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Bioaccessibility of Phenols in Common Beans (*Phaseolus vulgaris* L.) and Iron (Fe) Availability to Caco-2 Cells

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Samples of common and biofortified beans (*Phaseolus vulgaris*), both raw and cooked (autoclaved at 120 °C for 20 min) were analyzed for their polyphenol composition. Polyphenols were identified via HPLC-UV/diode array detection. Cooking favored the extraction of polyphenols without the need of a hydrolysis step, a fact that is of interest because this is the usual form in which beans are consumed. The main differences between white and colored beans were the presence of free kaempferol (13.5−29.9 μg g^{-1}) and derivatives (kaempferol-3-*O*-glucoside) (12.5−167.5 μg g^{-1}), only in red and black beans. An in vitro digestion (pepsin, pH2; pancreatin−bile extract, pH 7) was applied to beans to estimate bioaccessibility of individual polyphenols. Kaempferol from seed coats exhibited high bioaccessibility (45.4−62.1%) and a potent inhibitor effect on Fe uptake at concentrations ranging from 0.37 to 1.30 μM. Caco-2 cell ferritin formation was used to evaluate Fe uptake. Cell Fe uptake was significant only from white beans.

KEYWORDS: Polyphenols; bioaccessibility; iron bioavailability; Caco-2

INTRODUCTION

Legumes constitute the main source of proteins and are a good source of different mineral elements such as iron (Fe) for large groups of populations in many Latin American and African countries. However, Fe bioavailability from beans (*Phaseolus vulgaris* L.) is low (1−2%) to humans (*1, 2*). Common beans are a source of phytochemicals such as phytic acid and polyphenols (*3, 4*), which are known inhibitors of Fe absorption (*4*). From our previous in vitro studies it was concluded that polyphenols had a greater inhibitory effect than phytate on Caco-2 Fe bioavailability (*5*). It is important to mention that although differences in the bean seed color have been attributed to the presence and quantity of polyphenols, Fe bioavailability to humans was similar from white and colored beans (*2*). Strategies for combating Fe deficiency include supplementation, dietary diversification, and food fortification. In addition, plant breeding programs have been shown to successfully increase Fe content (biofortification) in beans and other staple crops (*6, 7*). Presently, breeders at Centro Internacional de Agricultura Tropical (CIAT, Colombia) have developed biofortified bean genotypes with Fe content up to 100 μg g^{-1} of bean. Thus, consumption of biofortified beans having improved nutritional value with significantly higher amounts of Fe than conventional beans might contribute to the alleviation of Fe deficiency (*6, 7*). However, a previous human study reported no significant differences between Fe absorption from a typical common bean-based diet (50.4 μg of Fe g^{-1} of bean) and a high Fe bean-based diet (82.9 μg of Fe g^{-1} of bean) (*1*).

Presently, it is still unclear how polyphenols affect Fe bioavailability from beans, and further research is needed. Among the polyphenols found in beans, inhibition of Fe uptake to Caco-2 cells has been related mainly to the presence of kaempferol (*8*). Any evaluation of the potential inhibitory effect of polyphenols on Fe absorption should consider not only the content in bean seeds but also their bioavailability from foods prepared in the way that they are consumed. Bioavailability depends on, among others, the solubility of food-derived components in the gut. Presently, there are scarce and only in vitro data in this respect (*9, 10*). The latter studies indicate that polyphenols are poorly bioavailable (*9*) and/or unstable during gastrointestinal conditions (*10*).

Studies of Fe bioavailability in humans are very expensive, and this factor alone hampers the alleviation of Fe deficiency. To accelerate knowledge of Fe bioavailability, in vitro models that combine simulated gastrointestinal digestion of foods with culture of a human intestinal epithelial cell line (Caco-2) constitute a valuable tool to understand the mechanism(s) or process(es) in the gut that can occur in in vivo situation. The Caco-2 cell model has demonstrated strong correlations with human studies regarding the direction of response of different food-derived components (i.e., enhancers and inhibitors) affecting Fe absorption at the intestinal level (*11*). This model has been shown to be highly sensitive to variation on soluble

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Fe concentrations (*12, 13*). The in vitro models offer a good approach to evaluate relative indices for bioavailability, making comparisons to determine effects caused by different factors.

Therefore, the aims of this study were (1) to quantify polyphenols in different commercial white and colored commercial and biofortified beans (*P. vulgaris* L.), (2) to estimate their bioaccessibility (maximum solubility in simulated gastrointestinal media) from beans to better understand their effects on Fe uptake, and (3) to evaluate Fe bioavailability to Caco-2 cells.

