Kaempferol in Red and Pinto Bean Seed (*Phaseolus vulgaris* L.) Coats Inhibits Iron Bioavailability Using an in Vitro Digestion/Human Caco-2 Cell Model

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Four different colored beans (white, red, pinto, and black beans) were investigated for factors affecting iron bioavailability using an in vitro digestion/human Caco-2 cell model. Iron bioavailability from whole beans, dehulled beans, and their hulls was determined. The results show that white beans contained higher levels of bioavailable iron compared to red, pinto, and black beans. These differences in bioavailable iron were not due to bean-iron and bean-phytate concentrations. Flavonoids in the colored bean hulls were found to be contributing to the low bioavailability of iron in the non-white colored beans. White bean hulls contained no detectable flavonoids but did contain an unknown factor that may promote iron bioavailability. The flavonoids, kaempferol and astragalin (kaempferol-3-*O*-glucoside), were identified in red and pinto bean hulls via HPLC and MS. Some unidentified anthocyanins were also detected in the black bean hulls but not in the other colored bean hulls. Kaempferol, but not astragalin, was shown to inhibit iron bioavailability. Treating in vitro bean digests with 40, 100, 200, 300, 400, 500, and 1000 μM kaempferol significantly inhibited iron bioavailability (e.g., 15.5% at 40 μM and 62.8% at 1000 μM) in a concentration-dependent fashion. Thus, seed coat kaempferol was identified as a potent inhibitory factor affecting iron bioavailability in the red and pinto beans studied. Results comparing the inhibitory effects of kaempferol, quercitrin, and astragalin on iron bioavailability suggest that the 3′,4′-dihydroxy group on the B-ring in flavonoids contributes to the lower iron bioavailability.

KEYWORDS: Iron bioavailability; bean; polyphenols; flavonoids; kaempferol; astragalin; quercitrin; phytate; Caco-2 cell; in vitro digestion; HPLC-MS

INTRODUCTION

Iron deficiency is the most universal nutrient deficiency worldwide with over two billion people currently afflicted (*1, 2*). Iron deficiency results in increased mortality and morbidity rates, decreased labor productivity, and impaired mental development, which reduces the capacity of people to live healthy and productive lives and, thereby, stagnates national development efforts in some developing nations. To significantly reduce iron deficiency among the poor in developing nations, significantly increasing the bioavailable concentrations of iron in staple plant foods through genetic selection for improved iron bioavailability via plant breeding is an agricultural tool that can be used to improve the iron status of the poor. Doing so would be a sustainable food-based approach to the problem (*3−5*).

The common bean (*Phaseolus vulgaris* L.) is an important staple food crop providing important quantities of protein,

calories, minerals, and vitamins. Common beans are widely consumed throughout the world, and people have acquired specific preferences for various combinations of size, shape, and color of bean seeds. In many countries these preferences distinguish the various commercial market classes, which must meet specific consumer expectations and industry standards. Seed coat color and seed size are the two main criteria that identify the numerous market classes recognized worldwide (*6*).

Bean hull (i.e., seed coat) color is determined by the presence and amounts of flavonol glycosides, anthocyanins, and condensed tannins (proanthocyanidins). Various bean genotypes with different hull colors have been studied, and the phenolic compounds responsible for the bean color were isolated and identified (*7, 8*). Polyphenols in bean seed hulls have been reported to have both health-promoting affects (as antioxidants) and antinutritional effects as inhibitors of iron bioavailability. The antioxidant effects of polyphenolic compounds have led to claims that the consumption of foods and beverages rich in polyphenolics is beneficial to human health. Polyphenols can play an important role as antioxidants by inhibiting the formation

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of damaging free radicals that result from the digestion of foods (*9*, *10*). For example, flavonoids obtained commercially and isolated from plant species are known to be effective free radical scavengers (*11*). However, polyphenols also have antinutritional effects interfering with both protein and iron absorption, for example, the condensed tannins in seed hulls. Poor digestibility of cooked colored beans has also been directly related to the tannin content of colored bean hulls (*12*, *13*). Nevertheless, the biochemical bases for the effects of polyphenol consumption on iron bioavailability are not well understood. Moreover, the determination of what factors in beans contribute to low iron bioavailability is needed to find ways to improve the iron nutritional value of this important staple food crop that feeds numerous poor in many developing nations (*14*).

We investigated iron bioavailability in different commercially available colored beans (white, red, pinto, and black). We also determined the amounts of bioavailable iron in dehulled beans and in their hulls. Iron bioavailability was determined using an in vitro digestion Caco-2 human cell bioassay model (*15*, *16*). The following questions were addressed: (1) What are the differences in iron bioavailability between white and non-white beans? (2) Which bean flavonoids affect iron bioavailability? (3) What is the relationship between the concentrations of these flavonoids to the reduction of iron bioavailability in in vitro bean digestates?

MATERIALS AND METHODS

Sample Preparation. Four U.S. common bean samples of white, red, pinto, and black beans (Goya Foods, Inc., Secaucus, NJ) were purchased at a local supermarket. Subsamples of each bean type were dehulled. Dehulling was facilitated by soaking the bean in deionized water overnight at 20 °C followed by manual removal of the seed coat. Samples of whole beans, dehulled beans, and bean hulls were autoclaved (121 °C for 20 min) with sufficient deionized water. After cooling to room temperature, the samples were lyophilized, ground, and stored in an airtight container at room temperature. All samples were analyzed for iron via inductively coupled argon plasma emission spectroscopy (ICP-ES; ICAP model 61E trace analyzer, Thermo Jarrell Ash) and for phytate via a modification of a high-pressure ion chromatography method (see below).

Experimental Design. All four bean types (white, red, pinto, and black) were assayed for iron bioavailability using an in vitro Caco-2 cell model including the whole beans, dehulled beans, and bean hulls. In this model, Caco-2 cell ferritin formation following exposure to an in vitro digested food sample serves as a proxy for food iron bioavailability. In the experiments using whole beans, the samples were also compared with or without the addition of ascorbic acid (AA; 400 μM), a known promoter of iron bioavailability. The polyphenols from in vitro digestates and acid extracts were studied using HPLC or HPLC-MS to identify those compounds in beans that inhibit iron bioavailability. Further studies were performed to examine the effect of increasing concentrations of polyphenols on iron bioavailability. Solutions containing 40−1000 μM concentrations of these polyphenols (dissolved in DMSO; final DMSO concentration was 2.2%) were added to the white bean digests (1 g of sample in 15 mL of digestion buffer).

