, amaranth is an indispensable part of the diet in India (Shukla, Bhargava et al. 2006). In Nigeria, people normally consume amaranth leaf in soup or stew form, in dishes such as melon soup and yam porridge. Expectant mothers in Nigeria are commonly advised by herbalists to consume boiled amaranth (Onyeike, Ihugba et al. 2003).

The promise of amaranth leaf as a possible food source of iron has led to many investigations on characterizing its iron content and iron bioavailability. These studies have shown variation in the total iron content due to location of growth, cooking losses, and variation in the amount of bioavailable iron from amaranth.

Variations were found in the total iron content of raw amaranth. Iron content of fresh amaranth ranged from 3.9, 15.01, 34.14 and 123 mg per 100 g depending on the location (Wills, Wong et al. 1984; Yadav and Sehgal 2002; Gupta, Jyothi Lakshmi et al. 2005; Shukla, Bhargava et al. 2006). In Hawaii, amaranth has been previously shown to

contain 1.66 mg Fe per 100 g fresh EP (Louis 1947) and 2.8 mg Fe per 100 g FW (Evensen and Standal 1984).

The iron content of amaranth after boiling is also variable. Five minutes of boiling significantly reduced the iron content in amaranth grown in Nigeria from 9.71 to 9.54 mg per 100 g DW (Onyeike, Ihugba et al. 2003). Using the moisture content found in these leaves (85.61%), the iron content of FW leaves was 1.40 and 1.37 mg Fe per 100 g, which may not be significantly different. In a separate study, amaranth grown in India was boiled for 5, 10, and 15 minutes and the iron content assessed. The amount of iron decreased from 27.33 to 17.10 mg per 100 g DW with increased boiling time (Yadav and Sehgal 2002; Onyeike, Ihugba et al. 2003). These researchers also found significant reductions in oxalic acid, phytic acid, and polyphenolic contents with boiling, which are inhibitors of iron absorption and therefore suggest that boiling may increase bioavailable iron (Yadav and Sehgal 2002). Alternatively, one study found a non-significant difference in total iron content when amaranth was boiled for 22 minutes in deionized water (Kala and Prakash 2004). In Hawaii, amaranth steamed for 10 minutes contained 2.97 mg Fe per 100 g EP (Louis 1947).

Measuring ionizable iron is a technique that has been used to assess the iron bioavailability of foods. Following an in-vitro digestion, the measurement of ionizable iron (the free form of Fe that reacts with α, α'-diprydidyl reagent) correlates well with in-vivo iron bioavailability data (Rao and Prabhavathi 1978). In one study, the ionizable iron of amaranth was measured before and after blanching. 28 percent of the total iron was ionizable after blanching for 10-15 seconds, but the researchers did not use an in-vitro digestion that replicates gastrointestinal physiology (Singh, Kawatra et al. 2001). In

another study that used a HCl/pepsin digestion with amaranth, ionizable iron was 50% of the total iron (Das, Raghuramulu et al. 2005). Yadav and Sehgal (2002) found that 27% of the total iron was ionizable when amaranth was boiled and 22% was ionizable when fresh. These researchers also found that boiling led to increases in soluble iron. Findings that show cooking amaranth increased ionizable iron are not universal. Kala and Prakash (2004) found that 20-48% of ionizable iron was lost due to boiling, pressure cooking, or microwave cooking.

Iron dialyzability is another indicator of iron bioavailability. Following an in-vitro enzymatic digestion, the digest containing 'bioavailable' iron is applied to a dialysis membrane and the iron found in the filtrate can be quantitatively measured (Miller, Schricker et al. 1981). Rangarajan and Kelly (1998) found that amaranth contained 705 µg Fe per g DW and then using this methodology found that eight percent of the total Fe was dialyzable. Using an in-vivo model, the iron bioavailability of steamed amaranth in Hawaii was 32% using the hemoglobin regeneration assay in iron-depleted rats (Miller and Louis 1945).

1.3.2. Bitter melon leaf (*Momordica charantia)*

Bitter melon leaf is considered a good source of Ca, Mg, K, P, Fe, and the B-vitamins thiamin, riboflavin, and niacin (Rashmi, Kamlesh et al. 2011). While the fruit is the most known and commonly consumed edible part of bitter melon, the leaf is an edible, nutritious part of the plant. Bitter melon leaves are widely consumed in Asia (Zhang, Hettiarachchy et al. 2009). Bitter melon is cultivated all over the world, but thrives especially in tropical regions such as India, Malaysia, China, South America and

the Caribbean (Scartezzini and Speroni 2000). The plant also propagates well in hot, arid areas, such as Africa and the Middle East (Assubaie 2004).

Cultures have developed unique ways in preparing bitter melon leaf. Many Asian dishes use the young shoots as components in meals (Behera, Behera et al. 2010). In the Western world, bitter melon leaf is used in soups. The leaf or leaf extract is used to make beverages such as tea and beer (Assubaie 2004). In Western Africa, bitter melon leaves have supplemented the diet during times of famine (Bakare, Magbagbeola et al. 2010).

Limited studies have investigated bitter melon leaf as a food source of iron. In Nigeria, bitter melon leaves contained 98.00 +/- 0.23 μg Fe per g DW (Bakare, Magbagbeola et al. 2010), while in Hawaii, the iron content was 2.38 mg Fe per 100 g FW (Miller, Louis et al. 1946). The age of bitter melon leaf has shown to play an important role in iron uptake. Investigators found that the iron content of a Sri Lankan cultivar of bitter melon leaf increased with maturation. The iron content increased from 8.4 to 16.7 mg Fe per 100 g DW as the leaf increased in age (Zhang, Hettiarachchy et al. 2009). The type of soil may also play a role in the iron content of bitter melon leaf. Researchers from Saudi Arabia found that bitter melon leaf contained 11.7 μg Fe per g DW in fertilized soil and 9.1 μg Fe per g DW in non-fertilized soil (Assubaie 2004).

1.3.3. Garland chrysanthemum (*Chrysanthemum coronarium*)

Garland Chrysanthemum has been proposed to contain good amounts of vitamins and minerals, especially potassium and carotene (Chuda, Suzuki et al. 1998). The vegetable originated in the Mediterranean and then became widely distributed in Europe,

Africa, and Asia. Presently, chrysanthemum is commonly consumed in China, Japan, Korea, and Southeast Asia (Larkcom 1991).

Chrysanthemum is prepared in many diverse ways, such as steaming, blanching, lightly boiling or stir-frying. The young leaves are eaten raw, blanched for salads, and used in soups in China. Similarly to the Chinese, Koreans generally eat chrysanthemum in raw and blanched preparations. In Japan, chrysanthemum is eaten as a component of many traditional stews and one-pot dishes (Larkcom 1991). The iron content of chrysanthemum has not been extensively studied. One researcher found that chrysanthemum contained 3.2 mg Fe per 100 g edible portion (EP) (Wills, Wong et al. 1984).

1.3.4. Edible Hibiscus (*Hibiscus manihot*)

Information regarding the nutrient content for edible hibiscus leaves is limited. Edible hibiscus leaves are indigenous to Southeast Asia. They were first introduced to Melanesia and then afterwards dispersed into Western Polynesia. Hibiscus leaves serve as a central component in the diets of Vanuata, Fiji and Tonga and are eaten to a lesser extent in Samoa and the Cook Islands. Traditionally, edible hibiscus leaves are simply prepared. The leaves are usually boiled, baked or steamed in coconut milk with or without meat (Fa'anunu 2009). The iron content of edible hibiscus leaves is limited. In Papa New Guinea, edible hibiscus leaves contained 3.32 mg Fe per 100 g FW (Rao, Dominic et al. 1990) and 4.86 mg Fe per 100 g EP (Ohtsuka, Kawabe et al. 1984). The iron content of edible hibiscus leaves grown in Hawaii is 2.2 mg per 100 g FW (Evensen

and Standal 1984). None of the studies have published data on the iron content of cooked edible hibiscus leaves.

1.3.5. Kale (Brassica oleracea)

Kale is usually regarded as a good source of vitamin A, calcium and iron (Greenwood and Salerno 1949). It is easy to cultivate because it can grow year-round in a multitude of different climates. Because kale is easily cultivated, kale is widespread and eaten all over the world. While the entire kale plant is edible, the leaves are usually reserved for human consumption. The remaining plant not consumed by humans is portioned off for animal fodder (Vilar, Cartea et al. 2008). Kale is prepared in a variety of ways, but most often, it is used as a component of soup and fermented to make pickles (Ayaz, Glew et al. 2006).

The iron content of kale is variable and depends on many factors. Iron content is somewhat dependent on whether it is fresh or cooked. Miller, Schricker et al. (1981) found that fresh kale contained 18.58 mg Fe per 100 g DW. The iron content in boiled kale was 2.0 mg Fe per 100 g FW (Vilar, Cartea et al. 2008) and 4.6 mg Fe per 100 g FW (Umeta, West et al. 2005). Geographical location can affect the iron content in kale. Kale harvested in Turkey contained 72.6 μg Fe per g DW (Ayaz, Glew et al. 2006), while kale grown in New Zealand contained 258 μg Fe per g DW (Cornforth, Stephen et al. 1978). The date of harvesting has also shown to significantly affect the iron content of kale. Rosa and Heaney (1996) discovered that kale harvested late in the season contained more Fe than kale harvested earlier in the season. At 94 days old, harvested kale

contained 239 μg Fe per g DW and at 247 days old, the iron content increased to 347 μg Fe per g DW. The season and year of harvest can also affect the iron content in kale. Researchers harvested kale in two separate seasons, spring-summer (SS) and summer-winter (SW) using fertilizer containing 62 kg of nitrogen, 126 kg P_2O_5, 240 kg K_2O per hectare. Kale harvested in SS contained 0.134 mg Fe per kg DW and kale harvested in SW contained 0.137 mg Fe per kg DW. The following year, the iron content of harvested kale decreased to 0.055 mg Fe per kg DW in SS and 0.124 mg Fe per kg DW in SW. In both seasons, kale was harvested using the same land under the same conditions. Furthermore, they discovered that the leaves contained more iron than the stems.

One study examined the effects of cooking on the iron content of kale. They did not find a significant difference in iron content between fresh and cooked kale. Kale was cooked using dry heat in an open glass pan without the addition of water for three minutes. Fresh kale contained 0.4 mg Fe per 100 g FW and cooked kale, when recalculated to fresh weight, contained 0.5 mg Fe per 100 g FW (Kawashima and Valente Soares 2003).

The iron content of kale has shown to be affected by the cooking method and the freezing process. Researchers compared two cooking methods in kale; a 'basic' method that resulted in blanching kale for 3 minutes and an alternative method in which kale was cooked for 15 minutes in a brine solution (a method that produced a readymade cooked product). The iron content of kale ranged from: 0.96 mg Fe per 100 g FW, 0.91 blanched, and 0.89 brined. Cooked kale from both treatments were then frozen and defrosted to reflect the total amount of iron available to the consumer. Blanched, frozen and defrosted kale contained 0.84 mg Fe per 100 g and cooked in brine, frozen and

defrosted kale contained 0.90 mg Fe per 100 g. No statistical difference in iron content between the cooking methods either before or after freezing was found (Lisiewska, Gebczynski et al. 2009).

In a common Ethiopian dish of fresh kale sautéed in spices, the iron content was 6.7 mg Fe per 100 g cooked FW. The kale dish also contained large quantities of calcium and tannins, known inhibitors of iron absorption (Umeta, West et al. 2005).

Lucarini, Di Lullo et al. (2000) assessed the iron bioavailability of kale using the in-vitro dialysability method. Fresh kale was pressure-cooked for 8 min in 150 ml of deionized water and then freeze-dried. The pressure-cooked kale contained 0.56 mg Fe per 100 g cooked FW. The freeze-dried powder was then subsequently exposed to an in-vitro digestion, and the digest was placed onto a dialysis membrane. 15% of the total iron in the digest was able to 'dialyze' and was therefore considered bioavailable. The researchers found no correlation between polyphenol content and iron dialysability. They proposed that the organic acids present in kale were resistant to the digestive process, and aided the high dialysability of iron.

1.3.6. Moringa leaf (*Moringa oleifera)*

Moringa is thought to be a good source of beta carotene, protein, vitamin C, calcium and potassium (Anwar, Latif et al. 2007) and has been traditionally consumed for its medicinal properties. It has been used extensively to cure ailments such as headaches, sore throat, and ear and eye infections. Moringa has also been used nutritionally to control glucose levels (Anwar, Latif et al. 2007). Moringa was originally cultivated in India and has successively spread throughout Southeast Asia and Africa. It thrives equally well in hot, humid areas as well as in arid environments. The culinary uses of

moringa are diverse. It is eaten fresh, cooked, or dried into a powder (Agte, Tarwadi et al. 2000). Moringa leaves are prepared similarly to spinach, either fresh as a component of a salad or cooked as an ingredient for soup (Yameogo, Bengaly et al. 2011). It can also be consumed in the form of stews and curries (Anwar and Bhanger 2003).

The iron content of moringa has extensively been studied. Yang, Tsou et al. (2006) found that moringa contained 9.2 mg Fe per 100 g FW. Freiberger, Vanderjagt et al. (1998) found that moringa grown in the Western African country of Niger contained 226 μg Fe per g DW. The iron content of moringa grown in Thailand ranged from 203 to 376 μg Fe per g DW (Jongrungruangchok, Bunrathep et al. 2010), while Ndong, Guiro et al. (2006) found that moringa contained 184 μg Fe per g DW. Investigators from West Africa propagated moringa at three different locations and found that the iron content ranged from 100 to 311 μg Fe per g FW and 129 to 521 μg Fe per g DW (Yameogo, Bengaly et al. 2011). Curiously, there was only a small difference in iron content from fresh to dry leaves. The iron content of moringa propagated in three different plots located in Pakistan ranged from 205, 397, and 573 μg Fe per g DW (Aslam, Anwar et al. 2005). Fresh, dried, moringa leaves from Nigeria contained 287 μg Fe per g DW (Barminas, Charles et al. 1998). The iron content of moringa found in Hawaii was 37 and 39 mg Fe per g FW (Miller, Louis et al. 1946; Evensen and Standal 1984). While measurement of the iron content in moringa has usually been conducted using fresh leaves, Oduro, Ellis et al. (2008) found that moringa contained 283 μg Fe per g DW after five minutes of steam blanching. Agte, Tarwadi et al. (2000) used a traditional shallow pan-frying technique for cooking moringa and found that it contained 2.39 μg Fe per g cooked FW.

Because moringa contains a fair amount of iron, several investigators have attempted to study its iron bioavailability. Yang, Tsou et al. (2006) found that boiling moringa increased its iron bioavailability relative to raw moringa. Fresh leaves and dried powder were boiled separately for ten minutes, and the iron dialyzability of boiled leaves and boiled dried powder were determined. Boiling increased the iron dialyzability of fresh leaves by 3.5 times and dried powder by 3 times. They hypothesized that boiling moringa increased its iron bioavailability in both fresh leaves and dried powder because cooking decreased its oxalate content (0.99 vs 25-45 mg per g). Although the polyphenols in spinach have been identified as one of the main contributors to its poor iron bioavailability, the oxalate content may be the more profound inhibitor in moringa. Agte, Tarwadi et al. (2000) also assessed the iron bioavailability of moringa and included an in-vitro digestion. In the experiment, moringa was extrinsically tagged with ^{59}Fe, subjected to an in-vitro digestion, and the digest applied to a dialysis membrane. 8.11% of the total iron was dialyzable using this method.