MATERIALS AND METHODS

Reagents. Unless otherwise stated, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO). All glassware used in the sample preparation and analyses was treated with 10% (v/v) of HCl concentrated (37%) for 24 h and then rinsed with 18 MΩ cm^{-1} deionized water before use.

Instruments. Total Fe content was determined with an inductively coupled argon plasma emission spectrometer (ICP-ES, model 61E Trace Analyzer, Thermo Jarrell Ash Corp., Franklin, MA). Other equipment used included a spectrophotometer (DU 520 UV−vis, Beckman Coulter, BC) and an automatic gamma counter Wizard 3 Wallac 1480 (Perkin-Elmer).

Sample Preparation. Commercial samples of white (great northern) and red (kidney) beans (*P. vulgaris* L.) were obtained from local supermarkets in Ithaca (NY) in the form of a raw product and were kept at room temperature. Two different lines of red (CAL96 and NUA35) and black (DOR500 and MIB465) beans (*P. vulgaris* L.) were provided by CIAT (Cali, Colombia). All bean samples were cooked in an autoclave (at 120 °C for 20 min) with deionized water (25 g of beans in 100 mL of water). After cooling to room temperature, the samples were frozen and lyophilized, finely ground, and stored in an airtight container at 4 °C until use. All samples were analyzed for Fe content via ICP-ES.

Total Iron Determination. Aliquots (0.3 g) of cooked beans were acid digested in 1.0 mL of HNO_3 with 1.5 mL of $HClO_4$ at 120 °C for 1 h and then at 220 °C until $HClO_4$ fumes were observed. The samples were diluted with 5% HNO_3 to 6 mL. The instrument was calibrated with 10% $HClO_4$ as the low standard and 1 μg g^{-1} Fe in a multielement standard as the high standard. The Fe was determined using the 238.2 nm line.

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 (Costar, Cambridge, MA) and maintained with Dulbecco's modified Eagle's medium (DMEM) under conditions previously described (*12*). The cells were used for Fe bioavailability experiments at 13 days postseeding. On the day prior to the in vitro digestion experiment, the DMEM medium was removed and cell cultures were washed with 2 mL of minimal essential medium (MEM, Gibco). Then, 2 mL of MEM was added to cell cultures, and the latter was returned to the incubator.

In Vitro Digestion. Porcine pepsin (P-7000) (800−2500 units/mg of protein), pancreatin (P1750) (activity, 4× USP specifications), and bile extract (B8631) (glycine and taurine conjugates of hyodeoxycholic and other bile salts) were demineralized with Chelex-100 (Bio-Rad Laboratories, Hercules, CA) before use.

Samples (0.3 g) of cooked white and red beans were subjected to in vitro digestion, as previously described (*12*). Briefly, the samples were mixed with 10 mL of isotonic saline solution (140 mM NaCl, 5 mM KCl) and then acidified to pH 2 with 0.1 M HCl. After gastric step (pepsin at pH 2, 1 h, 37 °C), 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 a pancreatin−bile solution (pH 7). Then, 1.5 mL of each digest was placed in the upper chamber of a bicameral chamber system designed to separate the "intestinal digest" from the Caco-2 cell monolayer with a 15000 Da molecular weight cutoff dialysis membrane. The intestinal digest was incubated at 37 °C for 2 h (gastrointestinal digestion), and then the insert and digest were removed.

Table 1. Iron (Fe) Concentrations Quantified (ICP-ES) in Commercial (White, Red, Black) and Biofortified Beans (*Phaseolus vulgaris* L)

beans		
genotype	color	Fe[a] (μg g^{-1} of bean, dw)
commercial	white	67.9 ± 0.6a
commercial	red	87.4 ± 1.2d
CAL96		75.3 ± 0.2c
NUA35		78.5 ± 1.4c
commercial	black	81.9 ± 3.3cd
DOR500		71.4 ± 1.4b
MIB465		106.9 ± 1.8e

[a] Different letters indicate significant ($p < 0.05$) statistical differences.

An additional 1 mL of MEM was then added, and the cells were incubated for 22 h. The next day, the cells from each well were washed twice with the isotonic saline solution and harvested in 2 mL of deionized−distilled water. Cell ferritin formation was used as a measure of cell iron uptake.