Cell Cultures. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passage 25−33. Cells were seeded at a density of 50000 cells/cm^2 in collagen-treated six-well plates (six-well cell culture cluster dishes, Costar, Cambridge, MA). The cells were grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) with 10% (v/v) fetal calf serum (GIBCO), 25 mmol/L HEPES, and 1% antibiotic−antimycotic solution (GIBCO). The cells were maintained at 37 °C in an incubator with a 5% CO$_2$/95% air atmosphere at constant humidity, and the medium was changed every 2 days. The cells were used for iron bioavailability experiments at 13-day postseeding (*15*). On the day prior to the in vitro digestion experiment, the DMEM medium was removed and washed with 2 mL of minimal essential medium (MEM,

GIBCO), and then the cells with another 2 mL of MEM were returned to the incubator. Under these conditions, baseline cell ferritin levels averaged 2.1 ng/mg of cell protein. From experience these levels indicate that only trace background levels of iron were present in the growth medium and that the cells were at maximal responsiveness to bioavailable iron in the test samples. A positive control (i.e., 50 μmol/L iron with 500 μmol/L ascorbic acid added to the MEM) was used to verify responsiveness of the Caco-2 cells to bioavailable iron. Three replications of this treatment were included for each experiment.

In Vitro Digestion/Caco-2 Cell Iron Uptake. Details of the in vitro digestion method have been described elsewhere (*15*). Briefly, 1 g of sample was mixed with 10 mL of saline buffer (140 mM NaCl, 5 mM KCl) and then acidified to pH 2 with 0.1 M HCl. Then, the sample was mixed with 0.5 mL of pepsin solution (0.8 g of pepsin in 20 mL of 0.1 M HCl) and incubated on a gently rocking shaker for 1 h at 37 °C. After 1 h of incubation (gastric digestion), the digest was adjusted to pH 5.5−6.0 with 1 M NaHCO$_3$. The intestinal phase of digestion was then initiated with the addition of 2.5 mL of pancreatin−bile solution and adjusted to pH 7. Then, 1.5 mL of each digestate was placed in the upper chamber of a dual chamber system designed to separate the "intestinal digestate" from the Caco-2 cell monolayer. A 15000 Da molecular weight cutoff dialysis membrane attached to a plastic insert ring was used to create the two chambers, thereby allowing food digestion to occur without loss of cells. In this model, soluble iron in the digestate can dialyze across the membrane for potential absorption by the Caco-2 cell monolayer. The intestinal digestate was allowed to incubate at 37 °C for 2 h, and then the insert and digestate were removed. An additional volume of cell culture medium was then added, and the cells were allowed to incubate overnight. The next day, the cells from each well were harvested in 2.00 mL of deionized−distilled water. Cell ferritin formation was used as a measure of cell-iron uptake.

Ferritin and Protein Assays. Caco-2 cell ferritin assays were performed with a one-stage, two-site immunoradiometric assay (FER-IRON II ferritin assay, RAMCO Laboratories, Houston, TX). A 10 μL sample of each harvested cell sample was used for ferritin determination. The ferritin formation was expressed per unit of cell protein (nanograms of ferritin per milligram of cell protein). Caco-2 cell protein was determined using a semimicro adaptation of the Bio-Rad DC protein assay kit (Bio-Rad Laboratories). A 25 μL sample of harvested cells, diluted in an equal volume of 0.5 M NaOH, was used for each protein measurement. The absorbance at 750 nm was determined using a Thermo Spectronic BioMate 3 spectrophotometer.

Bean Hull Polyphenol Extractions. Samples of colored bean hulls (1.0 g) were extracted in 10 mL of acidified methanol (methanol and 1.0 M HCl, 85:15, v/v). The sample solutions were vortexed and shaken for 1.5 h. The extracts were centrifuged at 2500 rpm for 15 min, and the supernatants were prepared for further HPLC analysis.

HPLC and ICP-ES Analyses. Polyphenols were chromatographed using a Waters HPLC system equipped with a 996 photodiode array detector (PDA), a 600S controller, a 626 quaternary gradient pump, a 717 autosampler, and data acquisition software (Millennium^{32}, ver. 4.0). Details of the method were described elsewhere (*17*, *18*). Briefly, chromatographic separations were carried out using a Symmetry C$_{18}$ reverse-phase column (5 μm, 4.6 × 250 mm) at 25 °C. The elution rate was 1.0 mL/min using a tertiary gradient consisting of (A) 50 mM (NH$_4$)H$_2$PO$_4$, pH 2.6; (B) 80:20 (v/v) acetonitrile/50 mM (NH$_4$)H$_2$-PO$_4$, pH 2.6; and (C) 200 mM H$_3$PO$_4$, pH 1.5. The linear gradient used was as follows: 0−4 min, linear gradient from 100 to 92% A (v/v) and from 0 to 8% B (v/v); 4−10 min, linear gradient from 92 to 0% A, from 8 to 14% B, and from 0 to 86% C (v/v); 10−22.5 min, isocratic elution of 0% A, linear gradient from 14 to 16.5% B and from 86 to 83.5% C; 22.5−27.5 min, linear gradient from 16.5 to 25% B and from 83.5 to 75% C; 27.5−50 min, linear gradient from 25 to 80% B and from 86 to 75 to 20% C; 50−55 min, linear gradient from 0 to 100% A, from 80 to 0% B, and from 20 to 0% C; 55−60 min, column wash with 100% A. The UV−vis spectra were recorded simultaneously at 280, 320, 370, and 520 nm. Samples (1.5 mL) of extracts or in vitro digestions were further centrifuged at 13200 rpm for 10 min and then filtered through a 0.45 μm filter. Samples of 50−100 μL were injected onto the column for polyphenol analysis. Iron