Not all studies have reached consensus that moringa is a highly bioavailable source of iron. Using a double pepsin and pancreatic digestion followed by exposure of the digest to a dialysis membrane, iron bioavailability of moringa was only 2.2 percent (Ndong, Guiro et al. 2006).

1.3.7. Sweet potato leaf (*Ipomoea batatas*)

Many consider sweet potato leaves a good source of the B-vitamins, specifically riboflavin, thiamin, niacin, and pyroxidine, and other nutrients such as beta carotene, iron, calcium, zinc and protein (Islam 2006; Woolfe 1992). Sweet potatoes are the

seventh most important food crop in the world (FAO 1992), but the leaves are largely neglected as a source of human food. Many cultures around the world incorporate sweet potato leaves in their diet, but the majority of the leaves are used as livestock feed for domesticated animals such as cattle, sheep, goats and pigs (Akwaowo, Ndon et al. 2000; Ishiguro, Toyama et al. 2004; Islam 2006). Sweet potato leaves are generally consumed by the impoverished, predominantly in Southeast Asia and Sub-Saharan Africa (Villareal, Tsou et al. 1982; Nwinyi 1992). In Southeast Asia, countries such as Indonesia, Korea, Malaysia, Philippines, Southern China, Thailand, Taiwan, and Vietnam all consume sweet potato leaf. Liberia, Sierra Leone, Tanzania, and Zaire are some examples of sub-Saharan African countries that consume sweet potato leaf (Woolfe 1992).

Sweet potato leaves are usually consumed in cooked form, either boiled, steamed, or in soups. In the Philippines, a mixture of rice and sweet potato leaves is a commonly eaten dish. In Tonga and Fiji, sweet potato leaves are eaten as a cold salad with onions and coconut milk. In Japan, the petioles of sweet potato leaf are braised in soy sauce or battered and fried (Woolfe 1992). In Nigeria, sweet potato leaves are eaten frequently in porridges (Antia, Akpan et al. 2006).

Many researchers have documented the iron content in sweet potato leaf. The iron content in sweet potato leaves range from 1.8, 2.4, and 3.9 mg Fe per 100 g FW (Villareal, Tsou et al. 1979; Pace, Sibiya et al. 1985). In Hawaii, sweet potato leaves contained 1.02 and 4.6 mg Fe per 100 g FW (Miller, Louis et al. 1946; Evensen and Standal 1984). Two varieties of sweet potato leaves grown in Japan contained 5.43 and 5.54 mg Fe per 100 g FW (Ishida, Suzuno et al. 2000) while a novel, high yielding Japanese cultivar of sweet potato leaf named Suioh contained 17.9 mg Fe per 100 g DW

(Ishiguro, Toyama et al. 2004). Antia, Akpan et al. (2006) found that sweet potato leaves grown in Nigeria contained 16.0 mg Fe per 100 g DW. Researchers compared the iron content of three different cultivars of sweet potato leaf grown in the southern United States using two separate growing techniques, either grown in a greenhouse or grown using a nutrient film technique (NFT), a specialized hydroponics system. The iron content of sweet potato leaves grown in a greenhouse ranged from 11 to 17 mg per 100 g DW. The iron content of sweet potato leaves grown using NFT ranged from 15 to 18 mg per 100 g DW. No significant differences in iron content were discovered between the two different growing techniques (Almazan, Begum et al. 1997).

The effects of cooking on the iron content of sweet potato leaves have also been investigated. Examining the effects of cooking on the iron content of seven different cultivars of sweet potato leaf grown in Ghana, after five minutes of steam blanching, the iron content ranged from 9.62 to 23.02 mg Fe per 100 g DW (Oduro, Ellis et al. 2008). It has been suggested that the iron content in sweet potato leaves do not have good bioavailability due to the presence of the inhibitors, oxalate, phytate, and tannins. Almazan (1995) examined the effects of conventional blanching (2.5, 5, 10 minutes) and microwave blanching (30, 45, 60 seconds) on these inhibitors contained within sweet potato leaves. They found that blanching decreased the oxalate, phytate, and tannic contents of the leaves, but that increased blanching time beyond 2.5 minutes for conventional blanching and beyond 30 seconds for microwave blanching did not induce further reduction.

1.3.8. Taro leaves (*Colocasia esculenta*)

Fresh taro leaves are a possible good source of vitamins A and C, thiamin and riboflavin (Matthews 2004). However, taro leaves are always cooked before consumption; therefore it is possible they may not be good sources of some of these nutrients. Taro leaves originated from a tropical area between India and Indonesia. They are widely eaten throughout Asia and the Pacific. They are considered a staple food of the people in the South Pacific. The leaves contain 0.1-0.4% FW of raphides, needle-like calcium oxalate crystals. These raphides cause swelling in the throat when not degraded to limited quantities. Therefore, taro leaves are always cooked to reduce raphide content (Aregheore and Perera 2003). Cooking treatments of taro leaves include boiling, blanching, steaming, stewing, frying, and pressure cooking (Lewu, Adebola et al. 2010). In the South Pacific, they are eaten boiled or baked with coconut or cows milk (Savage, Martensson et al. 2009). Taro leaves in Nigeria are consumed as a part of soups and sauces (Aregheore and Perera 2003).

The iron content of taro leaves varies by location. Taro leaves from Nigerian local markets contained 12.4 mg Fe per 100 g DW (Barminas, Charles et al. 1998) and taro leaves grown in India contained 18.22 mg Fe per 100 g DW (Borah, Baruah et al. 2009). Taro leaves grown in 4 different gardens in Cameroon contained an average of 11.7 mg Fe per 100 g DW (Ejoh, Mbiapo et al. 1996). The iron content of taro leaves grown in Western Kenya was 0.54 mg Fe per g 100 g FW (Orech, Christensen et al. 2007), while in Hawaii, taro leaves contained 0.95 mg Fe per 100 g FW (Evensen and Standal 1984)

Since taro leaves must be cooked prior to consumption, the iron content reflecting cooked data should be more relevant to consumers than fresh data, yet information regarding the iron content of cooked taro leaves is limited. In one study, the ash content of taro leaves was shown to decrease when taro leaves were boiled for 5 minutes, but specific minerals, such as iron, were not determined (Lewu, Adebola et al. 2010). In Hawaii, it was previously shown that the iron content of steamed taro leaves were 1.25 and 1.42 mg Fe per 100 g cooked EP (Louis 1947).

Using an in-vivo model to assess the iron bioavailability of taro leaves, Miller and Louis (1945) found that the iron bioavailability of steamed taro leaves was 93% using the hemoglobin regeneration assay in iron-depleted rats.

1.3.9. Ung Choi (*Ipomoea aquatica*)

Ung choi is considered as a good source of protein, carotenoids, vitamins, and minerals such as sodium, potassium, calcium, iron, magnesium, and zinc (Prasad, Shivamurthy et al. 2008). It is native to Africa, Asia, and the southwestern Pacific Islands. While ung choi easily propagates in the wild, its cultivation first began in Southeast Asia and is currently grown and consumed throughout the world (Westphal 1993). In many parts of the world, ung choi is considered an invasive species because of its rampant growth and proliferation and efforts have been made to impede its growth (Austin 2007). Usually, ung choi is cooked before consumed. Some standards of preparation of ung choi are frying of the tips, stems and leaves (Westphal 1993). The tips themselves are also boiled and steamed or added to soups, stews, and curries (Austin 2007).

The iron content of ung choi has been studied all throughout the world. The iron content of ung choi from Nigeria was 210 mg Fe per 100 g DW (Umar, Hassan et al. 2007). In Vietnam, ung choi contained 1.5 mg Fe per 100 g FW (Ogle M. 2001). Ung choi purchased from markets in Australia contained 2.4 mg Fe per 100 g EP. The edible portion included the entire plant, both leaf and stalk (Wills, Wong et al. 1984). In Papa New Guinea, researchers found that ung choi contained 4.43 mg Fe per 100 g FW basis (Rao, Dominic et al. 1990). In Hawaii, ung choi contained 1.58 and 2.5 mg Fe per 100 g FW (Miller, Louis et al. 1946; Evensen and Standal 1984).

Two studies have investigated the iron bioavailability of ung choi. In one study, researchers first determined the effects of seasonal change on the iron content of ung choi. The iron content of ung choi was 1.9 mg Fe per 100 g FW and did not vary significantly from month to month. They then compared differences in the iron dialyzability of ung choi after two different cooking treatments. In the first treatment, fresh ung choi was cooked in boiling water (1 part ung choi: 5 parts water) for 10 minutes. In the second treatment, ung choi from the first treatment was lyophilized, grounded into a powder, and boiled again in water (1:10) for ten minutes. Dialyzable iron from lyophilized-cooked treatment was higher (4.1% of total iron) than cooked treatment (3.1%), displaying evidence that increasing the cooking time of ung choi may increase its iron bioavailability (Gooneratne and Kumarapperuma 2007). In-vivo iron bioavailability data shows that the iron bioavailability of steamed ung choi was 59% using the hemoglobin regeneration assay in iron-depleted rats (Miller and Louis 1945).

1.3.10. Watercress (*Nasturtium officinale*)

Watercress has been suggested as a good source of vitamins A and C, niacin,

thiamine, riboflavin, and iron (Stephens 2009). Originally cultivated in Europe, watercress is currently cultivated and eaten around the world. Watercress is predominantly eaten fresh in salads, but is also steamed or added to soups (Cruz, Vieira et al. 2006; Goncalves, Cruz et al. 2009).

The iron content of watercress ranges from 0.9 mg Fe per 100 g FW (Kawashima and Valente Soares 2003), 1.2 mg Fe per 100 g FW (Ogle M. 2001), 2.2 mg Fe per 100 g FW (Gibson, Donovan et al. 1997), and 3.0 mg Fe per 100 g EP (Wills, Wong et al. 1984). Researchers from Pakistan found watercress contained 138 mg Fe per 100 g DW (Imran, Talpur et al. 2011). In Hawaii, the iron content of watercress was 1.05 mg Fe per 100 g EP, and when steamed, the iron content increased to 1.37 mg Fe per 100 g cooked EP (Louis 1947).

Sotelo, Gonzalez-Osnaya et al. (2010) investigated differences in iron content and iron bioavailability of fresh and cooked watercress found in Mexican markets. Cooked watercress was prepared by boiling watercress 1:1 v/v until all water was evaporated. Boiling significantly decreased the total iron content from 35.1 mg Fe per 100 g DW to 34.4 mg Fe per 100 g DW. Iron bioavailability of raw and boiled watercress was assessed using iron dialyzability. They did not find any significant differences in iron dialyzability between raw (1.90 mg per 100 g DW) and boiled watercress (1.98 mg per 100 g DW).

In Hawaii, iron bioavailability was measured using iron deficient rats. The iron bioavailability of steamed watercress was 31% when using the hemoglobin regeneration assay (Miller and Louis 1945).

1.3.11. Conclusions and Proposed Research

Iron bioavailability of spinach has consistently been proven to be low. The identification of vegetarian sources which have both high iron content and high iron bioavailability would be of great benefit to groups consuming predominantly vegetarian diets. Few studies presently have assessed the iron content and iron bioavailability of underutilized, uncommon green leafy vegetables that have the potential to be important iron sources in the diet. Furthermore, the studies that have investigated iron bioavailability of green leafy vegetables using in-vitro digestion methods are outdated and not as informative as newer methodologies to measure bioavailability. The purpose of this research is to measure the mineral content of cooked green leafy vegetables found locally in Hawaii, and secondly, to evaluate iron bioavailability relative to spinach using the in-vitro digestion Caco-2 cell model.

CHAPTER 2: EVALUATION OF MINERAL COMPOSITION IN HAWAIIAN GROWN GREEN LEAFY VEGETABLES

2.1. Introduction

Minerals are essential inorganic nutrients required by the body for growth and repair and to maintain human health. Green leafy vegetables may be able to provide a dietary strategy in improving micronutrient intakes in mineral deficient populations (Agte, Tarwadi et al. 2000). The determination of mineral content of local green leafy vegetables provides important nutritional information to ethnic populations and expands local and national nutrient database information. The Hawaiian Islands provide a unique climate and geography to investigate the mineral content of several underutilized green leafy vegetables that have not been well characterized in terms of nutritional value.

The mineral content of green leafy vegetables has been shown to be highly variable. This variability may be a result of the variety (Almazan, Begum et al. 1997; Ishida, Suzuno et al. 2000), season (Rosa and Heaney 1996; Citak and Sonmez 2009), region (Aslam, Anwar et al. 2005; Oduro, Ellis et al. 2008; Jongrungruangchok, Bunrathep et al. 2010) and agricultural practices such as the type of fertilizer (Reddy, Sondge et al. 1993; Reddy and Bhatt 2001), maturation stage of the plant (Zhang, Hettiarachchy et al. 2009), harvesting time (Savage, Martensson et al. 2009), production method (Almazan, Begum et al. 1997), or other unaccounted for factors in each green leafy vegetable. Investigating the mineral content of GLV found in Hawaii will improve local nutrient databases and provide nutrition information for consumers of Hawaiian-grown produce.

Furthermore, in the United States, it is not uncommon for nutrient analysis values for cooked produce to be imputed from fresh produce values. The mineral content in cooked produce does not accurately reflect the amount found in raw produce. Although it has been estimated that mineral losses due to various types of cooking are minimal and no more than 3 percent of the total amount (Watzke 1998), the magnitude of mineral loss has been shown to vary depending on cooking method and time duration. In several studies, cooking was shown to both increase (Kawashima and Valente Soares 2003; Kala and Prakash 2004) and decrease (Onyeike, Ihugba et al. 2003; Sotelo, Gonzalez-Osnaya et al. 2010) the mineral content in green leafy vegetables. The discrepancies found in the mineral content are probably due to differences in the method and duration of cooking.

The mineral content in green leafy vegetables after cooking remains important for several reasons. First, many green leafy vegetables are primarily consumed in the cooked form. Therefore, the mineral content based upon cooked vegetables is more relevant to consumers than fresh. Secondly, cooking has been shown to improve the mineral bioavailability of green leafy vegetables. It has been hypothesized that cooking decreases non-nutrient components found in green leafy vegetables that are known to inhibit mineral absorption (Watzke 1998; Yang and Tsou 2006). For example in one study, applying various cooking methods to cruciferous vegetables greatly enhanced iron dialyzability compared to raw cruciferous vegetables, many of which are green leafy vegetables such as kale and watercress (Kapanidis and Lee 1995). Analyzing green leafy vegetables for mineral content in their cooked form provides more useful information than fresh and, therefore, warrants further investigation.

The objectives of this study were to 1) evaluate ten green leafy vegetables found in Hawaii for their mineral content after cooking. These minerals include calcium, copper, magnesium, manganese, phosphorus, potassium, iron, sodium, zinc and 2) determine whether each cooked vegetable meets the U.S. dietary guidelines for good or excellent source of minerals.

2.2. Methods and Materials

Source of Samples

Ten green leafy vegetables were investigated: amaranth leaf, bitter melon leaf, chrysanthemum, edible hibiscus, kale, moringa, sweet potato leaf, taro leaf, ung choy and watercress. Amaranth, chrysanthemum, sweet potato leaves, ung choi, and watercress were purchased in local ethnic markets. Bitter melon leaf, kale, moringa, and taro leaf were purchased from local grocery stores. Edible hibiscus was obtained from a local grower on the Big Island. These green leafy vegetables were procured in the summer of 2010. Once obtained, samples were stored at 4°C for 24 hours to maintain freshness until cooked and prepared for analysis.