Ferritin and Total Protein Assays. Caco-2 cell ferritin assays were performed with a one-stage, two-site immunoradiometric assay (FERIRON II ferritin assay, RAMCO Laboratories, Houston, TX). A 10 μL sample of each harvested cell sample was used for ferritin determination. Baseline cell ferritin levels in cultures grown in MEM averaged 2.1 ng mg^{-1} of cell protein. The ferritin formation was expressed per unit of cell protein (ng $mg^{-1}$of protein). Caco-2 cell protein was determined using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories). The experiments were conducted in triplicate on two different days.

Extraction and Determination of Phenolic Acids and Flavonoids. Samples of cooked whole beans (1.0 g) were extracted in 5 mL of acidified methanol (methanol in 1.0 M HCl, 85:15, v/v), acidified water (water in 1.0 M HCl, 85:15, v/v), or aqueous methanol (methanol in deionized water, 85:15, v/v). Aliquots (5 mL) of bioaccessible fractions were centrifuged (3214*g*, 4 °C, 10 min). Then the supernatants were placed in new tubes and lyophilized. All of the samples were shaken and vortexed for 1 h. Afterward, samples were centrifuged (3214*g*, 4 °C, 10 min) to separate the supernatants, and those were filtered through 0.45 μm (MillexGN, Millipore). Then, 75 μL of the filtrates was injected into the HPLC system.

Phenols were analyzed according to a previously described method (*3*) with slight modifications. The analysis was carried out on a HPLC system (Waters, Milford, MA) consisting of a 600E multisolvent pump, a 717 Plus autosampler, and a 996 photodiode array detector, operated using Empower software. The separation was performed on a Vydac 5u 300A C18 column (Phenomenex), 250 × 4.6 mm. The gradient program started with 100% of solvent A (deionized water adjusted to pH 2.8 with glacial acetic acid), and solvent B (70:30 v/v, water/acetronitrile; HPLC grade) was increased linearly to reach 10% in 2.5 min. From 2.5 to 6 min the flow was increased linearly to 12% of solvent B. From 6 to 16 min the flow was increased linearly to 23% of solvent B. From 16 to 22 min the flow was increased linearly to 35% of solvent B. From 22 to 24 min the flow was increased linearly to 95% of solvent B. From 24 to 30 min the flow rate was maintained constant at 95% of solvent B. The column was equilibrated using the initial conditions during 10 min. UV absorbance was recorded at 282 nm.

Statistical Analysis. A one-factor analysis of variance (ANOVA) and the Tukey test (*14*) were applied to determine statistical differences in the mineral native and dialyzable contents, polyphenols in analyzed samples, bioaccessible phenols contents, and ferritin concentrations between exposed and control cultures. A significance level of $p < 0.05$ was adopted for all comparisons. Statgraphics Plus version 5.0 (Rockville, MD) was used for the statistical analysis.

RESULTS AND DISCUSSION

The concentrations of Fe (μg g^{-1} of beans, dry basis, dw) in the analyzed cooked beans are shown in **Table 1**. Iron

Table 2. Concentration of Phenolic Acids and Flavonoids in Cooked White and Commercial Red Beans by Using Different Extractants[a]

	white beans (μg g^{-1} of sample, dry basis)			red beans (μg g^{-1} of sample, dry basis)		
	MeOH/H_2O	MeOH/HCl	water/HCl	MeOH/H_2O	MeOH/HCl	water/HCl
(+)-catechin	0.63 ± 0.02	0.41 ± 0.16		0.38 ± 0.01	0.75 ± 0.12	
p-coumaric	0.17 ± 0.01	3.30 ± 0.35		5.56 ± 0.71	7.04 ± 1.52	
hydroxybenzoic	1.72 ± 0.15	6.77 ± 1.56		2.75 ± 0.18	2.39 ± 0.73	3.71 ± 0.89
ferulic	0.75 ± 0.002	1.23 ± 0.48		1.06 ± 0.11	3.46 ± 0.68	
caffeic	0.15 ± 0.03	0.44 ± 0.09			0.95 ± 0.18	
kaempferol				30.13 ± 2.79	29.95 ± 3.19	
astragalin				214.11 ± 15.58	167.55 ± 20.61	
Σphenols[b]	3.4 ± 0.2	12.2 ± 2.6		254.0 ± 19.4	212.1 ± 27.0	3.7 ± 0.9

[a] Results are expressed as mean ± standard deviation; $n = 3$). Samples of cooked whole beans (1.0 g) were extracted in 5 mL of aqueous methanol (methanol in deionized water, 85:15, v/v), acidified methanol (methanol in 1.0 M HCl, 85:15, v/v), or acidified water (water in 1.0 M HCl, 85:15, v/v). [b] Σphenols represents the sum of the individual compounds quantified in the extract.