Table 1. Concentrations (Dry Weight Basis) of Iron, IP5, IP6 (Phytic Acid), IP5 + IP6, and Bioavailable Iron (As Determined by Cell Ferritin Formation) with and without the Addition of Ascorbic Acid Using an in Vitro Digestion/Caco-2 Cell Model in Four Different Colored Commercial Whole Beans, Dehulled Beans, and Hulls[a]

bean color	iron (μg g^{-1})	ferritin[b] (ng mg^{-1})[c]	IP5 (μmol g^{-1})	IP6 phytate (μmol g^{-1})	IP5 + IP6[c] (μmol g^{-1})	phytate/iron molar ratio[d]
			Whole Bean			
white	58.0 ± 0.3	21.1 ± 1.1	0.1 ± 0.0	16.8 ± 0.2	16.9 ± 0.2	16.2
red	58.2 ± 1.3	1.6 ± 0.1	0.0 ± 0.0	12.2 ± 0.3	12.2 ± 0.3	11.7
pinto	64.1 ± 0.6	1.8 ± 0.3	0.1 ± 0.0	18.1 ± 0.1	18.2 ± 0.1	15.9
black	73.3 ± 1.2	0.9 ± 0.1	0.1 ± 0.0	16.0 ± 2.0	16.1 ± 2.0	12.3
white + AA[e]		103.5 ± 7.0				
red + AA		1.6 ± 0.1				
pinto + AA		1.2 ± 0.4				
black + AA		1.2 ± 0.1				
			Dehulled Bean			
white	50.6 ± 0.3	12.2 ± 0.3	0.0 ± 0.0	14.9 ± 0.5	14.9 ± 0.5	16.4
red	54.4 ± 0.5	17.6 ± 2.2	0.0 ± 0.0	13.2 ± 0.5	13.2 ± 0.5	13.6
pinto	61.5 ± 0.8	13.7 ± 1.3	0.0 ± 0.0	17.4 ± 0.3	17.4 ± 0.4	15.8
black	67.5 ± 0.8	5.5 ± 0.6	0.1 ± 0.0	25.8 ± 0.4	25.9 ± 0.4	21.4
			Hull			
white	80.5 ± 3.9	42.6 ± 1.6	0.1 ± 0.0	3.4 ± 0.1	3.5 ± 0.9	2.4
red	69.3 ± 0.4	1.4 ± 0.1	0.1 ± 0.0	3.9 ± 1.3	4.0 ± 1.3	3.2
pinto	133.9 ± 2.9	1.2 ± 0.2	0.2 ± 0.0	1.8 ± 0.1	2.0 ± 0.1	0.8
black	192.6 ± 1.7	2.3 ± 0.2	0.0 ± 0.0	2.9 ± 0.1	2.9 ± 0.1	0.8

[a] Data are means ± SEM (standard error of mean, $n = 3$ except for ferritin values, where $n = 6$). The cells grown in MEM alone had baseline ferritins of 2.1 ng mg^{-1}. [b] Bioavailable iron (ferritin formation) was determined using a Caco-2 cell model incubated with in vitro digests of bean samples. Data are presented as means ± SEM ($n = 6$). [c] IP5 + IP6 = concentration of *myo*-inositol pentaphosphoric acid plus phytic acid. [d] Data are presented as [IP6 (μmol)]/[(iron (μg)/55.845]. [e] AA = 400 μM ascorbic acid added to in vitro digestates.

concentrations in the samples were determined using an ICP-ES after dissolving 1.5 mL of digestate in 3 mL of a 5% HNO$_3$ solution. All glassware used in the sample preparation and analyses was acid-washed and rinsed with deionized water (18 MΩ resistance).

HPLC-MS. HPLC-MS analysis of red bean extract for the identification of astragalin was carried out using a Waters 2690 HPLC using a reversed phase C18 column (Waters Symmetry; 5 μm, 2.1 × 150 mm) using a binary mobile phase consisting of (A) acetonitrile plus 0.1% formic acid and (B) 0.1% formic acid at a flow rate of 0.25 mL/min. Percent A was ramped in a linear fashion from an initial level of 6% to 20% at 10 min, then to 40% at 40 min, then to 100% at 46 min, and back to 6% at 48 min. The flow was introduced first into a Waters 996 photodiode array detector (PDA) providing UV–visible absorption spectra in the range of 210–550 nm detectors. Flow was then introduced into a Micromass ZMD 4000 spectrometer using the electrospray probe with capillary and cone voltages set at 4.0 kV and 40 V, respectively. Centroided ESI-MS spectra were acquired in positive ion mode using a 1 s scan time and a 0.1 s interscan delay for the mass range 100–500 amu. Spectra for standards were acquired similarly by the ZMD 4000 by direct infusion of solutions in 1:5 A/B (see above) by syringe pump (Harvard Apparatus) at 5 μL/min (needle, 3.6 kV; cone, 25 V; desolvation gas temperature, 150 °C; mass range, 10–1010 amu; scan time, 3.47 s; interscan delay, 0.13 s in continuum mode with averaging of four scans). MS-MS spectra of standard astragalin were also acquired on an API-Sciex Q-trap 2000 in triple-quadrupole mode. Product ion spectra were acquired for the range of 20–500 amu in 1.5 s (declustering potential, 80 V; collision energy, 50 V).

Statistical Analysis. Data were analyzed using the software package GraphPad Prism version 4.02 (GraphPad Software, San Diego, CA) and Statistical Analysis System (SAS) version 9.1 (SAS Institute Inc., Cary, NC). Data for the relative ferritin concentrations of bean samples were normalized by using white bean ferritin formation as 100%. The data were further logarithmically transformed to perform the multiple linear model statistical analyses. Differences between means were determined by Bonferroni post-test if the P value was ≤0.05.

RESULTS

Concentration of Iron in Whole Beans, Bean Hulls, and Dehulled Beans. **Table 1** shows the bean-iron concentrations,

bioavailable iron (as determined by cell ferritin formation), *myo*-inositolpentaphosphoric acid (IP5), phytic acid (IP6), IP5 + IP6, and the molar ratio of phytate to iron in the samples. The concentrations of iron in the whole beans ranged from 58.0 to 73.3 μg g^{-1}. As expected, the iron concentrations in the dehulled beans were slightly lower, 50.6–67.5 μg g^{-1}, compared to the whole bean. The concentrations of iron in the hulls were as follows: red (69.3 μg g^{-1}) < white (80.5 μg g^{-1}) < pinto (133.9 μg g^{-1}) < black (192.6 μg g^{-1}). Overall, iron concentrations were highest in the hulls, and non-white colored beans exhibited higher iron concentrations than the white beans.

Iron Bioavailability from Whole Beans and the Effect of Ascorbic Acid. To compare the bioavailable bean-iron results between various experiments, iron bioavailable values (i.e., ferritin formation) were normalized by expressing the results as a percentage of the bioavailable iron in the whole white bean digests. **Figure 1** shows the bioavailable iron in the in vitro digests of the bean samples tested. Within the whole beans studied, the white beans contained significantly higher bioavailable iron than that of the red, pinto, and black bean samples (>10-fold higher), even though the white beans contained the lowest iron concentrations.