Preparation of Samples

Green leafy vegetables were removed from a 4°C walk-in refrigerator and allowed to equilibrate to room temperature for 15 minutes. Edible portions (EP) of green leafy vegetables consisted of leaves only, and were removed of stalks and stems except for ung choy. The stems of ung choy remained as part of the edible portion because they are normally consumed with the leaves. EP's were weighed on a scale prior to cooking

to determine moisture content. EP's were first rinsed in deionized water (Millipore) and then boiled in deionized water. Each green leafy vegetable was boiled separately in a stainless steel pot using fresh water. Cooking times for each leaf ranged from five to ten minutes, determined by the tenderness of each leaf, except for taro and kale. Taro leaf was boiled for 60 minutes and kale was boiled for 40 minutes, modified from recipes (Harrington and Meyer 1994; Von Starkloff Rombauer, Becker et al. 1997).

After boiling, samples were removed and placed on ice immediately for up to five minutes to prevent further cooking. Food samples were then drained for five minutes to remove any residual water. Once drained, each GLV was placed in a Cuisinart food processor with stainless steel blades and fully homogenized. Approximately 100 g of homogenous food samples were aliquoted into clean, large plastic weigh boats (135 mm x 135 mm x 20 mm), covered with plastic wrap, and frozen overnight at -40°C. Samples were then lyophilized (VirTis Virtual 50xl, SP Scientific) using a 36-hour drying cycle. A temperature probe was placed into one representative sample during lyophilization to confirm complete dryness. Moisture content of the cooked GLV's was calculated.

Dried aliquots of the same GLV were pooled together and finely ground using a stainless steel coffee grinder (Black and Decker). Pooled, ground samples were aliquoted into 50 ml centrifuge tubes (BD BioSciences), sealed with parafilm, and stored in dessicant until further use.

Mineral analysis

Three, one-gram samples of each dry, green leafy vegetable were sent to Louisiana State University Agricultural Center (LSU) for analysis of 9 minerals: calcium,

copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc. Briefly, 0.5 gram aliquots of each sample were used for analysis; 5 ml concentrated trace metal grade HNO_3 was added to each aliquot and digested for 50 minutes using an automated block digester (Thomas Cain Inc. DEENA, Omaha, Nebraska). 3 ml of H_2O_2 was added and further digested for 2.75 hours. The final mixture was cooled and diluted to 20 ml final volume. The mixture was filtered through a 1-micron filter and analyzed using Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (Spectro Arcos EOP ICAP, Germany). National Institutes of Standards and Technology (NIST) peach samples were digested every 20 samples at the end of each set as a quality control. Calibration of the ICP was self checked every 15 samples and three integrations taken on the ICP for each sample. One replicate was run for each sample. Dry matter mineral content (expressed as mg per kg or g per 100 grams) obtained from LSU was converted to mg of mineral per 100 grams cooked edible portion (cooked EP). The mineral content was further expressed as percent DV based upon the NLEA RACC of 85 grams cooked weight for cooked vegetable. For the purposes of this experiment, we consider our food samples that were boiled as synonymous with cooked, and thereby the term "boiled" will be referred to as "cooked" from this point forward.

2.3. Results

Mineral content expressed as mg per 100 grams cooked edible portion (Table 1)

The total minerals assayed varied from 7 to 10 fold among these GLVs, indicating that not all green leafy vegetables contribute equal amounts of minerals, and some green leafy vegetables are better sources of certain minerals than others. The iron content

varied the least, about 3-4 fold among the samples. There was not a particular mineral that was consistently high in all of the GLV's, but calcium, copper, manganese and iron were notably higher in some of the samples as described below.

The calcium content of amaranth leaf, 351 mg Ca per 100 grams cooked EP, was especially high in comparison to other green leafy vegetables. Bitter melon leaf, edible hibiscus, moringa, and taro leaf contained about 200 mg Ca, while all other leaves contained less than 100 mg Ca per 100 g cooked weight.

The copper content of bitter melon leaves (0.445 mg/100 g cooked EP) was high compared to all other GLV's investigated. Its copper content was greater than four times higher than the second highest copper containing leaf (Chrysanthemum).

The manganese content was highest in moringa and taro leaves (1.25 and 1.08 mg/100 g cooked EP, respectively). All other GLV had less than 100 mg Mn per 100 g cooked EP.

The highest amount of iron was found in bitter melon leaves and chrysanthemum with 2.5 and 2.4 mg Fe per 100 g cooked EP, respectively. Amaranth, edible hibiscus and moringa contained between 1.1-1.6 mg Fe per 100 g. All other GLVs contained less that 100 mg Fe per 100 g.

Among all the green leafy vegetables investigated, moringa contained the most phosphorus (56 mg P per 100 g cooked EP). Sweet potato leaf had the highest potassium content (229 mg K per 100 g cooked EP). Edible hibiscus contained the highest amount of zinc (0.49 mg Zn per 100 g cooked EP).

Mineral content of cooked green leafy vegetables expressed as percent DV (Table 2)

The labeling of good (≥10%<20%/RACC) and excellent (>20%/RACC) sources of nutrients is an easy and simple method for informing consumers of the nutritional content of a particular food. As shown in Table 2, bitter melon leaf was a good source of over half the minerals assayed, meeting 29.9% of the DV for calcium, 19.0% of the DV for copper, 11.7% of the DV for iron, 10.0% of the DV for magnesium, and 33% of the DV manganese. Amaranth leaf, edible hibiscus, moringa, and taro leaf were good sources of calcium. Bitter melon leaves and chrysanthemum were good sources of iron. Eight of ten green leafy vegetables were good or excellent sources of manganese. Amaranth and chrysanthemum were good sources of manganese while bitter melon leaf, edible hibiscus, moringa, sweet potato leaf, taro, and ung choi were all excellent sources. Moringa and taro leaf exceeded twice the amount of manganese considered as an excellent source (53% and 46%). Bitter melon and amaranth were the only green leafy vegetables to provide 10% of the DV for magnesium. None of the green leafy vegetables were good sources of phosphorus, potassium, sodium, and zinc. Among all the green leafy vegetables investigated, sweet potato leaf had the highest potassium content. Kale and watercress were the only green leafy vegetables that did not meet the definitions for good or excellent sources in any of the minerals.

2.4. Discussion

The goal of the 2010 Dietary Guidelines for Americans (DG) is to improve national health by promoting healthy eating behaviors and are targeted primarily for growing children and individuals at risk for diet-related chronic diseases. One of the objectives of the DG is to promote the increased consumption of particular nutritious

foods, such as fruits and vegetables. In particular, the DG recommends increasing the consumption of green leafy vegetables. Green leafy vegetables (GLV) are nutrient-dense foods that can provide certain nutrients required to maintain health. Furthermore, GLV are low in calories, and therefore can meet certain nutrient needs without the consumption of excess calories (DG Committee 2010).

This study has determined the mineral content of cooked GLV found in Hawaii and has demonstrated that many of the cooked GLV are good or excellent sources of a number of minerals as defined by the NLEA Nutrient Content Claim Definitions. Six of the GLV studied contained good or excellent levels of manganese (amaranth, bitter melon leafy, chrysanthemum, edible hibiscus, moringa, sweet potato leaf, taro leaf, ung choi), 5 were good or excellent in calcium content (amaranth, bitter melon leaf, edible hibiscus leaf, moringa, taro leaf), 2 had good or excellent levels of iron (bitter melon leaf and chrysanthemum), and 1 contained good levels of copper (bitter melon leaf).

The calcium content of amaranth leaves in this study was the highest of all GLV. Moreover, in comparison to multiple studies, the calcium content of amaranth investigated in this study was also the highest of all. Previous studies found that amaranth leaves contained 236 and 183 mg Ca per 100 g FW (Kala and Prakash 2004; Gupta, Jyothi Lakshmi et al. 2005). Cooked amaranth leaves contained 182-195 mg Ca per 100 g cooked weight, using conventional boiling, pressure cooking, and microwave cooking (Kala and Prakash 2004). These values are a little more than half the amount of the calcium measured in this study (351 mg Ca per 100 g cooked EP). It has been shown that the bioavailability of calcium from amaranth can be quite high. Gupta, Lakshmi A et al. (2006) measured calcium bioavailability of *Amaranthus tricolor*. Their obtained

amaranth contained 249 mg Ca per 100 g FW and using an in-vitro digestion and dialysis method, calcium bioavailability was approximately 51%. This evidence suggests that amaranth may be an excellent plant source of bioavailable calcium.

Among the ten green leafy vegetables investigated, bitter melon leaf and chrysanthemum had the highest iron content. The iron content of bitter melon leaf in this study (359.99 µg Fe per g DW) was four times greater than the highest amount found in previous studies. Bitter melon leaf from other locations ranged from 11.7 to 98 µg Fe per g DW (Assubaie 2004; Zhang, Hettiarachchy et al. 2009; Bakare, Magbagbeola et al. 2010).

The iron content of chrysanthemum (2.4 mg Fe per 100 g cooked EP) in our study was less than the value found in Australia (3.2 mg Fe per 100 g EP) (Wills, Wong et al. 1984). Although bitter melon leaf and chrysanthemum were particularly good sources of iron content, whether they are good sources of bioavailable iron is still unclear.

The manganese content of moringa in this study (124 mg Mn per kg DW) was higher than that found by others: 76.9-112.8 mg Mn per kg DW (Aslam, Anwar et al. 2005), 77 mg Mn per kg DW (Barminas, Charles et al. 1998), and 62 mg Mn per kg DW (Freiberger, Vanderjagt et al. 1998). The manganese content of taro leaf (154 mg Mn per kg DW) was lower than that found by others: 189 mg Mn per kg DW (Barminas, Charles et al. 1998) and 259 mg Mn per kg DW (Ejoh, Mbiapo et al. 1996).

The copper content of bitter melon leaf (65 mg Cu per kg) was twice the amount (32 mg Cu per kg) found by Bakare, Magbagbeola et al. (2010) and approximately 6 times the amount (11-14 mg Cu per kg) found by Zhang, Hettiarachchy et al. (2009). We also found that chrysanthemum contained sodium in excess of two fold greater than

watercress, the second largest sodium containing green leafy vegetable in our study. We were surprised to identify this relatively large amount of sodium in chrysanthemum, even after cooking. In comparison, one study found that chrysanthemum contained 0.2 mg Na per 100 g edible portion, compared to the 47.3 mg Na per 100 g cooked EP found in our study (Wills, Wong et al. 1984). Further investigation is warranted to confirm the high levels of sodium found in chrysanthemum in our study. It is important to note that the chrysanthemum in this study would still be considered a low source of sodium in the diet. Our chrysanthemum contained 40 mg Na per RA, which meets the definition of low sodium (less than 140 mg Na per serving).

In comparison with other researchers, we found that the potassium content of sweet potato leaves found in Hawaii (229 mg K per 100 g cooked EP) were more consistent with the values, 357 and 639, found in a Japanese study (Ishida, Suzuno et al. 2000) rather than the low amount of 4.50 found in Pakistan (Antia, Akpan et al. 2006). Using units of mg K per 100 g DW, the potassium content of 2820 mg K per 100 g DW in our sweet potato leaves was consistent with the values of 2880, 3380, and 3440 mg K per 100 g DW found by another researcher (Almazan, Begum et al. 1997). While sweet potato leaves had the highest potassium content, it still only met 5.5% of the DV and therefore does not qualify as a good source of potassium.

The zinc content of edible hibiscus found by other researchers ranged between 0.92 and 1.25 mg Zn per 100 g FW (Ohtsuka, Kawabe et al. 1984; Rao, Dominic et al. 1990), both of which are higher than the values found in this experiment (0.49 mg Zn per 100 grams cooked EP). All boiled GLV in this experiment were poor sources of zinc.

Edible hibiscus was only 2.8% of the DV, while all other GLV ranged from 0.5% to 1.8% of the DV.

2.5. Conclusions

Our data suggests that cooked green leafy vegetables are good to excellent sources of many minerals. In our study, the majority of GLV were excellent sources of manganese. Cooked amaranth, bitter melon leaf, and edible hibiscus were good sources of magnesium. Cooked amaranth, bitter melon leaf, edible hibiscus, moringa, and taro leaf were good sources of calcium. Cooked bitter melon leaf and chrysanthemum were good sources of iron.

The results present new information on the mineral content of Hawaiian-grown boiled green leafy vegetables. Furthermore, data from this study may be able to provide additional data to the National Nutrient Database for Standard Reference Version 24. The mineral content of edible hibiscus is currently not among the foods analyzed for nutrient content in the National Nutrient Database. Raw moringa and raw watercress are found in the database, but their cooked form is not. The remaining cooked green leafy vegetables are found in the database, but the majority of minerals listed either have only one data point or the data is imputed.

The present investigation into the mineral content of underutilized green leafy vegetables found in Hawaii will provide important nutrient information for consumers. We found that many of the green leafy vegetables were good and excellent sources of many nutritionally relevant minerals. This information could be used to improve the nutritional quality of the diet, in particular plant-based diets.

CHAPTER 3: ASSESSMENT OF IRON BIOAVAILABILITY IN GREEN LEAFY VEGETABLES USING THE IN-VITRO DIGESTION/CACO-2 CELL METHOD.

3.1. Introduction

Iron deficiency is the most common nutritional deficiency in the United States and the leading cause of anemia (Looker, Cogswell et al. 2002). Iron deficiency is caused by increased iron requirements and/or decreased iron intake or absorption. In order to decrease the prevalence of iron deficiency, it is important to identify food sources that have good iron content, and more importantly, contain iron that is readily absorbed, i.e. are good bioavailable sources.

It is well established that the form of iron in food, as well as the presence of food constituents, collectively known as inhibitors or enhancers, plays a pivotal role in whether a particular food is a good bioavailable source of iron. Sources of heme iron, found as hemoglobin and myoglobin in animal tissues is the most bioavailable form of iron. In contrast, non-heme iron found in plants is generally less bioavailable due to the form of iron and the presence of inhibitors that reduce its absorption. There remains a glaring need to identify vegetarian sources of iron that are highly bioavailable, or at the least, compare vegetable sources of iron based upon iron bioavailability.

Green leafy vegetables are nutrient-dense foods with some high in iron content. Previous investigations into the iron bioavailability of GLV suggest they may be good bioavailable sources of iron (Chiplonkar, Tarwadi et al. 1999; Agte, Tarwadi et al. 2000;

Yang, Tsou et al. 2006). However, there is little information on iron bioavailability in local, ethnic GLVs consumed in Hawaii.

In-vivo clinical studies are the most accurate measurement of iron bioavailability but the extensive length of time required and its high cost limit its usefulness. Therefore, other in-vitro methods have been developed that can accurately predict iron bioavailability similar to in-vivo. One particular in-vitro method that has been utilized to measure iron bioavailability has been the use of the in-vitro digestion / Caco-2 cell model. Caco-2 cells (colon adenocarcinoma), a human derived cell line, model the intestinal epithelial barrier and are primarily used for small intestinal absorption studies. It has been shown that Caco-2 cells cultured on collagen coated Transwell polycarbonate membranes rapidly differentiate into enterocytic-like monolayers, making this cell type suitable for small intestinal transport studies (Hidalgo, Raub et al. 1989). The transition of Caco-2 cells from a purely colonic cell line to enterolytic-type cells is well characterized by differentiation of the brush border microvilli. This differentiation exhibits expression of the enzymes alkaline phosphatase, sucrose-isomaltase, and aminopeptidase. Furthermore, differentiated Caco-2 cells are polarized, and exhibit both dome and tight junction formation (Pinto, Robine-Leon et al. 1983).