Table 3. Concentration of Phenolic Acids and Flavonoids in the Methanol/HCl Extract from Cooked White, Different Red, and Black Beans[a]

	beans (μg g^{-1} of sample, dry basis)						
seed color:	white	C-red	red	red	C-black	black	black
genotype:			CAL96	NUA35		DOR500	MIB465
(+)-catechin	0.41 ± 0.16a	0.75 ± 0.12b	1.53 ± 0.03c	0.62 ± 0.01b		1.83 ± 0.45c	
p-coumaric	3.30 ± 0.35d	7.04 ± 1.52e	1.76 ± 0.16cd	2.43 ± 0.50d	1.59 ± 0.07c	0.63 ± 0.04a	0.94 ± 0.07b
hydroxybenzoic	6.77 ± 1.56b	2.39 ± 0.73a	9.49 ± 0.41c	11.33 ± 0.09d	12.78 ± 2.69de	13.06 ± 0.92e	9.07 ± 0.05c
ferulic	1.23 ± 0.48a	3.46 ± 0.68b	12.90 ± 0.08d	10.92 ± 0.37c	14.81 ± 0.12e	17.54 ± 0.54f	20.30 ± 0.28g
caffeic	0.44 ± 0.09a	0.95 ± 0.18b	1.15 ± 0.01b	1.00 ± 0.17b		0.93 ± 0.22b	1.37 ± 0.05c
kaempferol		29.95 ± 3.19c	17.29 ± 1.67ab	18.31 ± 0.81b	14.18 ± 0.52a	14.45 ± 0.43a	13.53 ± 0.18a
astragalin		167.55 ± 20.61c	22.69 ± 1.25b	21.71 ± 1.01b	12.49 ± 0.09a	12.68 ± 0.33a	12.87 ± 0.36a
Σphenols[b]	12.2 ± 2.6a	212.1 ± 27.0d	66.8 ± 3.6c	66.3 ± 3.0c	55.9 ± 3.5b	61.1 ± 2.9b	58.1 ± 1.0b

[a] Results are expressed as mean ± standard deviation; $n = 3$). [b] Σphenols represents the sum of the individual compounds quantified in the extract.

concentrations in dry beans typically range between 50 and 80 μg g^{-1} (*15*). Recently, selective breeding strategies have been indicated as useful strategies to increase the concentration of Fe in staple crops to reduce micronutrient malnutrition and deficiency (*16*). As shown, breeders at CIAT have been able to provide a biofortified black bean (MIB465) with a Fe content of up to 100 μg g^{-1} (dw). This fact supposes a significant increase in Fe content relative to conventional beans. However, it is well-known that Fe bioavailability in beans is low (*1, 2*). The latter observation has been attributed to several antinutritional factors present in beans (phytate, polyphenols, lectins, etc.), which lower the digestibility and/or bioavailability of nutrients (*17*). In this sense, several studies of our group have shown an inhibition of Fe uptake by Caco-2 cells when phytic acid (*18*) and polyphenols (*8, 18*) are present.

Extraction of Polyphenols. Phenolics and flavonoids in the analyzed bean (*P. vulgaris* L.) samples have been extracted using different solvents: methanol/water (85:15, v/v) (*3, 19*), acidic methanol (methanol/1 M HCl, 85:15, v/v) (*8, 19*), and acidic water (deionized water/1 M HCl, 85:15, v/v). Both methanol mixtures were compared to acidic water, which represents an acidic environment similar to the gastric media. The extraction efficiencies of the solvents were compared on samples of commercial white and red beans, settling on 1 g of beans (dry weight, dw) and 5 mL of the solvent in each case (**Table 2**). The comparison of the extraction mixtures was done according to the sum of the individual phenols quantified in the extract (Σphenols).

In white beans, the Σphenols quantified was highly variable when using methanol/water (3.4 μg g^{-1} of bean, dw) or methanol/HCl mixtures (12.2 μg g^{-1} of bean, dw). The extraction of phenols by using acidic water was negligible, and the concentrations of individual phenols were below the quantification limit of the methodology used. When considering red beans, the Σphenols quantified in both methanol/water and methanol/HCl mixtures were markedly higher than calculated in the acidic water. Taking into account the Σphenols calculated in the extracts, and to preserve the chemical stability of polyphenols, the acidic methanol mixture was chosen for further analysis throughout.