The addition of 400 μM ascorbic acid (a known promoter of iron uptake) significantly enhanced iron bioavailability from whole white bean digests but had no effect on the whole non-white colored bean samples (**Figure 1**). Ferritin levels from these non-white colored bean samples ranged from 1.2 to 1.6 ng of ferritin (mg of protein)$^{-1}$, and ferritin values were below the control baseline ferritin values [baseline control value of 2.1 ng of ferritin (mg of protein)$^{-1}$ for cells not receiving bean digests]. These results show that non-white colored bean hulls contain potent inhibitors of iron bioavailability.

Effect of Bean Color and Dehulling on Iron Bioavailability. Iron bioavailability from in vitro digests of the bean hulls showed a pattern similar to that of digests of whole beans; that is, the hulls of the white beans contained significantly higher
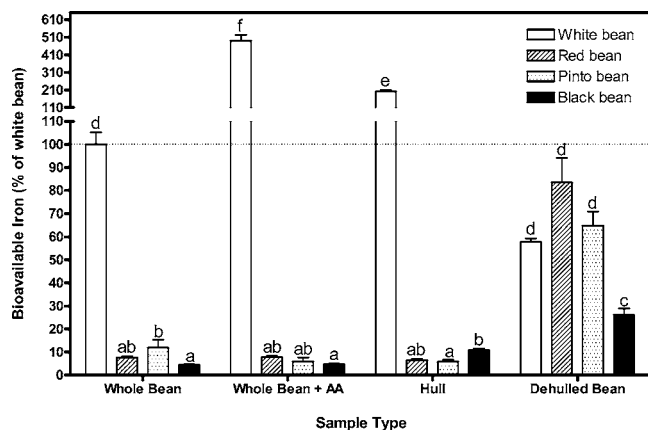
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Figure 1. Comparison of bioavailable iron using an in vitro Caco-2 cell digestion model (cell ferritin formation was used as a proxy for iron absorption) in four different colored commercial bean samples (white, red, pinto, and black). Samples included whole beans, whole beans with ascorbic acid (AA, 400 μM) added to the digests, bean hulls, and beans with hulls removed. All values of cell ferritin formation (ng mg^{-1}) are expressed as percent of whole white bean ferritin formation. Error bars represent means $\pm$ SEM ($n = 6$). Bars with no letter in common are significantly different ($P \leq 0.05$). Dashed line represents 100% for the white bean reference sample.

bioavailable iron compared to the hulls of the red, pinto, and black beans. The white bean hulls contained about twice the level of bioavailable iron as whole white beans. The hulls of the non-white colored beans contained no observable bioavailable iron as indicated by the results showing that the ferritin values for these samples were at or below baseline levels [i.e., ~2.1 ng of ferritin (mg of protein^{-1})]. Removing the non-white colored bean hulls significantly increased bioavailable iron levels. The highest bioavailable iron levels for dehulled beans were observed in cells incubated with digests from dehulled red beans followed by dehulled pinto beans and white beans. Removing the seed coats from black beans also greatly increased the amount of bioavailable iron they contained. Thus, it appears that certain phytochemicals associated with red, pinto, and black hull color are negatively correlated with iron bioavailability in common beans.

Concentrations of Phytate and IP5 in Whole Beans, Dehulled Beans, and Bean Hulls. Table 1 also shows the concentrations of IP5, IP6, the sum of IP5 + IP6, and the molar ratio of phytate to iron in the samples. The bean IP6 concentrations are much higher than bean IP5 concentrations. Both IP5 and IP6 are known to inhibit iron bioavailability from foods. There were no large differences in IP5 + IP6 concentrations among the four different colored beans or between the whole bean and dehulled bean samples studied. The IP6 concentration of bean hulls was very low (2.0−4.0 μmol g^{-1}) compared to whole beans (12.2−18.2 μmol g^{-1}) and dehulled beans (13.2−25.9 μmol g^{-1}). There were no significant relationships between bean-iron concentration and IP6 or IP5 + IP6 concentrations or between iron bioavailability and IP6 or IP5 + IP6 concentrations. Thus, iron bioavailability in the beans tested was not related to IP6 or IP5 + IP6 or to bean-iron concentrations.

Identification of Polyphenols in Bean Hulls. Figure 2 shows the HPLC elution profiles of polyphenols (UV absorbance, 370 nm) in the hulls of white, red, pinto, and black beans. The largest differences in chromatograms for polyphenols were found at wavelength 370 nm, where flavonones, flavones, and flavonols (classified as flavonoids) primarily absorb (*17, 18*). **Figure 2A** shows the results for the methanol/HCl extracts of
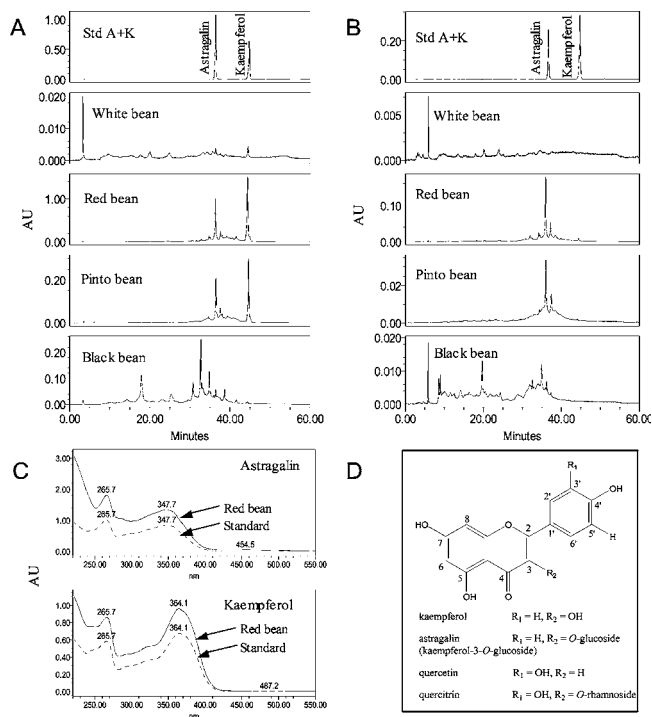