The use of the Caco-2 cell culture system is a widely accepted model for use in iron absorption studies. They feature structural and functional characteristics localized and distributed along apical membrane of the enterocyte that are unique to iron absorption, such as divalent metal transporter expression (DMT-1), heme carrier protein transporter expression (HCP-1), and ferrireductase activity (Dyctb) (Gunshin, Mackenzie

et al. 1997; McKie, Barrow et al. 2001; Shayeghi, Latunde-Dada et al. 2005). Moreover, the coupling of an in-vitro digestion to the Caco-2 cell culture accurately predicts iron bioavailability from foods (Garcia, Flowers et al. 1996; Glahn, Lee et al. 1998). Following in-vitro digestion, a food digest is applied to a dialysis membrane in contact with medium covering a Caco-2 cell monolayer. Caco-2 monolayers do not contain a mucus layer; therefore the mucus layer is replicated by placement of a dialysis membrane directly on top of each monolayer. Dialyzable iron that is taken up into the Caco-2 cell monolayer is understood as 'bioavailable'. Quantification of bioavailable iron can be detected by the presence of ferritin, an iron storage protein. The rate of ferritin formation is proportional to the amount of iron absorbed into the cells. Therefore, ferritin is used as a biomarker of iron absorption (Glahn, Lee et al. 1998).

The in-vitro digestion / Caco-2 model is currently the most robust and accurate in-vitro technique for the assessment of iron bioavailability from foods. Solubility and dialyzability are two other in-vitro techniques used to assess iron bioavailability, but are not as reliable. These techniques tend to vary upon experimental conditions and do not measure cell uptake. In contrast, the in-vitro digestion / Caco-2 cell model provides the most useful information on iron bioavailability from food digests (Fairweather-Tait, Phillips et al. 2007). For example, investigators compared the results of iron bioavailability from foods using the iron solubility test and Caco-2 cell method. In comparing their results with in-vivo data, they discovered that the Caco-2 cell method was closer in accurately predicting iron bioavailability (Pynaert, Armah et al. 2006). Furthermore, the Caco-2 cell method system correlates well with in-vivo human

absorption data, and responds appropriately to varying concentrations of enhancers (ascorbic acid) and inhibitors (tannic acid) (Yun, Habicht et al. 2004).

Using the in-vitro digestion/Caco-2 cell model, iron bioavailability can be measured by radiolabeled iron uptake or ferritin synthesis. Food radiolabeling is not only difficult, but an inherent weakness of iron radiolabeling is that unabsorbed radiolabeled iron can attach onto the cell surface. The measurement of unabsorbed iron would provide erroneous false positives in iron absorption. Ferritin forms in response to iron uptake into the cells and eliminates the need for radiolabeling (Glahn, Lee et al. 1998).

Food scarcity and lack of dietary diversification are some of the main contributors to the prevalence of iron deficiency. One particular strategy for acquiring the necessary amount of iron in iron deficient populations is iron biofortification of staple crops. The in-vitro digestion / Caco-2 cell culture method has been used to investigate iron bioavailability in biofortified staple crops such as corn, beans, and rice. This method helps screen for the most promising cultivars, eliminating the cost and expense of human trials (Oikeh, Menkir et al. 2004). In one study using the in-vitro digestion / Caco-2 cell model, differences in iron bioavailability were demonstrated among bean genotypes (*Phaseolus vulgaris*). Iron bioavailability was lower in genotypes that contained darker seed coats. A follow-up study identified certain polyphenolic compounds, kaempferol and quercetin, found in the seed coats of beans, as primarily responsible for the low bioavailability in these darker bean genotypes (Hu, Cheng et al. 2006). These particular polyphenols found in the seed coats exhibited stronger inhibitory effects on iron absorption compared to phytate (Ariza-Nieto, Blair et al. 2007).

Differences in iron bioavailability were also evident in 15 rice genotypes. Rice genotypes with the lowest iron bioavailability were brown to purple in color (Glahn, Cheng et al. 2002). In the assessment of iron bioavailability in 20 late-maturing tropical maize varieties grown in Africa, researchers discovered two particular maize varieties that had both high iron content and high bioavailability. The identifications were promising, but researchers cautioned that future research is required to assess whether these varieties have the ability to improve the iron status of iron-deficient individuals (Oikeh, Menkir et al. 2004).

The in-vitro digestion / Caco-2 cell method has been used on other types of foods besides staple crops, in particular green leafy vegetables. The method was used to assess the iron bioavailability of spinach and the results correlated well with previous in-vivo iron bioavailability data (Rutzke, Glahn et al. 2004). Therefore, the use of this method is both appropriate and valid for investigations of iron bioavailability in green leafy vegetables that have similar properties and composition as spinach.

Green leafy vegetables are promising food sources of iron that have not been previously investigated for iron bioavailability using the in-vitro digestion / Caco-2 cell method, except for spinach. The objective of this study was to screen green leafy vegetables found in Hawaii for iron bioavailability using the in-vitro digestion / Caco-2 cell method. The goal of this study is to find relatively novel and good sources of bioavailable iron. We aimed to distinguish differences in iron bioavailability between GLV with different iron content and hope to provide insight on whether GLV are good sources of bioavailable iron using spinach as a reference.

3.2. Materials and Methods

Amaranth (*Amaranthus tricolor*), bitter melon leaf (*Momorida charantia*), chrysanthemum (*Chrysanthemum coronarium*), moringa (*Moringa oleifera*), and spinach (*Spinacea oleracea*) were used for iron bioavailability experiments. Sample preparation and analysis of iron content was described in Chapter 2. Briefly, fresh green leafy vegetables were obtained in local markets in Honolulu and were boiled in deionized water. Boiled samples were homogenized, lyophilized, and ground using stainless steel equipment. Aliquots of dried, ground GLV were analyzed for iron content using ICP-AES. Iron content expressed as ppm DM (see appendix) was recalculated to mg Fe per 100 g cooked EP using the percent dry matter (Table 1). Fresh spinach was obtained separately from a national distributor, cooked and processed similarly to GLV. The beef was obtained from a local supermarket, cooked on both sides in a stainless steel pan (water and oil excluded), and processed similarly to GLV.

Cell culture

Unless otherwise stated, all chemicals, hormones and enzymes were purchased from Sigma Chemical (St. Louis, MO). All glassware used in iron bioavailability experiments was previously treated in 10% HCl overnight, 10% HNO_3 overnight, and rinsed with deionized water. All the water used in experiments was cell culture grade, deionized, ultrapure water (Milli-Q, Millipore Corp). Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17. Cells were seeded at a density of 2,250,000 cells in 75 cm^2 cell culture flasks containing DMEM [Dulbecco's Modified Eagle Medium/High Glucose (1x) supplemented with 25 mM

HEPES, 4.5 g/L glucose, 4.00 mM L-glutamine and 10% v/v fetal bovine serum, Hyclone, Logan, UT]. Cells were passaged at 70-80% confluence with 0.5% trypsin-EDTA (MediaTech) and used at passages 29-36 for iron bioavailability experiments. The cells were maintained in 5% CO_2/95% air atmosphere at 37°C inside an incubator (New Brunswick Scientific). The medium was replaced every two days.

After sufficient numbers of cells were grown, they were seeded onto collagen coated, polystyrene, 6-well tissue-culture plates (BD Biosciences). To collagen coat each plate, 9.47 mg/ml rat tail Type-1 collagen (BD Biosciences) was diluted with 0.02N acetic acid to a working concentration of 50 ug/ml. 1.5 ml of collagen working solution was added to each well and incubated in a laminar flow hood for one hour. After one hour, the collagen working solution was removed, the wells were rinsed with 2 ml Hank's Buffered Salt Solution (HyClone), and each well was seeded at a density of 50,000 cells/cm^2 in 2 ml DMEM. At 12 days post-confluence, 2-4 wells were harvested and the protein content quantified to ensure sufficient growth and consistency between wells. Cells grown to 13-17 days post-confluence were used for experiments.

Day 1 - Pre experiment Preparation

The DMEM was removed, the wells were rinsed twice with fresh MEM (Minimal Essential Media, HyClone) supplemented with 10% FBS and 1% v/v antibiotic-antimycotic solution (pH 7.0), and 2 ml fresh MEM was added.

Cell culture inserts fitted with dialysis membrane were prepared: 2 inch length sections of 15 kDa metal free dialysis membrane (Membrane Filtration Products, Inc, Seguin, TX) were fitted onto 24 mm diameter Transwell plastic inserts (Costar) and

fastened with silicon O-rings (Web Seal Inc, Rochester NY). The completed inserts were stored in deionized water and placed in 4°C overnight.

Dried samples of green leafy vegetables, spinach, and beef were weighed to amounts equivalent to 67 μM Fe in the food digest (Experiment 1) or 0.5 grams (Experiment 2) and placed in weighed 50 ml centrifuge tubes overnight. Table 3 lists the amounts of iron and dry matter placed into each digest in experiments 1 and 2. In experiment 1, each digest was normalized to 67 μM Fe using different amounts of dry matter to obtain 55 μg Fe. The amounts of dry matter ranged from 1 g for beef to 0.13 g for chrysanthemum. In experiment 2, the gram amounts of GLV in the digests were equal (0.5 g DM), but the amounts of iron varied. Amounts of iron used ranged from 52 μg Fe in moringa digests to 215.31 μg Fe in chrysanthemum digests.

Day 2 - In-vitro digestion and exposure of food digests to Caco-2 cells

Preparation of pepsin solution:

To prepare the pepsin solution, 0.8 g pepsin was added to 20 ml of 0.1 M HCl. 10 grams of Chelex-100 was added to the solution and gently mixed on a tabletop shaker (New Brunswick Scientific) at 125 revolutions/min for 45 minutes. The slurry was poured into a 1.6 cm diameter filtration column (VWR) to collect the eluent and filter the Chelex. The column was further eluted with an additional 20 ml of 0.1 M HCl for a final volume of 40 ml pepsin solution.

Preparation of pancreatin-bile solution:

To prepare the pancreatin-bile solution, 0.3 g pancreatin and 1.8 g bile extract was added to 150 ml of 0.1 M $NaHCO_3$. 75 grams of Chelex-100 was added to this solution

and gently mixed on a tabletop shaker at 125 revolutions/min for 45 minutes. The slurry was poured into a 1.6 cm diameter filtration column (VWR) to collect the eluent and filter the Chelex. The column was further eluted with an additional 60 ml of 0.1 M $NaHCO_3$ for a final volume of 210 ml pancreatin-bile solution.

Sterilization of dialysis membrane and inserts:

The inserts were drained of water and sterilized in 0.5 M HCl for one hour. After one hour, the inserts were removed and rinsed with deionized water several times. The inserts remained in deionized water until use.

Preparation of quality control samples:

To make the ferrous sulfate quality control sample, first an iron stock solution was produced by adding 0.0556 g $FeSO_4 \bullet 7H_20$ to 10 ml of 1% H_2SO_4. 400 µl iron stock solution was added to 3.6 ml of 1% H_2SO_4 to make a working iron solution. 500 µl of the working iron solution was added to the digest solution to make a final concentration of 67 µM Fe.

To make the ferrous sulfate plus vitamin C positive control samples, first a vitamin C stock solution was made by adding 0.3522 g ascorbic acid (Macron Fine Chemicals) to 10 ml of deionized water and the solution was covered to protect from light. 800 µl of the vitamin C stock was added to 400 µl of the iron stock solution and 2.8 ml of 1% H_2SO_4. This solution was stirred for 15 minutes covered from light. 500 µl of this working ferrous sulfate and vitamin C solution was added to the digest solution to produce a final concentration of 1.3 mM Vitamin C and 67 µM Fe.

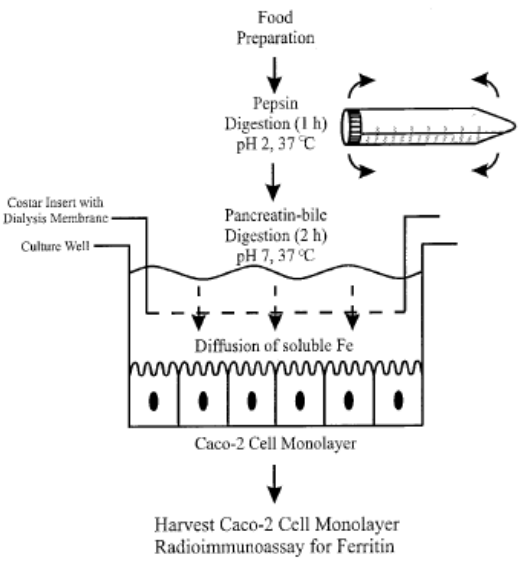

FIGURE 1 Diagram of in vitro digestion/Caco-2 cell culture model.

Picture adapted from Glahn, Lee et al. (1998)

Preparation of digests and exposure to cell monolayers:

10 ml of 140 mM NaCl, 5 mM KCl (pH 2.0) was added to each food and quality control sample, mixed, and readjusted to pH 2 with 0.1 M HCl. 0.5 ml pepsin solution was added to the digest, mixed, and the samples were placed in the incubator on a tabletop rocker (Boekel Scientific, Feasterville PA) at moderate speed for 1 hour. During incubation of the digests, MEM was removed from the plates, and replaced with 1 ml fresh MEM. The inserts were fitted onto each well and the plates were returned to the incubator.

After incubation, the pH of the digests was readjusted to 5.5 to 6.0 with 1 M $NaHCO_3$. 2.5 ml pancreatin-bile solution was added to the digest, mixed, and the pH was further readjusted to 6.7. The total volume of each digest was standardized to 15 ml by adding 140 mM NaCl, 5 mM KCl (pH 6.7). A 1.5 ml aliquot of each digest was placed

on top of the dialysis membrane in the insert of each well. The plates were gently rocked in the incubator for two hours. After 2 hours, the dialysis membrane inserts were removed from each well and a further 1 ml of MEM was added. The plates were returned to the incubator for a further 16 hours until harvesting.

Day 3 - Harvesting of cells

MEM was removed from all plates and the cells were rinsed twice with 2 ml of 130 mM, 5 mM KCl, 5 mM PIPES (pH 6.7). The cells were harvested by adding 2 ml of deionized water to each well, scraping the cells off the plastic substrate surface with a transfer pipette, and transferring the cells and 2 ml of water to sterile 5 ml culture tubes (VWR). The harvested cells were sonicated on high for 15 minutes using a bench top sonicator (VWR). Sonicated cells were kept at in -20°C until analysis.

Analysis

Lowry-Modified Protein Assay:

Protein concentrations were quantitatively measured using a modified Lowry protein assay kit (Thermo Scientific). Samples were defrosted in cold water and kept on ice during analysis. Reagents were brought to room temperature before the assay. An aliquot of 200 µl cell lysate in duplicate was placed in 5 ml disposable culture tubes (VWR). 1.0 ml Modified Lowry Reagent was added to each tube and mixed for 15 seconds. 100 µl of prepared 1x Folin-Ciocalteu Reagent was added and mixed for 15 seconds. The samples were covered to avoid light and incubated for 30 minutes. Absorbance was read at 750 nm (UV Visible Spectrophotometer, Shimadzu). A BSA

standard curve was generated at concentrations of 0, 1, 5, 25, 125, 250, 500, 750, and 1000 μg/ml using the same steps as the cell lysate.