It must be stressed that sequential hydrolysis steps, with a base followed by an acid, have been used to analyze the free and conjugated phenols in beans (*20*). Accordingly, we have experienced lower extraction efficiencies in both raw white and red beans by using the acidic methanol mixture (Σphenols were 1.6 ± 0.1 and 49.3 ± 3.6 μg g^{-1} of beans (dw) for white and red beans, respectively). However, in cooked beans the use of an additional hydrolysis (acid or base) step did not improve the extracted Σphenols. In the present study, to facilitate the analysis of polyphenols, no additional hydrolysis step has been used in the extraction procedure from cooked beans.

Polyphenol Profile in Cooked Beans. Contents of the phenolics and flavonoids found in commercial white, red, and black beans and in two different genotypes of red and black beans (*P. vulgaris* L.) are shown in **Table 3**. The identity of chromatographic signals was confirmed by comparison of their PDA spectra and retention time with those from standards.

In white beans the main phenolic acids found were hydroxybenzoic (HBA) > *p*-coumaric (CA) > ferulic (FA), and other minor phenolics present were caffeic acid and catechin. The distribution of these compounds follows the same pattern previously reported in different common beans (*P. vulgaris* L.) (*3*). In the present study the content of ferulic acid quantified was lower than reported by others in great northern navy beans: 17.0−26.2 mg/100 g (*20*).

With regard to red beans, the main phenolics found were CA, HBA, and FA. Among the phenolics, HBA and FA were higher in those varieties grown by CIAT (CAL96 and NUA35)

Table 4. Concentration of Phenolic Acids and Flavonoids in in Vitro Media from Cooked White, Different Red, and Black Beans[a]

	beans (μg g^{-1} of sample, dry basis)						
seed color: genotype:	white	C-red	red CAL96	red NUA35	C-black	black DOT500	black MIB465
(+)-catechin							
p-coumaric							
hydroxybenzoic							
ferulic		1.50 ± 0.07b	1.21 ± 0.09a	1.43 ± 0.10b	3.21 ± 0.59d	3.54 ± 0.11d	2.07 ± 0.18c
caffeic							
kaempferol		18.62 ± 1.12a	7.83 ± 0.85b	8.78 ± 0.19b	7.92 ± 0.98b	7.40 ± 1.33b	5.36 ± 0.65a
astragalin		173.83 ± 4.39e	9.38 ± 0.59bc	11.10 ± 0.16bc	18.39 ± 0.26d	6.87 ± 0.31a	6.85 ± 0.70a
Σphenols[b]		194.0 ± 5.6e	18.4 ± 1.5b	21.3 ± 0.5c	29.5 ± 1.8d	17.8 ± 1.8a	14.3 ± 1.5a

[a] Results are expressed as mean ± standard deviation; $n = 3$). Different letters within a row indicate significant ($p < 0.05$) statistical differences. [b] Σphenols represents the sum of the individual compounds quantified in the extract.

compared to the commercial red bean sample analyzed. The content for these main phenolics previously reported in wild and weedy beans was highly variable (mg/kg of flour): FA, 10.3−35.8 > HBA, 8.3−27.6 > CA, 0.0−28.3. Otherwise, the main flavonoids quantified were kaempferol (KA) and astragalin (kaempferol-3-*O*-glucoside) (AST). In the present study, the range of KA found is included in the interval reported for wild and weedy Mexican common beans, 5.2−98.6 mg/kg of flour (*3*). It is important to mention the higher content of KA and especially AST quantified in samples of the commercial red (kidney) beans analyzed compared to both CAL96 and NUA35 genotypes provided by CIAT. The presence of both flavonoids, KA and AST, has been previously confirmed in commercial red beans by using mass spectrometry techniques (*21*). The variation of phenolics in foods as a function of the genotype (cultivar or variety), climatic conditions and agronomic practices, harvest conditions, and postharvest storage has been documented (*22, 23*). The latter observations may explain the differences found in the present study between analyzed samples of red beans. Although the presence of quercetin 3-*O*-glucosides has been also reported in red beans (*21*), in the present study none of the chromatographic signals recorded in red bean extracts were identified as quercetin.