Figure 2. HPLC chromatograms (UV absorbance, 370 nm) and absorptions of flavonols for white and non-white colored bean hulls: (**A**) standard astragalin (kaempferol-3-*O*-glucoside) and kaempferol, and extracts of hulls using methanol/HCl (85:15); (**B**) standard astragalin and kaempferol, and digestates after in vitro digestion; (**C**) standard and red bean absorption spectra of astragalin and kaempferol; (**D**) structures of kaempferol, astragalin, quercetin, and quercitrin. Astragalin (RT = 36.8 min) and kaempferol (RT = 44.6 min) were identified in red and pinto bean hulls. Rutin (RT = 32.7 min) and other minor flavonols, including astragalin, were detected in the black bean hull. No flavonols were detected in the white bean hull extracts or their digestates. Std, standard; A, astragalin; K, kaempferol.

hulls, and **Figure 2B** shows the results for the in vitro digestates. The standards, astragalin (kaempferol-3-*O*-glucoside) and kaempferol, dissolved in the corresponding solvent and digestion buffer, are shown in the top panels. The retention times (RT) for astragalin (RT = 36.8 min) and kaempferol (RT = 44.6 min) are also shown. There were no flavonols detected in the extracts of white bean hulls or in their in vitro digestates. However, in the hulls of red and pinto beans, large peaks of astragalin and kaempferol could be identified in the hull extracts, whereas only astragalin was identified in the in vitro digestates of these samples (notice the different scale in the HPLC profiles). No kaempferol could be detected in black bean hulls, but another major flavonol peak (possibly rutin, RT = 32.7 min) and other minor flavonoids were detected in the black bean hulls. Some unknown anthocyanins were also found in the black bean hull (data not shown).

Figure 2C compares the absorption spectra of astragalin and kaempferol standards and unknown compounds in the red bean hull extracts. The patterns of the absorbance spectra confirm that astragalin and kaempferol were the main compounds in the red bean hull. **Figure 2D** shows the chemical structures of kaempferol, astragalin (kaempferol-3-*O*-glucoside), quercetin, and quercitrin (quercetin-3-*O*-rhamnoside). The numbering convention of these flavonoids is also depicted in the figure. In our present study, kaempferol and astragalin were the most abundant polyphenols identified in the hulls of the red and pinto beans.
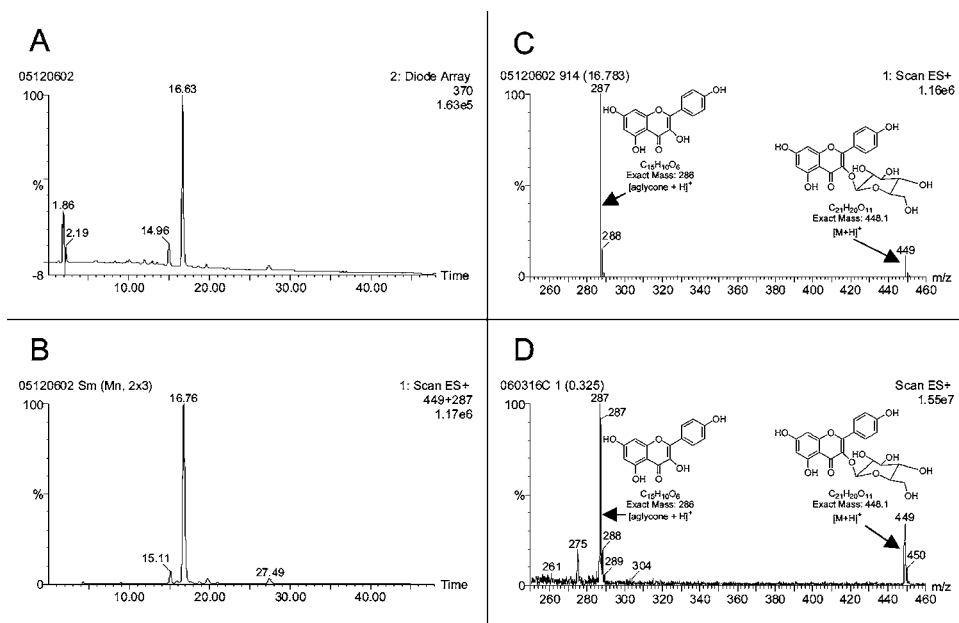
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Figure 3. (**A**) Extracted HPLC-UV chromatogram for 370 nm from diode array scan from 200 to 500 nm. (**B**) Extracted HPLC-MS chromatogram for the sum of 449 [M + H]$^+$ and 287 [aglycone + H]$^+$ ions scanning from 100 to 500 amu of astragalin peak from red bean. (**C**) Mass spectrum from a scan near the center of the peak at 16.76 min in (**B**). (**D**) ESI spectrum from standard astragalin (10 ng/mL solution, dissolved in 20% MeCN with 0.1% formic acid) infused by syringe pump at 5 mL/min.

HPLC-MS Identification of Astraglin from Red Bean Hull. Figure 3 shows the HPLC-ESI/MS results for mass spectra identification of astragalin in red bean hulls. **Figure 3A** shows the HPLC-UV−vis chromatogram of bean extracts detected at 370 nm using a diode array scan from 200 to 500 nm. The peak at the retention time of 16.63 min is astragalin that was identified using the HPLC-ESI/MS method. **Figure 3B** shows the HPLC-MS chromatogram for the sum of 449 [M + H]$^+$ and 287 [aglycone + H]$^+$ ions scanning from 100 to 500 amu for the red bean astragalin peak. **Figure 3C** is the mass spectrum from scanning near the center of the peak at 16.76 min in **Figure 3B**. **Figure 3D** shows the ESI/MS spectrum obtained from standard astragalin (10 ng/mL solution, dissolved in 20% acetonitrile with 0.1% formic acid) infused by syringe pump at 5 mL/min. **Figure 3C** exactly matches **Figure 3D**, confirming that the peak (RT = 16.63 min) in **Figure 3A** is astragalin.

Diagnostic aglycone fragments of 287 and 303 amu from astragalin and quercetrin, respectively, were readily observed in triple-quadrupole MS-MS experiments as products of the pseudomolecular ion [M + H]$^+$ (449 amu). The same fragment ions produced by in-source fragmentation were detected on a single-quadrupole ESI mass spectrometer, which was then used as a detector for LC-MS analysis of red bean extracts. The major chromatographic peak from the red bean extract matched both the UV−vis and mass spectral characteristics of astragalin, but not the isomeric quercetrin, showing the aglycone fragment at 287 amu but not the fragment at 303 amu (**Figure 3**).