Enzyme-linked immunosorbent assay (ELISA) for ferritin:

Ferritin concentrations were quantitatively measured using a Spectro Ferritin MT kit (Ramco Laboratories Inc., Houston, TX). Samples were defrosted in cold water and kept on ice during analysis. Reagents were all brought to room temperature before the assay. A 10 μl aliquot of cell lysate in duplicate was added to a 96 well plate. 200 μl of conjugated antihuman ferritin was placed onto each well and the plate was incubated on a platform shaker (New Brunswick Scientific) at 190 rpm for 2 hours. The plate was washed 3 times with deionized water and 200 μl of substrate solution was added to each well. The plate was further incubated for 30 minutes. 100 μl of 0.24% potassium ferricyanide was added to each well and the plate was shaken at 190 rpm for 1 minute to develop the color. Absorbance was read at 510 nm using a microplate reader (BioTek Instruments). A ferritin standard curve was generated using concentrations of 0, 6, 20, 60, 200, 600, 2000 ng/ml.

Statistics

Bioavailability was assessed in each well using the units, ng ferritin per mg cell protein. Data were expressed as means +/- SD, n = 4-6. To compare the efficiencies of iron uptake from foods, bioavailability was also expressed as ng ferritin / mg cell protein / μg Fe placed into each well. In order to compare bioavailability from foods per cooked EP serving size (RA), bioavailability expressed per μg of iron in the well was multiplied by the μg of iron in an RA of each food. The RA for each food was 85 grams cooked EP.

Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA). When necessary, the data were log transformed and averaged to achieve equal variance prior to analysis. One-way ANOVA followed by Tukey's post hoc test was used to compare means between each food digest. Means were considered significantly different if p values were ≤ 0.05.

3.3. Results

Iron content of samples used in Exp 1 and 2 (Table 4)

The amounts of iron found in all GLVs, as reported in Chapter 2, are listed in Table 3. Bitter melon (2.5 mg Fe per 100 g cooked weight) and chrysanthemum (2.4 mg Fe per 100 g cooked weight) contained the highest amount of iron. Sweet potato leaf, taro, ung choi, and watercress had the lowest amount of iron (0.6 to 0.8 mg per 100 g cooked weight). The iron content per 100 g cooked weight of amaranth, edible hibiscus, and moringa were in between the GLV listed above. GLVs used for iron bioavailability assays (amaranth, bitter melon leaf, chrysanthemum, and moringa) were chosen based upon their high iron content, or based upon the most promising GLVs found in the literature review in Chapter 1.

Experiment 1: Bioavailability of green leafy vegetables using equal amounts of iron in the digests (67 μM *Fe)*

Amaranth, bitter melon leaf, chrysanthemum and moringa were chosen for experiment one. Ferrous sulfate, ferrous sulfate and vitamin C, beef muscle, and spinach were included as well. Each digest contained 67 μM Fe (55 μg) and 5.5 μg was placed into each well. Since iron bioavailability was assessed using an equal amount of iron in

the digests, we consider these results to reflect the efficiency of iron absorption, or simply the 'quality of iron.'

The bioavailability of ferrous sulfate control sample was 31 ng ferritin / mg cell protein (Table 5). The addition of ascorbic acid significantly increased iron bioavailability to 235. The blank level was 23. These results indicate that the system was responding appropriately to the ferrous sulfate and Vitamin C positive control samples.

Spinach and moringa had the highest efficiency of iron bioavailability among all green leafy vegetables tested (Table 5). Amaranth and bitter melon leaf had the lowest efficiency, and chrysanthemum was intermediate. Beef muscle was also intermediate in efficiency and was not significantly different from any of the green leafy vegetables. Ferrous sulfate had a relatively high efficiency equal to spinach and moringa.

When the efficiency of absorption was used to calculate the bioavailability per RA on a cooked weight basis, all green leafy vegetables had equal iron bioavailability. Beef muscle had significantly higher iron bioavailability per RA than bitter melon leaf, moringa, and amaranth, but equal iron bioavailability to spinach and chrysanthemum, although these values were trending toward significance.

Experiment 2: Bioavailability of green leafy vegetables using equal weights

Spinach, moringa, and amaranth were chosen for experiment two. The methods and conditions were similar to experiment one. In this experiment, however, 0.05 g of dry matter (DM) constituted within 1.5 ml of digest was exposed to each well and, therefore, iron bioavailability was measured as ng ferritin / mg cell protein per 0.05 g of dry matter. The 0.05 g DM in each well contained 8.29 μg Fe for spinach, 5.21 μg Fe

for moringa, 10.49 µg Fe for amaranth, and 4.48 µg Fe for beef. The amount of iron from the ferrous sulfate control was the same as experiment one (5.5 µg Fe). Iron bioavailability per 0.05 g DM was then converted to iron bioavailability per µg Fe in the well, i.e. efficiency of iron bioavailability.

Among the GLVs, spinach and moringa had equal efficiencies of iron bioavailability (Table 6). Amaranth had significantly lower bioavailability per µg Fe than spinach, but was equal to moringa. Beef, although higher than spinach and moringa, was not significantly greater in bioavailability per µg Fe.

Comparing iron bioavailability per recommended amounts of food, a serving of moringa was equal to a serving of spinach, but a serving of amaranth had a lower iron bioavailability than spinach (Table 6). Beef had significantly greater iron bioavailability per RA than all of the GLVs.

3.4. Discussion

Appropriate and reproducible responses from the Caco-2 cell culture are critically important when measuring iron bioavailability and are dependent upon fully differentiated caco-2 cell monolayers. The concept of full differentiation is somewhat ambiguous and primarily depends on the particular aspect of differentiation being measured (Sambuy, De Angelis et al. 2005). It has been shown that DMT-1 transporters, ferrireductase activity, and heme carrier protein-1 transporters are expressed in fully differentiated cells (Ekmekcioglu and Marktl 1998; Follett, Suzuki et al. 2002; Sharp, Tandy et al. 2002). Confirmation of fully differentiated caco-2 cells is necessary to make comparisons of iron bioavailability between foods.

Using previous literature, we concluded that the Caco-2 cells in our studies were fully differentiated using the criteria of day of growth post-confluence. In both experiments, cells were used between 13-17 days post confluence, which is consistent with other studies published. The design of these experiments are modeled after Dr. Glahn's lab and they conduct experiments at 12 days post confluence (Glahn, Lee et al. 1998). Another researcher determined that Caco-2 cells were fully differentiated at 17 days post confluent (Han and Wessling-Resnick 2002). The levels of DMT-1 mRNA and protein increased during days 7 to 21 post seeding, with maximal iron uptake occurring at 14 days post seeding (Sharp, Tandy et al. 2002). 14 days post seeding is approximately equal to 11-12 days post- confluence, which is close to within the range of our experiments.

We further confirmed that our experiments were conducted with fully differentiated cells by using several controls. A reference amount of iron sulfate was used in each experiment to ensure an appropriate response from Caco-2 cells. Ferritin formation significantly increased above baseline with the addition of 5.5 μg Fe in iron sulfate in each study. Each experiment also included ascorbic acid added to ferrous sulfate as a positive control. The addition of ascorbic acid increased the bioavailability of ferrous sulfate by several fold.

In experiment 1, relative iron bioavailability was assessed among the green leafy vegetables that either contained the highest concentrations of iron per 100 grams cooked weight, or where the literature on a particular GLV showed potential for being a good source of bioavailable iron. 67 μM iron was used in each digest in order to measure iron absorption efficiency, or the quality of iron found in each leaf. This amount of iron has

been used previously in similar types of experiments and is known to be just below the saturation concentration for iron uptake in this system when using iron sulfate plus vitamin C in the digests (Lai, Dobbs et al. 2011). We found that the highest efficiency of iron bioavailability was in spinach and moringa, while amaranth and bitter melon had the lowest efficiency. These differences may be related to the different amounts and kinds of inhibitors found in these leaves.

The low efficiency of iron uptake in amaranth and bitter melon may be due to the presence of high levels of oxalates or polyphenols. In the investigation of several underutilized green leafy vegetables for iron bioavailability, amaranth was one of four GLV highest in oxalate content, a proposed inhibitor of iron bioavailability. Furthermore, the tannin concentration of amaranth were measured at 106 mg per 100 gram fresh weight, which may further negatively impact iron bioavailability (Gupta, Jyothi Lakshmi et al. 2005). Polyphenols are major inhibitors of iron bioavailability and green leafy vegetables are known to be rich sources of polyphenols. As an example, curly kale is a particularly rich source of flavanols, such as kaempferol and quercetin. A 200 gram serving of fresh curly kale averaged 300-600 mg/kg or 60-120 mg flavanol per serving. The distribution of flavanols within the same food can differ. In green leafy vegetables such as lettuce and cabbage, flavanol accumulation on the outer leaves exposed to direct sunlight is ten times greater than leaves found on the inside due to the UV biosynthesis of flavanols (Manach, Scalbert et al. 2004). The low iron bioavailability of bitter melon leaf may be due to its polyphenolic content. Phenolic content varies depending upon the different components of the plant. The leaf of bitter melon has the highest total phenolic content (mg gallic acid equivalents / 100 g dry sample) relative to

its stem, green fruit, and ripe fruit. Furthermore, the use of HPLC-DAD identified gallic acid as the predominant form of polyphenol found in bitter melon leaf (Kubola and Siriamornpun 2008).

Amaranth in our study contained a large amount of calcium relative to the other green leafy vegetables. Calcium is a known inhibitor of iron absorption. A 300 mg calcium supplement was shown to significantly inhibit non-heme iron absorption from a meal in human subjects (Cook, Dassenko et al. 1991). Moreover, the addition of 300 mg calcium to bread rolls dose-dependently decreased both heme and non-heme iron absorption. Increasing the dose of calcium in bread rolls from 300 mg to 600 mg decreased non-heme iron absorption by 50% (Hallberg, Brune et al. 1991). The mechanism behind the inhibitory effects of calcium on iron absorption is not fully understood. The inhibition of iron absorption by calcium may be due interference during mucosal uptake (Roughead, Zito et al. 2005) or at the efflux step (Gaitan, Flores et al. 2012). In a separate study, calcium is thought to inhibit non-heme iron absorption in Caco-2 cells by altering cellular localization of DMT-1, without effecting protein expression (Thompson, Sharp et al. 2010).

To help confirm the results of the first experiment, we conducted a follow-up study in experiment 2. We used spinach, moringa, and amaranth and rather than normalizing the iron content to 67 μM Fe, each digest contained 0.5 g amounts dry matter. Our objective was to investigate the reproducibility of experiment 1 when measuring iron bioavailability per g DM, and then compare the iron absorption efficiencies between experiments. Spinach was used as the reference green leafy vegetable and moringa and amaranth represented green leafy vegetables with the highest

and lowest absorption efficiencies, respectively, from the previous experiment. We again found that spinach and moringa had similar efficiencies of iron uptake, and that spinach had a higher efficiency than amaranth in experiment 2. This was possibly due to the higher concentration of iron in the amaranth digest (relative to moringa), which may have had a more robust uptake of iron. These results suggest that using 0.5 gram of food in the digests and calculating iron absorption efficiency give similar results as normalizing to 67 μM Fe, unless there are large differences in sample iron content and hence large differences in iron concentrations in the digests.

When bioavailability was expressed per RA, beef generally had higher bioavailability than GLVs due to beef having both a relatively high absorption efficiency and high iron content per RA. Experiment 1 suggests that the iron absorption efficiency of beef is equal to green leafy vegetables, contrary to what we had anticipated. It has been clearly demonstrated and widely accepted that heme iron is better absorbed than non-heme iron (Hallberg 1981). We expected that the iron efficiency of beef, which contains both heme and non-heme iron, would be significantly greater than the iron efficiency of green leafy vegetables, which contain only non-heme iron. Caco-2 cells express heme transport protein and have been shown to be useful as a model for intestinal heme iron absorption, therefore they should be able to display differences in iron absorption from heme and non-heme foods (Follett, Suzuki et al. 2002). We speculate that the reason for the low response of ferritin formation to beef was not attributable to the cell culture, but the food digest. Cooking is known to denature some of the heme iron in meat and therefore it is possible that in our samples, some of the heme could have

degraded leading to the release of free iron. Free iron is not absorbed as efficiently as intact heme (Schricker and Miller 1983).

Reviewers have voiced concerns that normalizing the iron concentration in different food samples may not be the most accurate method in measuring comparative iron absorption efficiencies. Normalizing Fe in each digest will result in different amounts of food in each digest and consequently affect the amount of iron absorbed. Our dried beef sample contained the least concentrated amount of iron compared to all green leafy vegetables and, therefore, the beef digest contained the largest amount of food DM. There is a possibility that the excess amount of food prevents iron absorption by preventing iron release from a 'food matrix,' or that the food matrix blocks available iron from dialyzing through the membrane. This may explain why the iron efficiency of beef compared to green leafy vegetables was trending toward significant difference in experiment 2.

Although there were different iron absorption efficiencies between green leafy vegetables, the bioavailability expressed per RA indicated few significant differences. Amaranth appeared to provide significantly less bioavailable iron per RA than spinach. We find that it is valuable to compare iron bioavailability using RA. The reason is that while there certainly may be significant differences in iron efficiency among foods, this measurement is limited in usefulness because it does not consider portion size and iron content per RA when comparing foods as sources of iron. We currently have no prior knowledge of other studies that express bioavailability per RA, except for Lai, Dobbs et al. (2011).

Although most of the foods tested in experiment 1 did not increase ferritin formation above the blank baseline, these results are not dissimilar to other studies using the in-vitro digestion / Caco-2 cell method. The limited responsiveness of ferritin formation to foods is not unique and has been exhibited in other studies. Comparing eight different bean genotypes for iron bioavailability, experimenters found only two genotypes which exhibited significantly higher bioavailability than baseline controls (Ariza-Nieto, Blair et al. 2007). Comparing the iron bioavailability of several rice genotypes, digested rice genotypes averaged 9.1 ng of ferritin per mg cell protein, while baseline values ranged from 3 to 9 ng ferritin per mg cell protein (Glahn, Cheng et al. 2002). It may be necessary to use 0.5 g or more of dry matter compared to using 67 μM Fe in the digests to achieve significant increases in ferritin formation past baseline when conducting studies of foods with low iron bioavailability. In experiment 2, all four food digests produced significantly greater ferritin than baseline and had greater concentrations of iron in the digest than the 67 μM Fe used in experiment 1.

In response to the apparent low ferritin response to foods, further studies investigating iron bioavailability of green leafy vegetables may warrant the addition of foods that have the ability to enhance ferritin production (increase iron bioavailability). Increasing ferritin production is beneficial because it is easier to distinguish differences in iron bioavailability. Using the in-vitro digestion / Caco-2 cell culture, one strategy to clearly elucidate differences in iron bioavailability among green leafy vegetables is to add physiologically relevant dosages of ascorbic acid to each digest to enhance ferritin production. Ascorbic acid at a 10:1 AA/Fe molar ratio was added to each rice digest. Ascorbic acid was able to increase the iron bioavailability of rice genotypes past baseline

in order to elucidate significant differences between genotypes (Glahn, Cheng et al. 2002).