In black beans, the differences in both phenolics or flavonoids contents were less variable between commercially available beans and both DOR500 and MIB465 genotypes. These results suggest that the genetic basis for increased Fe concentrations in bean seeds does not appear to be related to polyphenol contents. The phenolics quantified in black beans were FA > HBA ≫ CA. This same pattern has been previously found in different wild black beans, where FA and HBA ranged from 14.9 to 28.5 mg/kg of flour and from 8.32 to 17.4 mg/kg of flour, respectively (*3*). In the present study CA constitutes the lowest phenolic found in black beans, which is present in a range similar to that reported by the latter authors in wild common beans (2.1−8.7 mg/kg of flour) (*3*). We quantified a low content of caffeic acid, which is also included in the highly variable interval reported in wild beans, 0.3−20.6 mg/kg of flour (*3*). Interestingly, for flavonoids a similar pattern in commercial and both DOR500 and MIB465 genotypes was noted. The presence of flavonoids in colored beans has been also confirmed by mass spectrometry techniques (*21*). KA and AST were present in concentrations slightly lower than found in red bean genotypes provided by CIAT. The KA content was similar to the data previously reported for black beans: 3.0−17.5 mg/kg of flour (*3*). Interestingly, the marked difference quantified for AST in commercial red bean sample compared to those prepared by CIAT (CAL96 and NUA35) was not found between commercial black beans and those prepared by CIAT (DOR500 and MIB465).

Previous research studies have addressed the relationship between seed color and phenols content (*3, 19, 24*). In general, the results are highly variable, and converse correlations have been found in relating seed bean color to the content of tannins (*24*). It has been established that the wide variety of seed color in common beans is controlled by a group of well-defined genes (*24*). Otherwise, a common aspect in all of these studies is that seed coats exhibited higher concentrations of phenolic compounds than cotyledons (*19, 24*). Previous analyses in our laboratory have confirmed that Fe in beans is mainly found in the cotyledons (*18*).

Phenols are naturally occurring compounds in foods of plant origin, which have been reported to exhibit health-promoting effects due to their antioxidant (*25*), antimutagenic (*26*), and antiproliferative effects (*27*). However, there are aspects such as their stability in the gut and bioavailability of food-derived phenols that have been indicated as being of interest but on which there are only scarce in vitro studies (*10, 28*). Polyphenols, in addition to playing important roles in lowering chronic human diseases, can exert antinutritional effects on inhibiting Fe absorption due to their potential Fe-chelating ability in the gut (*17*). These aspects are discussed in the following sections.

Bioaccessibility of Phenols in Cooked Beans. The fact that changes in polyphenol structures and/or their solubility in the gastrointestinal media can take place during digestion might have marked repercussions in their potential antinutritional effect on Fe uptake. In vitro procedures, especially those that simulate the gastrointestinal digestion, allow us to screen multiple samples and provide data to improve the understanding of the processes that can occur in the gut in an in vivo situation. In this way, in vitro methods have been used to estimate the bioaccessible fraction (maximum concentration soluble in simulated gastrointestinal media that is available for subsequent absorption to the intestinal mucosa) of polyphenols in foods (*10, 28*). The bioaccessible concentrations of polyphenols from common beans are shown in **Table 4**.

After enzymatic digestion, none of the phenolics quantified in cooked white beans were detected in the in vitro digests. In contrast, the Σphenols in the digests of colored common and biofortified beans ranged between 14.3 and 194.0 μg g^{-1} of beans (dw). Regardless of bean color only FA, KA, and AST were detected in the bioaccessible fraction of analyzed beans. Although some compounds such as HBA and CA constitute a relatively high proportion of phenolics in common beans, they

were not detected in bioaccessible fractions from either red or black bean samples. Commercial red beans produced the highest soluble content of AST (173.8 μg g^{-1} of beans, dw) in in vitro media, which constitutes up to 89.6% of the Σphenols quantified in in vitro digests. In a similar way AST was also solubilized in a high proportion from the other red bean genotypes: 50.9 and 52.1% in CAL96 and NUA35 samples, respectively. The soluble content of KA from commercial red beans resulted in 62.1% relative to the content found in the acidic methanol extract. In all of the red beans analyzed the content of FA did not vary highly, although significant differences in the acidic methanol extract from commercial and both CAL96 and NUA35 genotypes were noted (**Table 2**). When the FA content in the in vitro digests was expressed as a percentage of its content found in the acidic methanol extracts (i.e., bioaccessibility), the values varied between 9.4 and 43.4%, being highest from commercial red beans. In black beans, the soluble FA content in in vitro media (range: 10.2−21.7%) was slightly higher than solubilized from red beans and in all cases was markedly lower than found in the methanol/HCl extract. On the other hand, soluble KA and AST contents represent mean proportion values of 45.4 and 43.3% relative to the soluble Σphenols in the methanol/HCL extracts, respectively.