Effects of Kaempferol, Astragalin, and Quercitrin on Iron Bioavailability from White Beans. Figure 4A shows the result of white bean in vitro digests treated with 40, 100, 200, 300, 400, 500, and 1000 μM kaempferol concentrations. Clearly, when the concentrations of kaempferol increased, bioavailable iron decreased. The reduction of iron bioavailability was from 15.5 to 62.8% with 40−1000 μM keampferol treatment, respectively. **Figure 4B** shows the result of white bean in vitro digests treated with 350, 500, and 700 μM astragalin concentrations. Astragalin did not inhibit iron bioavailability. Quercitrin

was shown to have greater effects on iron bioavailability compared to kaempferol (see **Figure 4A**,**C**). **Figure 4D** shows the effects of different concentrations of kaempferol on total soluble iron (micrograms per gram) in white bean in vitro digestates. When the concentrations of kaempferol increased in the digestates, the soluble iron in the digestates decreased. The inset of **Figure 4D** shows the linear correlation between the bioavailable iron and the concentration of soluble iron in the white bean digests; the correlation coefficient squared (R^2) was calculated to be 0.82.

DISCUSSION

Effects of Bean Color and Seed Parts on Iron Bioavailability. Comparison of iron bioavailabilities of the white, red, pinto, and black beans clearly shows that the bioavailable iron in white beans (whole, whole with ascorbate added, or bean hull alone) is significantly higher compared to those of the non-white colored bean samples. The hulls of white bean contained significantly more bioavailable iron than those of the whole white bean (>2-fold), whereas the whole non-white colored beans and their hulls contain very little if any bioavailable iron. These results agreed with those reported for the bioavailable iron in white soybean (*Glycine max* L.) hulls that were shown to contain very highly bioavailable iron to humans and to rats (*19, 20*). Potentially, both white beans and soybeans contain similar forms of highly bioavailable iron. Interestingly, removal of the hulls from non-white colored beans improved iron bioavailability, that is, increasing iron bioavailability by 75.9, 56, and 21.5% for red, pinto, and black beans, respectively (see **Figure 1**). These differences in bioavailable iron in the non-white colored beans occurred with only 6.5, 4.1, and 7.9% decreases in iron concentrations resulting from removal of the non-white colored bean hulls (see **Table 1**). These results demonstrate that non-white colored beans contain iron absorption inhibitors in their hulls that cannot be overcome by the addition of the iron bioavailability promoter, ascorbic acid. However, removal of the hulls from the white bean samples decreased iron bioavailability by 42.1% (see **Figure 1**). This
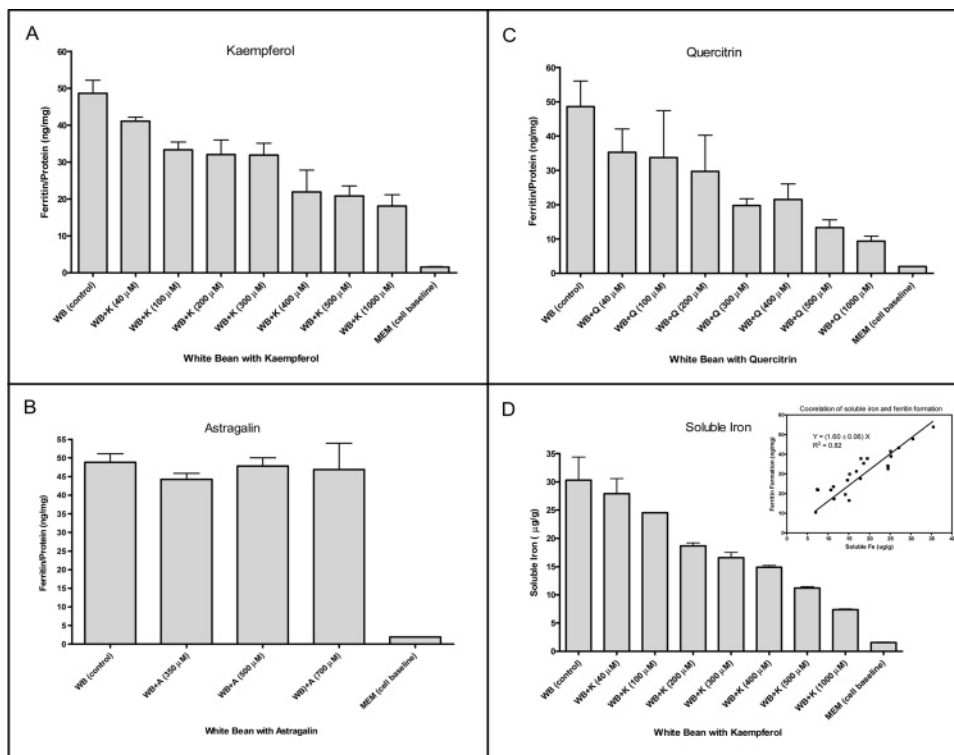
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Figure 4. Iron bioavailability from in vitro white bean digests treated with different concentrations of flavonoids: (**A**) kaempferol; (**B**) astragalin; (**C**) quercitrin. (**D**) Effects of increasing concentrations of kaempferol on soluble iron concentrations (μg g^{-1}) in white bean in vitro digestates. (Inset) Linear relationship between bioavailable iron and the concentration of soluble iron in the white bean digests. The calculated linear correlation coefficient squared (R^2) = 0.82.

decrease occurred with only a 12.8% decrease in iron concentration in the samples resulting from removal of the white bean hulls. These results suggest the presence of an iron bioavailability promoter in the white bean seed hull because significantly more iron was bioavailable from the white bean hull than from the dehulled white bean.

The differences in iron bioavailability found between the different beans and bean parts tested (whole, dehulled, and hull) were not explained by their phytate or iron concentrations, because the corresponding molar ratios of phytate/iron were in the ranges of 11.7−16.2, 13.6−21.4, and 0.8−3.2, respectively (see **Table 1**). These differences in phytate/iron ratios should have affected iron bioavailability according to published literature (*21−23*). Apparently, other factors in the bean seeds are controlling iron bioavailability. In our experiments the polyphenolic compounds associated with the seed coats appear to be the major factors affecting iron bioavailability from non-white colored beans.