Another strategy to further demonstrate differences in iron bioavailability between GLV is the addition of meat to each green leafy vegetable digest. Meat has been shown to increase non-heme iron bioavailability. There is evidence that proteins, and possibly other constituents found in beef or chicken, increase non-heme iron bioavailability (Hurrell, Reddy et al. 2006). Investigators have found that cysteine peptides derived from digested meat increase non-heme iron bioavailability by reducing Fe^{3+} to Fe^{2+} and increasing the solubility of iron (Kapsokefalou and Miller 1991). It is possible that heme alone or in combination with peptides in meat increase non-heme iron bioavailability. Purified heme was demonstrated to increase non-heme iron absorption in Caco-2 cells, by increasing ferroportin expression (Ma, Kim et al. 2011).

Although there is evidence to suggest that the iron bioavailability of GLV can be enhanced with ascorbic acid or meat, the polyphenols found in green leafy vegetables might limit ferritin response in Caco-2 cells. Using Caco-2 cells, the addition of ascorbic acid and meat was able to reverse the inhibitory effects of phytate on iron absorption, but not able to overcome the inhibitory effects of tannic acid (Engle-Stone, Yeung et al. 2005). The inhibition of iron bioavailability has recently been demonstrated using Caco-2 cells. Two dietary polyphenol extracts, epigallocatechin-3-gallate (ECGC) and grape seed extract (GSE) were applied to Caco-2 cells in physiological amounts. Investigators found that although polyphenols enhanced apical iron absorption, polyphenols inhibited basolateral iron release. They postulated that the formation of a polyphenol-iron complex in the cytosol of the enterocyte prevented iron efflux (Kim, Ham et al. 2008). In a

follow-up study, the addition of ascorbic acid to dietary polyphenol extracts were able to reduce the inhibition of iron bioavailability, possibly by increasing basolateral iron release through an identified polyphenol-iron and ascorbic acid interaction within the cell (Kim, Ham et al. 2011). Although the bioavailability of iron sulfate is enhanced by ascorbic acid, the presence of inhibitors may prevent the increase in iron bioavailability of green leafy vegetables even with the addition of ascorbic acid. Therefore, the addition of ascorbic acid or meat may or may not be able to enhance the iron bioavailability of green leafy vegetables. One of the inherent limitations of using the Caco-2 cell line is that reproducibility is very difficult. It is quite evident that conclusions of one experiment may be contrary to the conclusions of another experiment and is primarily due to the inherent morphological and characteristic variability of caco-2 cells, especially the passage number and time of cell growth (Briske-Anderson, Finley et al. 1997). Therefore, further experiments are required to confirm our results.

While none of the green leafy vegetables in our study were a better source of bioavailable iron than spinach, investigations must continue to search for plants with high iron content and bioavailability. Our data suggests that increasing the iron content does not necessarily translate into an increase in iron bioavailability. Research suggests that soybeans and peas are potential sources of bioavailable iron because they contain phytoferritin. Phytoferritin is a potentially bioavailable source of iron that can theoretically sequester thousands of iron atoms, and the surrounding protein nanocage prevents the influence of inhibitors. Purified soybean ferritin has been shown to be as bioavailable as ferrous sulfate in female subjects (Lonnerdal, Bryant et al. 2006) and is absorbed via AP-2 clathrin dependent endocytosis (San Martin et al 2008). Soybean

ferritin is unaffected by the non-heme iron inhibitors phytate, tannin, and calcium in Caco-2 cells, but it was shown that under the digestive conditions of pH 2, ferritin was degraded and phytate, tannin, and calcium inhibited iron bioavailability, most likely by binding the freely released iron (Kalgaonkar and Lonnerdal 2008). Moreover, it has also been shown that pea-ferritin is also degraded by gastric conditions. Using Caco-2 cells, the iron bioavailability of digested purified pea ferritin was enhanced by ascorbic acid but inhibited by phytate. Iron sulfate enhanced by ascorbic acid was significantly more bioavailable than pea-ferritin enhanced by ascorbic acid (Bejjani, Pullakhandam et al. 2007). While investigations on ferritin bioavailability from plants look promising, more research is required to study the genetic breeding of plants with phytoferritin and whether phytoferritin is actually a suitable source of iron bioavailability.

3.5. Conclusion

Some green leafy vegetables are good sources in iron content, but may not necessarily be good sources of bioavailable iron. In the first experiment, using equal amounts of iron in each digest, we discovered variability in the iron efficiency of green leafy vegetables. Moringa and chrysanthemum had equal iron efficiency to spinach, while amaranth and bitter melon leaf were less iron efficient than spinach, even though they had higher iron content. Although there were differences in iron efficiency among green leafy vegetables, when the dry matter was reexpressed to a cooked weight serving, iron bioavailability of green leafy vegetables was approximately equal. In the second experiment, 0.5 grams of DM food were used in each digest. The iron efficiency of moringa was equal to spinach, but amaranth was significantly lower in iron efficiency to

spinach. Reexpressed per RA, the same relationship as found. Iron bioavailability of moringa was equal to spinach but amaranth was significantly lower than spinach. We also discovered that comparing iron bioavailability using 0.5 gram food DM in each digest is preferable to 67 μM Fe in each digest as evidenced by the beef control and ferritin formation past baseline. More experiments are needed to confirm our findings. Enhancing iron bioavailability of green leafy vegetables with ascorbic acid and/or meat may help to elucidate differences in iron bioavailability among green leafy vegetables.

Table 1 Mineral composition of boiled green leafy vegetables expressed as mg per 100 grams cooked edible portion[1]

Name	Ca	Cu	Fe	Mg	Mn	P	K	Na	Zn
Amaranth Leaf	351 ± 5	0.065 ± 0.001	1.6 ± 0.0	64 ± 1	0.25 ± 0.01	27 ± 0.6	122 ± 1	4.9 ± 0.0	0.182 ± 0.002
Bitter Melon Leaf	195 ± 2	0.445 ± 0.007	2.5 ± 0.1	47 ± 1	0.80 ± 0.01	44 ± 0.7	46 ± 0.6	0.9 ± 0.0	0.087 ± 0.005
Chrysanthemum	73 ± 1	0.098 ± 0.001	2.4 ± 0.1	12 ± 0	0.30 ± 0.01	23 ± 0.4	135 ± 2.3	47.3 ± 0.9	0.121 ± 0.002
Edible Hibiscus	214 ± 4	0.062 ± 0.002	1.3 ± 0.1	54 ± 1	0.93 ± 0.02	40 ± 0.8	141 ± 1.5	0.7 ± 0.0	0.494 ± 0.007
Kale	99 ± 2	0.037 ± 0.001	0.8 ± 0.0	8 ± 0	0.11 ± 0.002	23 ± 0.3	57 ± 0.5	4.6 ± 0.0	0.149 ± 0.001
Moringa	207 ± 8	0.085 ± 0.003	1.1 ± 0.2	28 ± 1	1.25 ± 0.04	56 ± 2.0	31 ± 0.7	3.4 ± 0.1	0.316 ± 0.005
Sweet Potato Leaf	61 ± 0	0.064 ± 0.001	0.8 ± 0.0	27 ± 0	0.58 ± 0.002	41 ± 0.1	229 ± 1.4	1.4 ± 0.0	0.197 ± 0.002
Taro Leaf	191 ± 7	0.043 ± 0.002	0.8 ± 0.1	16 ± 1	1.08 ± 0.04	19 ± 0.6	80 ± 2.2	0.2 ± 0.0	0.119 ± 0.002
Ung Choy	35 ± 0	0.088 ± 0.002	0.7 ± 0.0	10 ± 0	0.89 ± 0.01	30 ± 0.6	156 ± 2.5	15.2 ± 0.3	0.126 ± 0.003
Watercress	48 ± 5	0.032 ± 0.004	0.6 ± 0.1	20 ± 1	0.14 ± 0.01	21 ± 1.3	97 ± 4.7	18.0 ± 1.2	0.112 ± 0.007

[1] Values are means ± SD (n = 3). 3 replicates sent to LSU Ag Sciences, NIST peach samples used as quality control. Data calculated as grams of mineral/100 g dry matter times % dry matter in cooked edible portion.

Table 2 Mineral composition of boiled green leafy vegetables expressed as % of DV[1]

	Ca	Cu	Fe	Mg	Mn	P	K	Na	Zn
Daily Value (DV)	1000	2	18	400	2	1000	3500	2400	15
Excellent (20% of DV)**	200	0.4	3.6	80	0.4	200	700	480	3
Good (10-19% of DV)*	100	0.2	1.8	40	0.2	100	500	240	1.5
Amaranth Leaf	29.9%	3.0%	7.7%	13.5%	10.5%	2.3%	3.0%	0.2%	1.1%
Bitter Melon Leaf	16.6%	19.0%	11.7%	10.0%	33.0%	3.7%	1.1%	0.0%	0.5%
Chrysanthemum	6.2%	4.0%	11.4%	2.5%	13.0%	2.0%	3.3%	1.7%	0.7%
Edible Hibiscus	18.2%	2.5%	6.1%	11.5%	39.5%	3.4%	3.4%	0.0%	2.8%
Kale	8.4%	1.5%	3.9%	1.8%	4.5%	2.0%	1.4%	0.2%	0.9%
Moringa	17.6%	3.5%	5.0%	5.8%	53.0%	4.7%	0.8%	0.1%	1.8%
Sweet Potato Leaf	5.2%	2.5%	3.6%	5.8%	25.0%	3.5%	5.5%	0.0%	1.1%
Taro Leaf	16.2%	2.0%	3.7%	3.5%	46.0%	1.6%	1.9%	0.0%	0.7%
Ung Choy	3.0%	4.0%	3.2%	2.0%	37.5%	2.5%	3.8%	0.5%	0.7%
Watercress	4.1%	1.5%	2.9%	4.3%	6.0%	1.8%	2.4%	0.6%	0.7%

[1]% of DV represents the ratio between the mean amount of the nutrient in an RA and the DV for the nutrient.

Table 3 Total iron content in green leafy vegetables and beef[1]

Food Sample	ug/g, dry weight	mg/ 100g, cooked weight
Amaranth	208 ± 4	1.6 ± 0.0[b**]
Bitter Melon leaf	360 ± 15	2.5 ± 0.1[a**]
Chrysanthemum	431 ± 9	2.4 ± 0.1[a**]
Moringa	104 ± 19	1.1 ± 0.2[d**]
Kale	145 ± 3	0.8 ± 0.0[de]
Edible hibiscus	133 ± 9	1.3 ± 0.1[c]
Sweet potato leaf	93 ± 0.3	0.8 ± 0.0[e]
Taro leaf	110 ± 10	0.8 ± 0.1[e]
Ung choi	143 ± 3	0.7 ± 0.0[e]
Watercress	145 ± 15	0.6 ± 0.1[e]
Reference food		
Spinach	166 ± 4	1.3 ± 0.0[cd**]
Beef Muscle	90 ± 2	3.1 ± 0.1[**]

[1] Values are means ± SD in triplicate. Means expressed in mg Fe per 100 grams cooked weight were compared using ANOVA followed by Tukey's post-hoc test. Means with different superscripts differ ($p \leq 0.05$)

[**] Foods chosen for in-vitro digestion/Caco-2 cell bioavailability studies

Table 4 Iron content and grams of food in digests applied to Caco-2 cells in experiments 1 and 2

		Experiment 1: Relative bioavailability using equal Fe		Experiment 2: Relative bioavailability using equal g food	
Sample		grams of DM per digest	µg Fe per digest	grams of DM per digest	µg Fe per digest
Green leafy Vegetables					
	Amaranth (cooked)	0.26	55	0.5	104.87
	Bitter Melon leaf (cooked)	0.15	55	0.5	180.00
	Chrysanthemum (cooked)	0.13	55	0.5	215.31
	Moringa (cooked)	0.45	55	0.5	52.13
	Spinach (cooked)	0.33	55	0.5	82.90
Beef					
	Beef Muscle (cooked)	1.00	55	0.5	44.80
Control	Ferrous sulfate		55		55

Table 5 Relative iron bioavailability using 67 μM Fe in each digest[1]

Sample		Relative efficiency of iron bioavailability	Relative iron bioavailability per serving (RA)[2]
		ng ferritin / mg cell protein / μg Fe	ng ferritin / mg cell protein / RA
Green leafy vegetables	Amaranth (cooked)	2.03 ± 0.80[a]	2805 ± 1106[a]
	Bitter Melon leaf (cooked)	1.71 ± 0.19[a]	3951 ± 401[a]
	Chrysanthemum (cooked)	2.55 ± 0.41[ab]	5233 ± 851[ab]
	Moringa (cooked)	4.76 ± 2.56[b]	4262 ± 2289[a]
	Spinach (cooked)	4.99 ± 2.03[b]	5376 ± 2187[ab]
Beef	Beef Muscle (cooked)	3.44 ± 0.65[ab]	9110 ± 1709[b]
Control	Ferrous sulfate	5.55 ± 0.38[b]	
		Relative bioavailability ng ferritin / mg cell protein	
Quality Controls	Blank	22.59 ± 1.97[a]	
	Ferrous sulfate	30.53 ± 2.10[b]	
	Ferrous sulfate plus Vit. C.	235.11 ± 15.30[c]	

[1]Values are means ± SD (n = 4).
[2]Per serving as recommended amount (RA) is 85 grams cooked weight.

Table 6 Relative iron bioavailability using 0.5 grams dry matter in each digest[1]

Sample		Relative efficiency of iron bioavailability	Relative iron bioavailability per serving (RA) [2]
		ng ferritin / mg cell protein / ug Fe	ng ferritin / mg cell protein / RA
Green leafy vegetables	Amaranth (cooked)	1.17 ± 0.40[a]	1616 ± 549[a]
	Moringa (cooked)	2.20 ± 0.73[ab]	1965 ± 651[ab]
	Spinach (cooked)	2.74 ± 0.36[b]	2951 ± 386[b]
Beef	Beef Muscle (cooked)	3.13 ± 1.30[b]	8303 ± 3443[c]
Control	Ferrous sulfate	1.83 ± 0.47[ab]	
		ng ferritin / mg cell protein	
Quality Controls	Blank	5.73 ± 0.58[a]	
	Ferrous sulfate	10.09 ± 2.58[b]	
	Ferrous sulfate plus Vit C	373.76 ± 48.91[c]	

[1]Values are means ± SD (n = 6).
[2]Per serving as recommended amount (RA) is 85 grams cooked weight.