When the Σphenols in in vitro digests were compared to the content found in the acidic methanol extracts from nondigested beans, the results obtained evidenced that not all polyphenols are equally released from the beans during digestion in the gut. This fact would affect in a relevant way their interaction with the bioavailable Fe. From a physiological point of view, after oral ingestion only the fraction of the polyphenols released by digestive enzymes from food would affect the Fe uptake in the intestinal epithelia. Several in vivo (*2, 6*) and in vitro (*8, 18*) studies have addressed the effect of phenols in beans affecting Fe absorption. It has been proposed that polyphenols in the hulls would impair Fe absorption, likely forming complexes unavailable for absorption due to their Fe-chelating ability (*8*). However, there are scarce data about the stability and concentration of polyphenols in simulated gastrointestinal media (*10, 28*). It was estimated that about 48% of dietary polyphenols from solid vegetable foods would be bioaccessible in the small intestine, whereas 42% become bioaccessible in the large intestine (*28*). The bioaccessibility values, in the small intestine, for polyphenols reported by the latter authors are in good agreement with the ones estimated in the present study (**Table 4**).

One important aspect that should be pointed out is the fact that only KA, but not AST, has been shown to inhibit Fe uptake to Caco-2 cells (*8*). In the latter study it was shown that KA inhibited Fe uptake by 16−63% when present in the media in concentrations of 40−1000 μM, respectively. In the present study, in considering the volume used in the in vitro digestion procedure, the soluble KA concentrations from beans ranged from 0.37 ± 0.05 μM (MIB465) to 1.30 ± 0.08 μM (commercial red beans). The experimental conditions used by Hu et al. (*8*) provided ratios of Fe/KA in in vitro media of 0.1 (Fe in digest of white beans was 17.4 μg and added KA was 171.7 μg) to Caco-2 cultures. However, in the present study, the concentrations of KA quantified in in vitro media are markedly lower than those used by Hu et al. (*8*) and provide Fe/KA ratios between 4.69 and 19.94 to Caco-2 cells. From these calculations we did not expect any, or at least a very low, inhibitory effect of KA on Fe uptake to Caco-2 cultures. Thus, we next evaluated Fe availability from common and biofortified beans to Caco-2 cells.

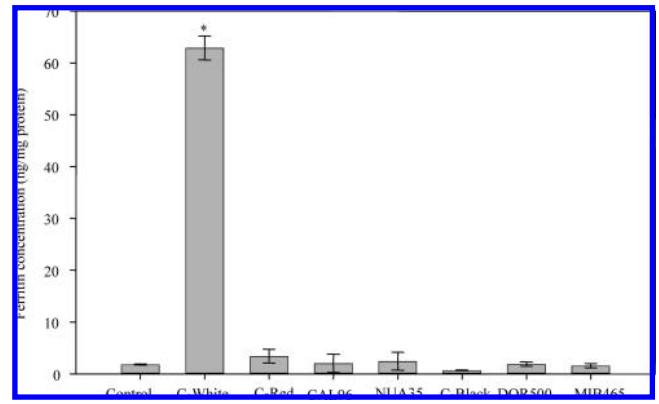

Figure 1. Iron bioavailability from beans (*P. vulgaris* L.) estimated by using an in vitro digestion/Caco-2 cell model. Values are expressed as mean ± standard deviation ($n = 6$). ∗ indicates significant ($p < 0.05$) statistical differences relative to the controls. Samples: C-White, commercial white beans; C-Red, commercial red beans; CAL96 and NUA35, red beans provided by CIAT; C-Black, commercial black beans; DOR500 and MIB465, black beans provided by CIAT. Iron content (μg g^{-1}): white, 67.9 ± 0.6; C-red, 87.4 ± 1.2; CAL96, 75.3 ± 0.2; NUA35, 78.5 ± 1.4; C-black, 81.9 ± 3.3; DOR500, 71.4 ± 1.4; MIB465, 106.9 ± 1.8.