Iron Bioavailability Assessment. The in vitro digestion/Caco-2 cell model method was used to assess the effects of seed coat polyphenols on iron bioavailability. Glahn et al. used the in vitro digestion/Caco-2 cell model and reported that a molar ratio 1:0.1 of iron/tannic acid resulted in a 92% reduction in iron uptake (*24*). Boato et al. studied iron bioavailability in several fruit juices using the Caco-2 cell culture model. They reported 31 and 67% decreases in ferritin formation from prune juice and red grape juice, respectively, because of the high levels of polyphenols in these juices. The remaining juices studied contained lower amounts of polyphenols and did not inhibit iron bioavailability (*25*).

Interestingly, some of the non-white colored bean samples decreased bioavailable iron levels in the digestates to below the baseline level in cells not treated with bean digests. The baseline levels in the cells are presumably due to the trace amounts of contaminant iron present in the cell culture media. Therefore, decreasing ferritin levels below the baseline value indicates that potent inhibitors of iron bioavailability were present in the digest, rendering any iron present in the cell culture media or digest unavailable for absorption. This large inhibition of iron absorption is typically reported for foods containing high levels of polyphenols. Our results suggest that non-white colored beans contain very low levels of bioavailable iron for humans and that the inhibitors they contain may also negatively affect iron bioavailability from other foods in the diet.

Isolation and Identification of Polyphenolic Compounds. The acidified methanol (85% methanol containing 15% 1 M HCl) was found to be most efficient for extracting total polyphenols from foods (*26*). Astragalin (RT = 36.8 min, λ_{max} = 347.7 nm) and kaempferol (RT = 44.6 min, λ_{max} = 364.1 nm) were identified in samples via HPLC by comparing the retention time and absorbance of pure standards (see **Figure 2**). Cochromatography of the flavonoids of red beans and standard samples showed that they have the same retention times (data not shown). Astragalin was reported by Beninger et al. to be in the seed coat of Manteca-type dry bean (*8*). The detected anthocyanins we observed in extracts of black bean hulls may be delphinidin-3-glucoside, petunidin-3-glucoside, or malvidin-3-glucoside, as reported by others in a previous study of the anthocyanins in black or purple bean seed coats from *P. vulgaris* (*7*).

Polyphenol Effect on Iron Bioavailability. Various subclasses of polyphenols impart color to bean hulls. In previous studies, polyphenol extracts from non-white colored bean hulls were shown to inhibit iron absorption in rats (*27*). Chang and Wong reported that in the black bean cultivar, Hakmeitau, the polyphenols (anthocyanins and flavonol glycosides) in the hull were about 6 times higher than that in the whole seed (*28*).

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These previous studies suggested that the iron bioavailability inhibitors in beans might be related to seed color, that is, the seed coat polyphenols. In another study certain white bean genotypes, G18372 and G18811 (selected by color to contain very low total tannin concentration), were reported to contain relatively high levels of bioavailable iron using a rat model (*29*). Additionally, Choung et al. studied 16 kidney beans cultivated in Korea having different seed coat colors and reported that the white bean seeds (cultivars KG96013, KG97128, and KG97621) contained no anthocyanins, which is in agreement with our results reported here (*30*). Our present results are also in agreement with the results of another study using various colored Kenyan beans; that is, the white bean varieties had much higher bioavailable iron compared to the non-white colored beans studied, such as brown, red, pinto, light brown, and black (*31*).

To further examine the inhibitory effect of polyphenols on iron bioavailability, we added kaempferol, astragalin, and quercitrin to white beans in vitro digests to examine their effect on iron bioavailability. The results clearly indicate that kaempferol and quercitrin are strong inhibitors of iron bioavailability from the white beans, but the astragalin did not inhibit iron bioavailability. The reason for the inhibitory effects of kaempferol and quercitrin could be attributed to their chemical structures. The inhibitory effect of polyphenols is probably due to the formation of insoluble complexes between polyphenols and ferric iron. In a typical flavonoid, such as quercetin (**Figure 2D**), there are three possible metal-complexing domains that can bind with metal ions, that is, the 3′,4′-dihydroxy group located on the B-ring and the 3- or the 5-hydroxy and 4-carbonyl group in the C-ring. Kaempfrol, with a 3- or a 5-hydroxy and 4-carbonyl group in the C-ring, could bind ferric ions at these sites and decrease iron bioavailability. Quercitrin, with a 3′,4′-dihydroxy group on the B-ring and a 5-hydroxy and 4-carbonyl group in the C-ring, could also bind ferric ions at these sites and thereby decrease iron bioavailability. Our Caco-2 cell in vitro digestion results show that quercitrin was a stronger inhibitor of iron bioavailability compared to kaempferol (**Figure 3A,C**). These findings suggest that the 3′,4′-dihydroxy group on the B-ring forms more stable ferric iron bonds compared to the 3-hydroxy and 4-carbonyl ligand group in the C-ring. The results are in agreement with a previous study of the complexation of metal ions by condensed tannins (*32*). The lack of an inhibitory effect of astragalin on iron bioavailability suggests that the 5-hydroxy and 4-carbonyl group in the C-ring of astragalin does not tightly bind ferric iron.

The different position of the hydroxy group in flavonoids was shown to affect the ability of polyphenols to reduce iron bioavailability. More research should be conducted to determine the binding properties of these ligand groups with ferric iron at pH values that occur in vivo in the digestive tract. This type of information could be used to develop mathematical models that would allow predictions of the effects of various polyphenols on iron bioavailability from plant foods. Such models would aid plant breeders interested in increasing the bioavailable levels of iron in staple food crops through changing the amount and type of polyphenols accumulated in edible seeds and seed products.

LITERATURE CITED

(1) United Nations Administrative Committee on Coordination, Subcommittee on Nutrition. In *Second Report on the World Nutrition Situation, Global and Regional Results*; World Health Organization: Geneva, Switzerland, 1992.

(2) World Bank. The challenge of dietary deficiencies of vitamins and minerals. In *Enriching Lives: Overcoming Vitamin and Mineral Malnutrition in Developing Countries*; World Bank: Washington, DC, 1994; Chapter 1, pp 6−13.

(3) Graham, R. D.; Welch, R. M. *Breeding for Staple-Food Crops with High Micronutrient Density*; Agricultural Strategies for Micronutrients, Working Paper 3; International Food Policy Research Institute: Washington, DC, 1996.

(4) Welch, R. M.; Graham, R. D. A new paradigm for world agriculture: meeting human needs. Productive, sustainable, nutritious. *Food Crops Res.* **1999**, *60*, 1−10.