APPENDIX

Table 7 Mineral composition of boiled[1] green leafy vegetables (mg per kg, dry weight basis)[2]

Name	Ca	Cu	Fe	Mg	Mn	P	K	Na	Zn
Amaranth Leaf	45053 ± 600	8.3 ± 1.8	208 ± 4	8220 ± 125	32.0 ± 0.7	3487 ± 76	15643 ± 142	616 ± 5	23.4 ± 0.3
Bitter Melon Leaf	28303 ± 300	65 ± 1	360 ± 15	6800 ± 87	113 ± 1	6317 ± 99	6597 ± 86	124 ± 1	12.6 ± 0.7
Chrysanthemum	13093 ± 200	17.6 ± 0.2	431 ± 9	2051 ± 39	54 ± 1	4155 ± 75	24131 ± 403	8439 ± 156	21.6 ± 0.4
Edible Hibiscus	22053 ± 400	6.4 ± 0.2	133 ± 9	5597 ± 106	96 ± 2	4077 ± 85	14570 ± 159	67 ± 3	50.9 ± 0.7
Kale[3]	17297 ± 300	6.5 ± 0.2	145 ± 3	1403 ± 21	19 ± 0.3	4030 ± 61	10060 ± 87	800 ± 5	26.1 ± 0.2
Moringa	20503 ± 400	8.4 ± 0.3	104 ± 20	2723 ± 91	124 ± 4	5517 ± 201	3103 ± 70	335 ± 5	31.3 ± 0.5
Sweet Potato Leaf	7503 ± 20	7.9 ± 0.1	93 ± 0.3	3268 ± 13	72 ± 0.3	5099 ± 17	28204 ± 167	178 ± 2	24.3 ± 0.2
Taro Leaf[4]	27270 ± 900	6.2 ± 0.3	110 ± 10	2347 ± 87	154 ± 6	2693 ± 86	11440 ± 318	21.8 ± 2.2	17.0 ± 0.2
Ung Choy	7326 ± 90	18.3 ± 0.4	143 ± 3	2073 ± 36	185 ± 3	6191 ± 118	32543 ± 527	3165± 65	26.3 ± 0.6
Watercress	11193 ±1000	7.4 ± 0.9	145 ± 15	4646 ± 242	32.7 ± 2.5	4872 ± 296	22622 ± 1095	4184 ± 273	26.1 ± 1.7

[1]Boiled for 5 minutes, lyophilized, and analyzed by ICP-ES.
[2]Values are means ± SD in triplicate.
[3]Sample cooked for 40 minutes.
[4]Sample cooked for 60 minutes.

Table 8 Mineral composition of boiled green leafy vegetables based on mg per NLEA[1] RACC[2] (85g)

	Ca (mg)	Cu (mg)	Fe (mg)	Mg (mg)	Mn (mg)	P (mg)	K (mg)	Na (mg)	Zn (mg)
Daily Value (DV)	100	2	18	400	2	1000	3500	2400	15
Excellent (20% of DV)**	200	0.4	3.6	80	0.4	200	700	480	3
Good (10-19% of DV)*	100	0.2	1.8	40	0.2	100	500	240	1.5
Amaranth Leaf	299**	0.06	1.38	54*	0.21*	23	104	4	0.16
Bitter Melon Leaf	166*	0.38*	2.11*	40*	0.66**	37	39	1	0.07
Chrysanthemum	62	0.08	2.05*	10	0.26*	20	115	40	0.1
Edible Hibiscus	182*	0.05	1.1	46*	0.79**	34	120	1	0.42
Kale	84	0.03	0.7	7	0.09	20	49	4	0.13
Moringa	176*	0.07	0.9	23	1.06**	47	27	3	0.27
Sweet Potato Leaf	52	0.05	0.64	23	0.50**	35	194	1	0.17
Taro Leaf	162*	0.04	0.66	14	0.92**	16	68	0	0.1
Ung Choy	30	0.08	0.58	8	0.75**	25	133	13	0.11
Watercress	41	0.03	0.53	17	0.12	18	83	15	0.1

[1]Nutrition Labeling and Education Act
[2]Recommended amounts customarily consumed per eating occasion

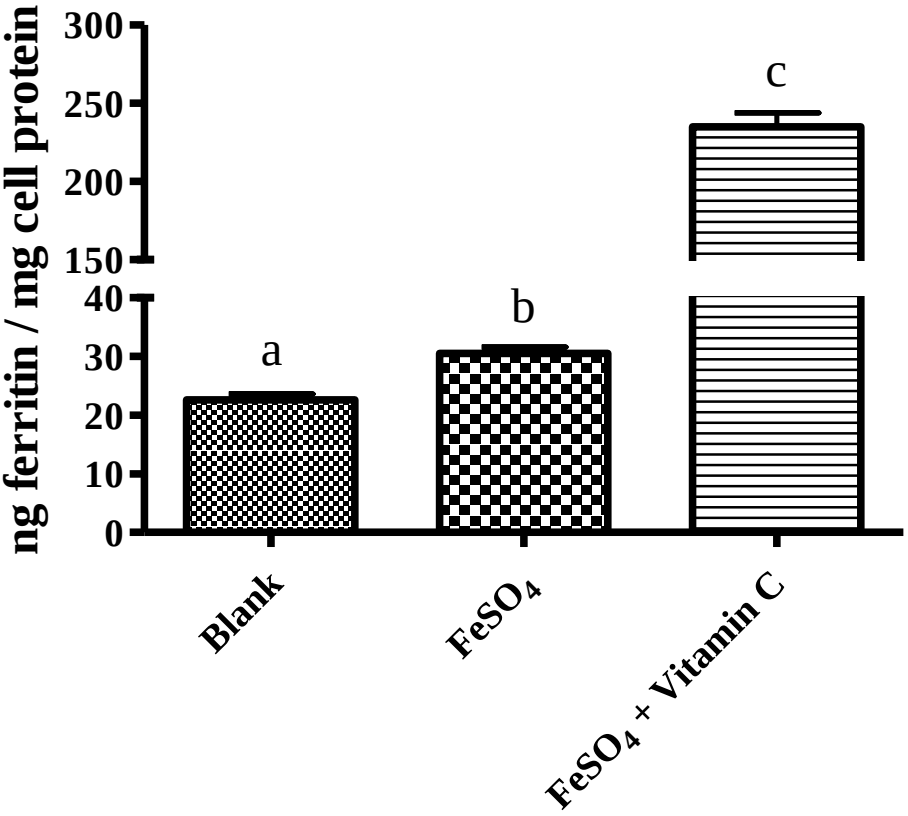

Figure 1. Controls for experiment 1: comparing relative iron bioavailability of green leafy vegetables and beef using 67 μM Fe**.**

Each digest was brought up to 15 ml and 1.5 ml of digest was placed into each well. The blank samples represent baseline ferritin formation from Fe present in MEM and digest enzymes. The ferrous sulfate samples represent a digest consisting of 67 μM Fe and the ferrous sulfate and vitamin C samples represent a digest consisting of 67 μM Fe and 1.3 mM vitamin C. Fe uptake in Caco-2 cells was measured by ferritin formation after 18 hr incubation using ELISA. Bar values indicate ± SD, n = 4. Data was log transformed prior to analysis to achieve equal variances. Bars with different letters are significantly different ($p \leq 0.05$).

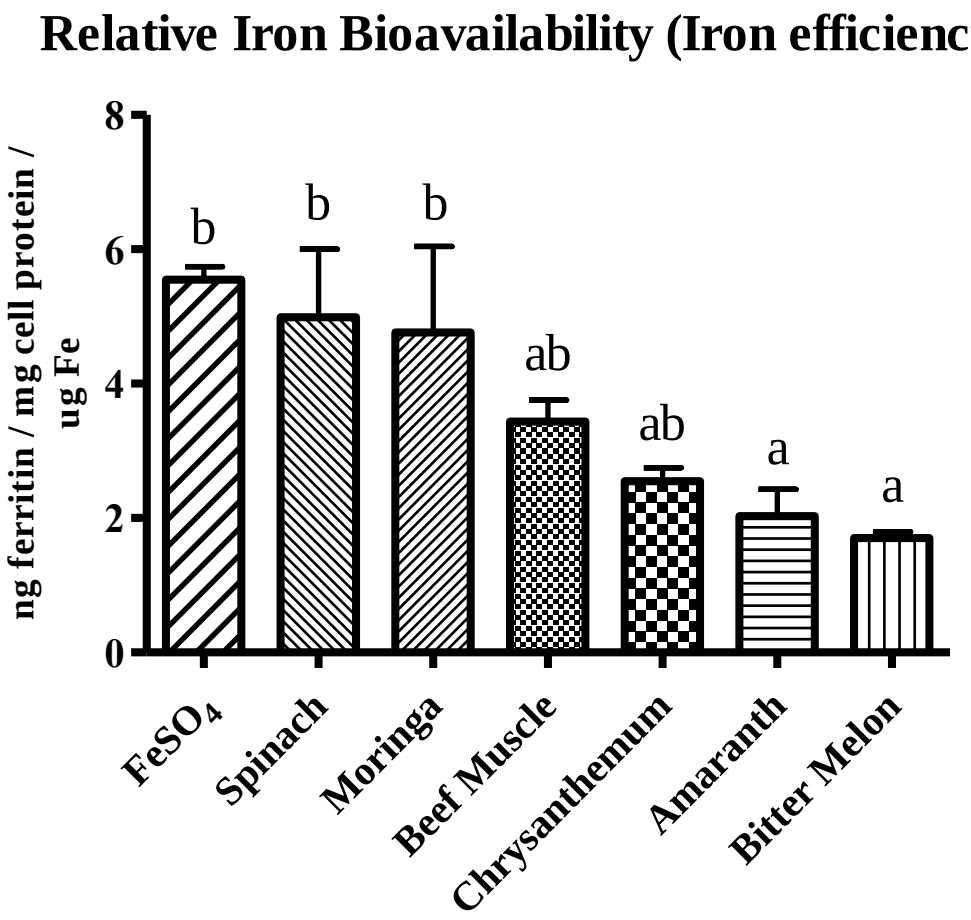

Figure 2. Experiment 1 - Relative Fe bioavailability from cooked green leafy vegetables and beef expressed per ug Fe using 67 μM in each digest**.**

Each digest was brought up to 15 ml and 1.5 ml of digest was placed into each well. Fe uptake in Caco-2 cells was measured by ferritin formation after 18 hr incubation using ELISA. Bioavailable Fe units (ng ferritin / mg cell protein) are per 5.5 μg Fe (actual exposure) but shown as per μg Fe. Bar values represent means ± SD, n = 4. Data were log transformed prior to analysis to achieve equal variances. Bars with no letters in common are significantly different ($p \leq 0.05$).

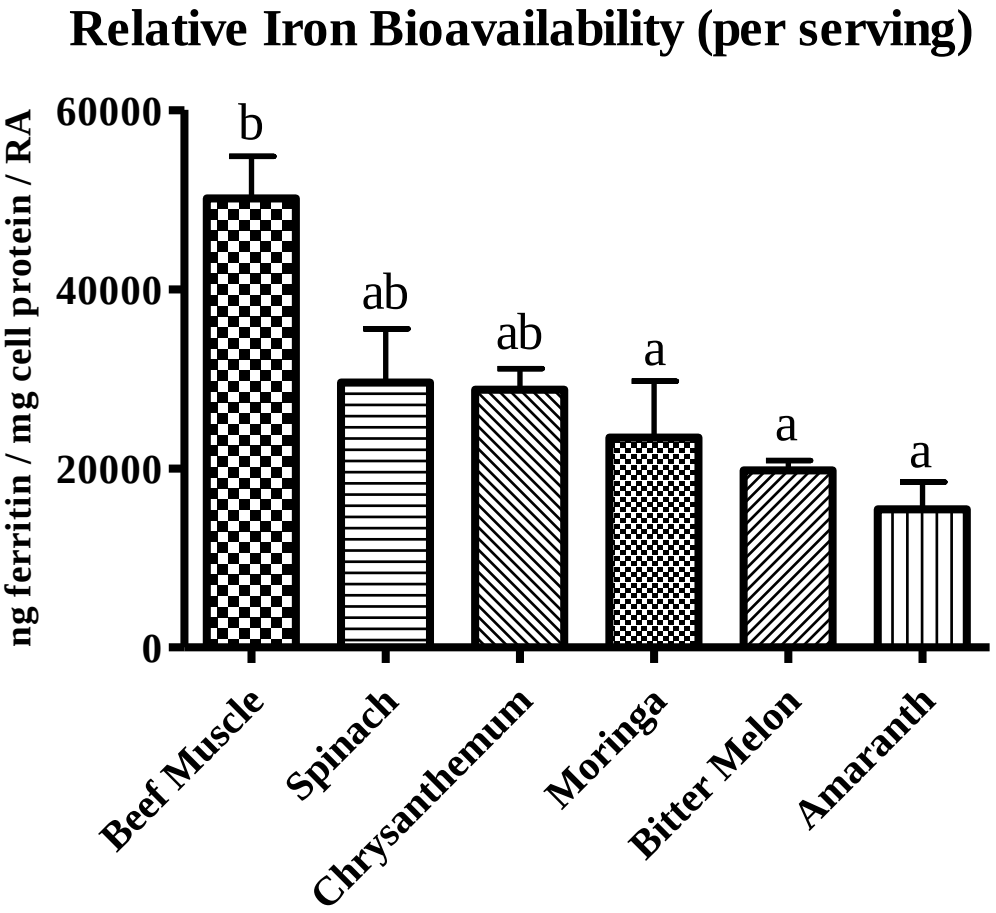

Figure 3. Experiment 1 - Relative Fe bioavailability from cooked green leafy vegetables and beef expressed per serving.

Each digest was brought up to 15 ml and 1.5 ml of digest was placed into each well. Fe uptake in Caco-2 cells was measured by ferritin formation after 18 hr incubation using ELISA. Bioavailable Fe units (ng ferritin / mg cell protein) are per 5.5 µg Fe (actual exposure) and reexpressed per recommended amount multiplying by the percent dry matter. A recommended amount consists of 85 grams cooked weight. Bar values represent means ± SD, n = 4. Data were log transformed prior to analysis to achieve equal variances. Bars with no letters in common are significantly different ($p \leq 0.05$).

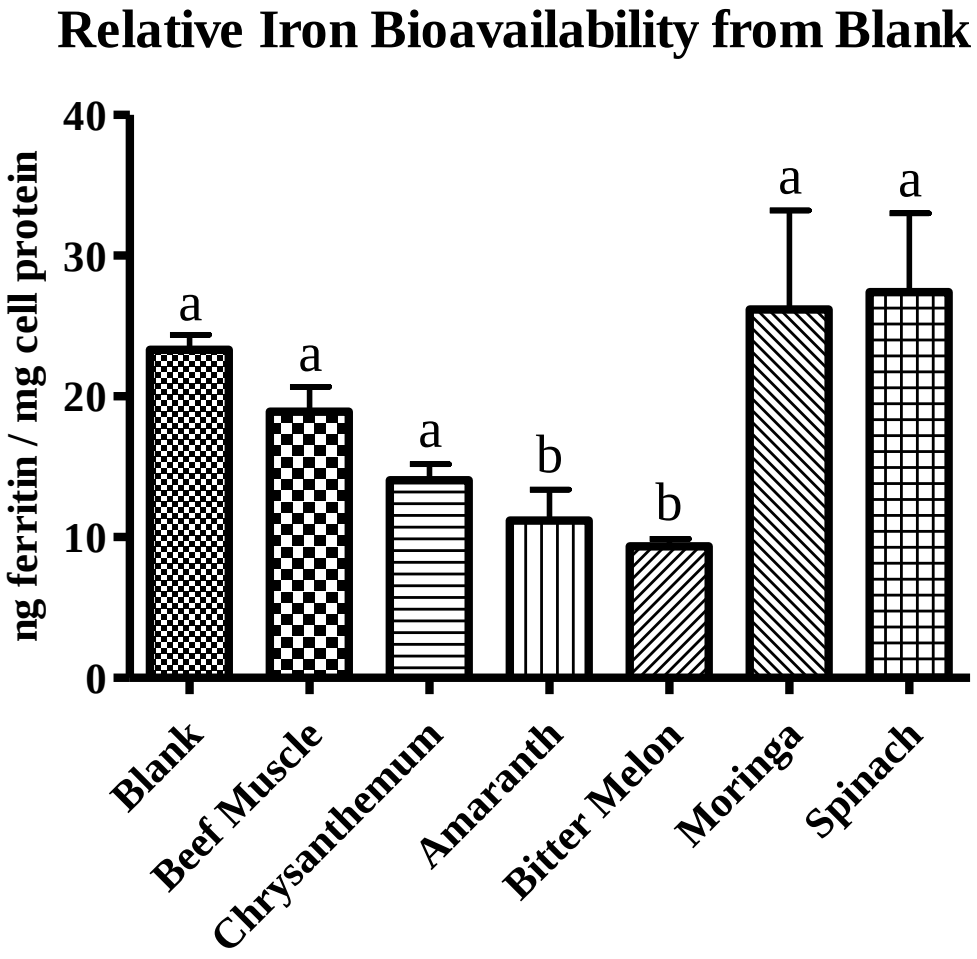

Figure 4. Experiment 1 - Relative Fe bioavailability from cooked green leafy vegetables and beef compared to blank expressed per ug Fe using 67 μM Fe in each digest.