Fe Bioavailabilty in Beans. The Caco-2 cell ferritin concentration values are shown in **Figure 1**. Only the digests from white beans provided a significant amount of bioavailable Fe, as concluded from the increased ferritin concentration in Caco-2 cultures compared to the controls. In contrast, neither red or black bean samples nor even biofortified MIB465 genotype produced higher ferritin concentrations than the controls. These results indicate a low Fe bioavailability from common beans to Caco-2 cells and are supported by the lower dialyzability of Fe from colored beans (1.5−2.7%) than white beans (12.1−18.8%) in the in vitro system used. Interestingly, there was no significant difference in Fe uptake from both red and black beans, even when the differences in Fe content in the beans and/or the soluble polyphenols concentration in in vitro media were considered (**Table 3**). As can be observed, MIB465 sample contained ∼49.7% more Fe (up to 30 μg g^{-1} of bean, dw) than DOR500 (106.9 ± 1.8 versus 71.4 ± 1.4 μg g^{-1}, respectively), but both of the black bean (DOR500 and MIB465) genotypes exhibited no significant ($p < 0.05$) differences in Fe uptake. An additional aspect that should be stressed is that MIB465 produced the lowest bioaccessible KA concentration; however, no response in ferritin formation was detected.

The in vitro digestion/Caco-2 cell culture model developed by Glahn et al. (*12, 29*) has demonstrated strong correlations with human studies, indicating that it is a useful, rapid, and cost-effective method to predict Fe uptake in humans. A recent expert consensus concluded that the Caco-2 cell culture model is useful for predicting the correct direction of intestinal response to enhancers and inhibitors of Fe absorption in humans but not its magnitude (*11*). In the present study, data from Caco-2 exposure to digests from beans suggest that Fe uptake is negligible or very low. The obtained results are in accordance with measurements of Fe bioavailability from colored beans by using human trials, which have shown that only about 1−2% of the Fe was absorbed (*1, 2*). Furthermore, in one of the latter studies there were no differences between the percent of Fe uptake from either common beans (Fe content of 50.4 μg g^{-1}, dry basis) or high Fe-containing beans (Fe content of 82.9 μg g^{-1}, dry basis) (*1*). All of these in vivo studies may indicate that physi-

ological adaptation to low Fe stores in in vivo situation would be detectable even at low levels of Fe absorption.

Although the applications of the in vitro digestion/Caco-2 cell culture model are diverse, it seems to be very sensitive to small changes in Fe availability and dietary factors affecting Fe bioavailability. For example, recent studies by our group showed that white and red bean-based diets provide similar Fe bioavailabilities to piglets (*30*). However, the Caco-2 cell model is able to detect the low KA concentrations in in vitro media and their inhibitory effect on Fe uptake. This observation pointed out the need to better understand the factors affecting Caco-2 cell culture model in predicting Fe uptake to better use its potential and understand the obtained results. Although in vitro methods constitute a good approach to the in vivo situation, they lack a series of physiological factors that can condition the absorption of nutrients. For example, the plausible "dilution or unspecific adsorption" of polyhenols that can occur in the larger surface area of small intestine will allow the Fe uptake in later areas of intestinal epithelia. This fact might explain the low Fe absorption found in vivo experiments and the nondetectable Fe uptake from colored beans in our in vitro model. In addition, when considering in vivo studies that fed subjects multiple meals, we must consider the physiological adaptation response to the antinutritional effect of polyphenols by increasing the secretion of proline-rich proteins in the saliva (*30, 31*), reducing the soluble concentration of polyphenols. From the latter in vivo response a reduced inhibitory effect of polyphenols on Fe absorption would be expected. However, it should be pointed out that urinary excretion of dietary polyphenols (*32*) demonstrates that a fraction of those results absorbed which indicate they potentially would affect Fe availability in the gut. These reasons might explain the overly sensitive response of the Caco-2 cell model to soluble polyphenol concentrations in the in vitro media and the differences compared to in vivo studies.

In summary, among different phenolics the flavonoids such as kaempferol and astragalin (kaempferol-3-*O*-glucoside) are highly prevalent in colored beans. Our results showed that these compounds are resistant to the cooking procedure. Following gastrointestinal digestion KA remains highly soluble and exhibits a high inhibition on Fe absorption, likely forming Fe complexes unavailable for absorption. Our data evidence that dietary polyphenols exhibit higher inhibition on Fe uptake than predicted using standards. The in vitro gastrointestinal models offer a simple and inexpensive approach to establishing relative indices for bioavailability, making comparisons, and obtaining information about different processes that can take place in the in vivo situation. However, further studies and complementary data within human trials are needed to better understand and use in vitro models.

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