(5) Graham, R. D.; Senadhira, D.; Beebe, S.; Iglesias, C.; Monasterio, I. Breeding for micronutrient density in edible portions of staple food crops: conventional approaches. *Field Crops Res.* **1999**, *60*, 57−80.

(6) Adams, M. W.; Bedford, C. L. Nutritional improvement of food legumes by breeding. In *Breeding Food Legumes for Improved Processing and Consumer Acceptance Properties*; symposium proceedings sponsored by the Protein Advisory Group, Rome, Italy, July 1972; United Nations: New York, 1972; pp 299−309.

(7) Takeoka, G. R.; Dao, L. T.; Full, G. H.; Wong, R. Y.; Harden, L. E.; Edwards, R. H.; Berrios, J. J. Characterization of black bean (*Phaseolus vulgaris*) anthocyanins. *J. Agric. Food Chem.* **1997**, *45*, 3395−3400.

(8) Beninger, C. W.; Hosfield, G. L.; Nair, M. G. Flavonol glycosides from the seed coat of a new Manteca type dry bean (*Phaseolus vulgaris* L.). *J. Agric. Food Chem.* **1998**, *46*, 2906−2910.

(9) Namiki, M. Antioxidants/antimutagens in food. *Food Sci. Nutr.* **1990**, *29*, 273−300.

(10) Beninger, C. W.; Hosfield, G. L. Antioxidant activity of extracts, condensed tannin fractions, and pure flavonoids from *Phaseolus vulgaris* L. seed coat color genotypes. *J. Agric. Food Chem.* **2003**, *51*, 7879−7883.

(11) Riedl, K. M.; Hagerman, A. E. Tannin−protein complexes as radical scavengers and radical sinks. *J. Agric. Food Chem.* **2001**, *49*, 4917−4923.

(12) Elias, I. G.; deFernandez, D. G.; Bresanni, R. Possible effects of seed coat polyphenolics on the nutritional quality of bean protein. *J. Food Sci.* **1979**, *44*, 524−52.

(13) Aw, T.; Swanson, B. G. Influence of tannin on *Phaseolus vulgaris* protein digestibility and quality. *J. Food Sci.* **1985**, *50*, 67−71.

(14) Welch, R. M.; Graham, R. D. Breeding for micronutrients in staple food crops from a human nutrition perspective. *J. Exp. Bot.* **2004**, *55*, 353−364.

(15) Glahn, R. P.; Lee, O. A.; Yeung, A.; Goldman, M. I.; Miller, D. D. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an *in vitro* digestion Caco-2 cell culture model. *J. Nutr.* **1998**, *128*, 1555−1561.

(16) Yun, S. M.; Habicht, J. P.; Miller, D. D.; Glahn, R. P. An in vitro digestion/Caco-2 cell culture system accurately predicts the effects of ascorbic acid and polyphenolic compounds on iron bioavailability in humans. *J. Nutr.* **2004**, *134*, 2717−2721.

(17) Kim, D.-O.; Lee, C. Y. HPLC separation of polyphenolics. In *Current Protocols in Food Analytical Chemistry*; Wiley: New York, 2002; pp I1.3.1−I1.3.16.

(18) Lamuela-Raventos, R. M.; Waterhouse, A. L. A direct HPLC separation of wine phenolics. *Am. J. Enol. Vitic.* **1994**, *45*, 1−5.

(19) Johnson, C. D.; Berry, M. F.; Weaver, C. M. Soybean hulls as an iron source for bread enrichment. *J. Food Sci.* **1985**, *50*, 1275−1277.

(20) Lykken, G. I.; Hunt, J. R.; Nielsen, E. J.; Dintzis, F. R. Availability of soybean hull iron fed to humans in a mixed, western meal. *J. Food Sci.* **1987**, *52*, 1545−1548.

Kaempferol Inhibition of Iron Bioavailability

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(21) Hurrell, R. F. Influence of vegetable protein sources on trace element and mineral bioavailability. *J. Nutr.* **2003**, *133*, 2973S−2977S.

(22) Sandberg, A. S. Bioavailability of minerals in legumes. *Br. J. Nutr.* **2002**, *88* (Suppl. 3), 281−285.

(23) Allen, L. H.; Ahluwalia, N. Improving iron status through diet. The application of knowledge concerning dietary iron bioavailability in human populations. *OMNI Opportunities for Micronutrients Interventions*; John Snow/OMNI Project: Washington, DC, 1997.

(24) Glahn, R. P.; Wortley, G. M.; South, P. K.; Miller, D. D. Inhibition of iron uptake by phytic acid, tannic acid, and ZnCl$_2$: studies using an *in vitro* digestion/Caco-2 cell model. *J. Agric. Food Chem.* **2002**, *50*, 390−395.

(25) Boato, F.; Wortley, G. M.; Liu, R. H.; Glahn, R. P. Red grape juice inhibits iron availability: Application of an in vitro digestion/Caco-2 cell model. *J. Agric. Food Chem.* **2002**, *50*, 6935−6938.

(26) Ju, Z. Y.; Howard, L. R. Effects of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin. *J. Agric. Food Chem.* **2003**, *51*, 5207−5213.

(27) House, W. A.; Campen, D. R. V. Iron absorption by rats fed tannins extracted from bean hulls. *Nutr. Res.* **1994**, *14*, 1043−1053.

(28) Chang, Q.; Wong, Y.-S. Identification of flavonoids in Hakmeitau beans (*Vigna sinensis*) by high-performance liquid chromatography−electrospray mass spectrometry (LC-ESI/MS). *J. Agric. Food Chem.* **2004**, *52*, 6694−6699.

(29) Welch, R. M.; House, W. A.; Beebe, S.; Cheng, Z. Genetic selection for enhanced bioavailable levels of iron in bean (*Phaseolus vulgaris* L.) seeds. *J. Agric. Food Chem.* **2000**, *48*, 3576−3580.

(30) Choung, M.-G.; Choi, B.-R.; An, Y.-N.; Chu, Y.-H.; Cho, Y.-S. Anthocyanin profile of Korean cultivated kidney bean (*Phaseolus vulgaris* L.). *J. Agric. Food Chem.* **2003**, *51*, 7040−7043.

(31) Hu, Y.; Cheng, Z.; Magnolia, A.-N.; Glahn, R. P.; Welch, R. M. Iron bioavailability of Kenyan bean. Unpublished results.

(32) Slabbert, N. Complexation of condensed tannins with metal ions. In *Plant Polyphenols*; Plenum Press: New York, 1991; pp 421−445.