Each digest was brought up to 15 ml and 1.5 ml of digest was placed into each well. The blank samples represent baseline ferritin formation from Fe present in MEM and digest enzymes. Fe uptake in Caco-2 cells was measured by ferritin formation after 18 hr incubation using ELISA. Bioavailable Fe units (ng ferritin / mg cell protein) are per 5.5 μg Fe (actual exposure). Bar values represent means ± SD, n = 4. Data were log transformed prior to analysis to achieve equal variances. Bars with no letters in common are significantly different from blank ($p \leq 0.05$).

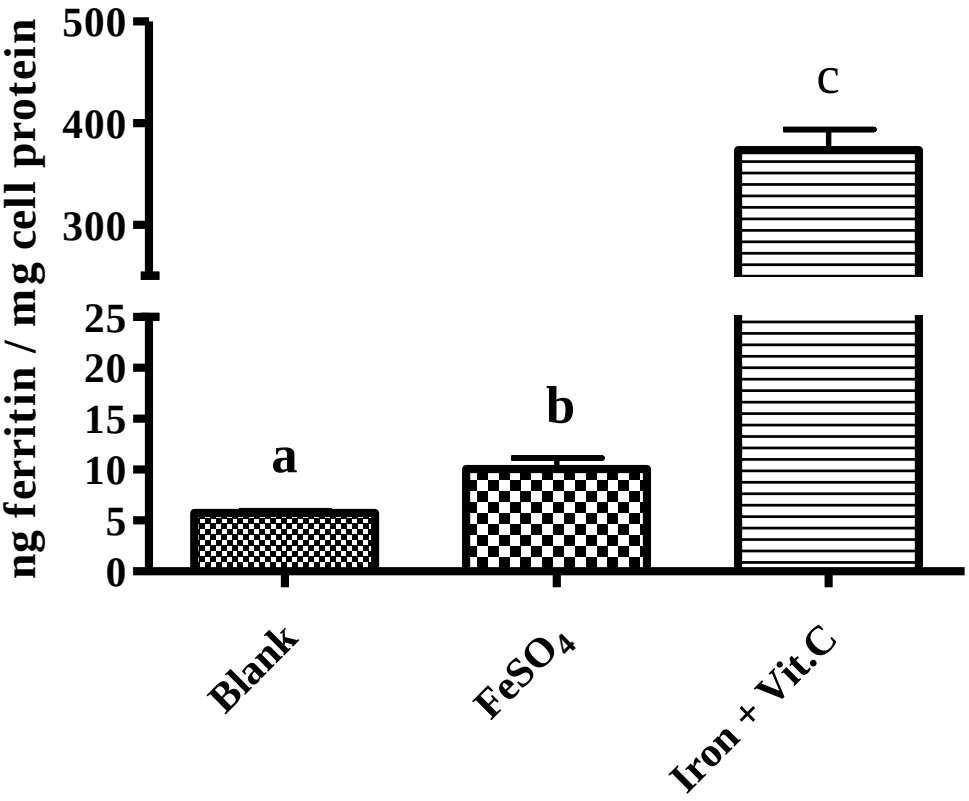

Figure 5. Controls for experiment 2: comparing relative Fe bioavailability of green leafy vegetables and beef using 0.5 g food DM

Each digest was brought up to 15 ml and 1.5 ml of digest was placed into each well. The blank samples represent baseline ferritin formation from Fe present in MEM and digest enzymes. The ferrous sulfate samples represent a digest consisting of 67 μM Fe and the ferrous sulfate and vitamin C samples represent a digest consisting of 67 μM Fe and 1.3 mM vitamin C. Fe uptake in Caco-2 cells was measured by ferritin formation after 18 hr incubation using ELISA. Bar values indicate ± SD, n = 6. Data were log transformed prior to analysis to achieve equal variances. Bars with different letters are significantly different ($p \leq 0.05$).

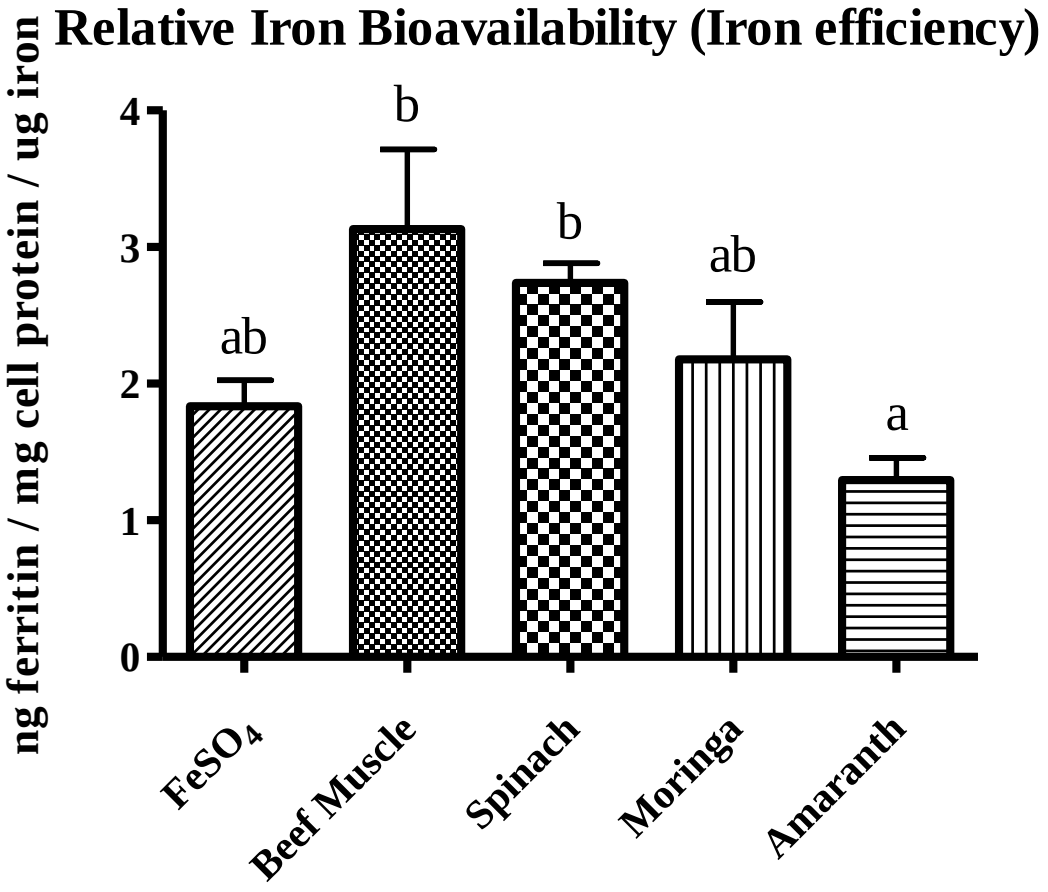

Figure 6. Experiment 2 - Relative Fe bioavailability from cooked green leafy vegetables and beef expressed per ug Fe using 0.5 g DM food in each digest.

Each digest was brought up to 15 ml and 1.5 ml of digest was placed into each well. Fe uptake in Caco-2 cells was measured by ferritin formation after 18 hr incubation using ELISA. Bioavailable Fe units (ng ferritin / mg cell protein) were per 0.05 g (actual exposure) and rexpressed per µg Fe. Bar values represent means ± SD, n = 6. Data were log transformed prior to analysis to achieve equal variances. Bars with no letters in common are significantly different ($p \leq 0.05$).

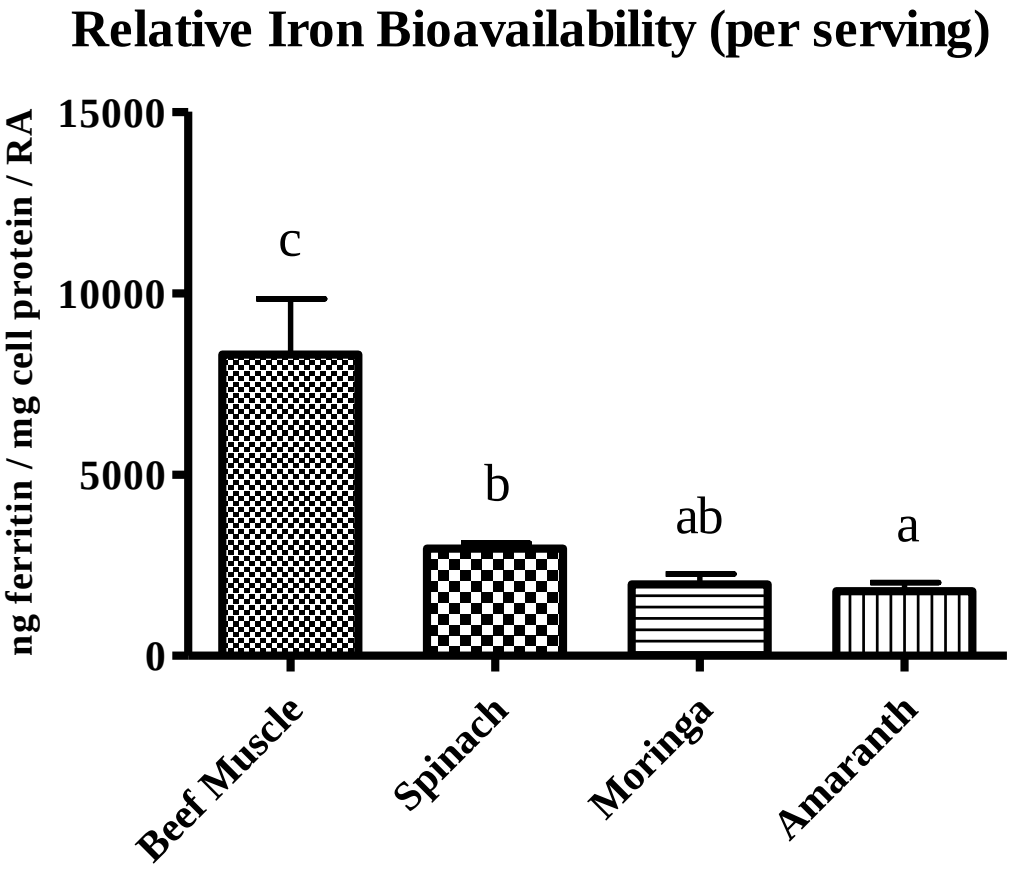

Figure 7. Experiment 2 - Relative Fe bioavailability from cooked green leafy vegetables and beef expressed per serving.

Each digest was brought up to 15 ml and 1.5 ml of digest was placed into each well. Fe uptake in Caco-2 cells was measured by ferritin formation after 18 hr incubation using ELISA. Bioavailable Fe units (ng ferritin / mg cell protein) were per 0.05 grams (actual exposure) and reexpressed per recommended amount by multiplying percent dry matter. A recommended amount consists of 85 grams cooked weight. Bar values represent means ± SD, n = 6. Data were log transformed prior to analysis to achieve equal variances. Bars with no letters in common are significantly different ($p \leq 0.05$).

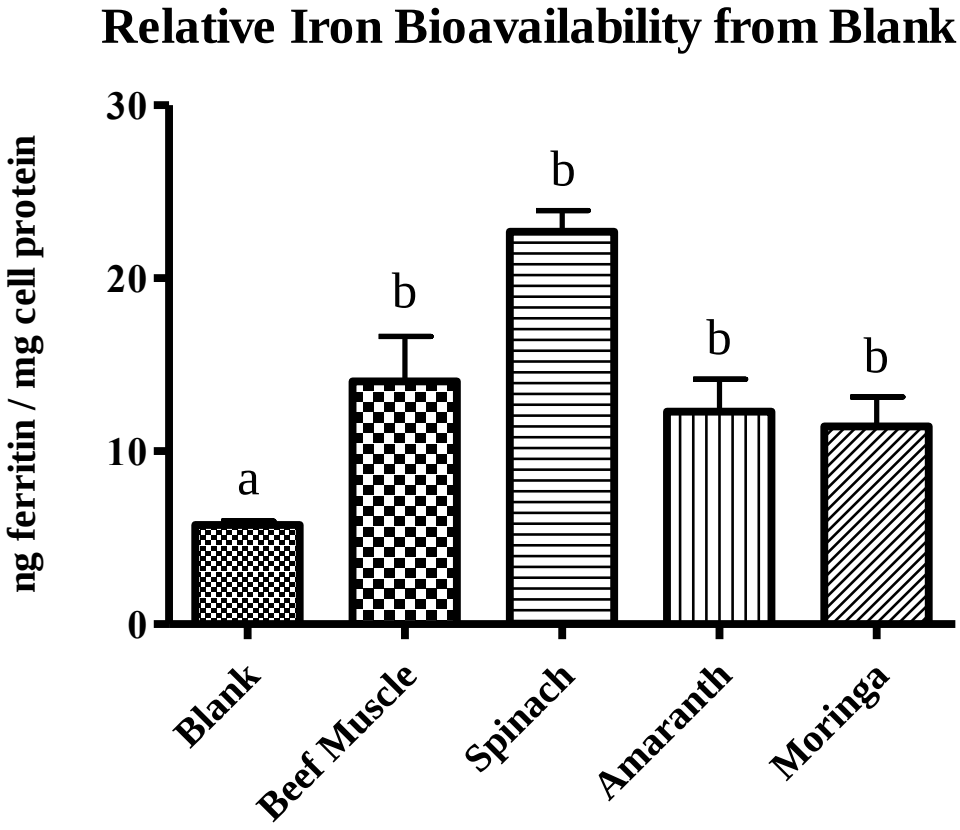

Figure 8. Experiment 2 - Relative Fe bioavailability from cooked green leafy vegetables and beef compared to blank using 0.5 g food DM in each digest.

Each digest was brought up to 15 ml and 1.5 ml of digest was placed into each well. The blank samples represent baseline ferritin formation from Fe present in MEM and digest enzymes. Fe uptake in Caco-2 cells was measured by ferritin formation after 18 hr incubation using ELISA. Bioavailable Fe units (ng ferritin / mg cell protein) were per 0.05 g (actual exposure). Bar values represent means ± SD, n = 6. Data were log transformed prior to analysis to achieve equal variances. Bars with no letters in common are significantly different ($p \leq 0.05$).

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