Iron (Fe) bioavailability from mung beans: Effects of household processing and form of Fe fortificants

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By:
Vidya Endraiyani
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Research mentors:
Dr. José Moisés Laparra Llopis
Dr. Dennis D. Miller

Abstract:
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Vidya Endraiyani

Under the supervision of José Moisés Laparra Llopis and Dennis D. Miller.
Department of Food Science

Mung beans (*Vigna radiata*) constitute a popular crop in Southeast Asian countries but contain phytates, and polyphenols that impair Fe absorption. One of the key causes of Fe deficiency is poor bioavailability of dietary Fe. Food fortification is a good strategy for combating Fe deficiency. This study compares the *in vitro* Fe bioavailability (bioaccessibility and cell uptake) from mung beans prepared by household cooking procedures, boiling and soak-boiling, and fortified with selected Fe fortificants. To estimate Fe bioavailability, an in vitro digestion/Caco-2 cell model was used. Mung beans can constitute a good source of Fe since their concentrations were 60.1±1.5 μg/g beans (dry basis). Although boiling did not affect Fe concentration in mung beans, the soak-boiling procedure decreased the Fe content by 16%. However, soak-boiling increased the Fe bioaccessibility up to 21% relative to boiled (9.1%) and raw beans (not detectable). Both cooking procedures decreased the content of phenolics, however, soak-boiling increased the soluble phytate in the digests. When comparing the effect of different fortificants, Fe bioavailability from beans fortified with Sprinkles™ was higher than either $FeSO_4$ or ferrous fumarate.

In conclusion, 1) cooking enhanced Fe bioavailability in non-fortified beans, and 2) fortification with Sprinkles™ was the most effective strategy.

Keywords: Mung beans, iron, fortification, bioavailability, Caco-2 cell.

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Summary

In Southeast Asian countries, mung beans (*Vigna radiata*) constitute the main source of protein and minerals for large groups of the population. Nevertheless, legumes also contain phytates, polyphenols, and other compounds that impair Fe bioavailability. One of the key causes of Fe deficiency is poor bioavailability of dietary Fe and its defficiency is a leading nutritional concern worldwide. Food fortification can be a good strategy for combating Fe deficiency. In view of the aforementioned, this study was designed to compare the *in vitro* Fe bioavailability from mung beans prepared by different cooking procedures (boiling and soak-boiling) commonly used by the consumers and fortified with selected Fe fortificants (0.63 µg as Fe/mg beans).

Mung beans were boiled (15 minutes) or soak-boiled (24h-15 minutes) before being subjected to an in vitro digestion/Caco-2 cell model to estimate the relative bioavailability of Fe. The in vitro/ Caco-2 cell model is a digestion procedure that simulates the gastrointestinal digestion, which consists of two-stage digestions: gastric with pepsin at pH 2.0 and intestinal with pancreatin-bile extract at pH 7.0. Caco-2 cell cultures were placed in the bottom of a two-chamber system separated by a dialysis membrane (15.000kDa), which allows only the soluble fraction of the Fe loaded in the upper chamber to reach the cell monolayer. Fe concentration in the bottom chamber at the end of the incubation indicated Fe dialyzability. Cell ferritin formation was used to estimate cell Fe uptake. Several rapid and simple spectrophotometric methods were used to quantify the total dialyzable Fe, and some of the well-known inhibitors of Fe absorption such as polyphenols (galloyl and catechol groups) and phytate.

Fe concentrations in the beans were as follows (µg/g dry weight): raw, 60.1 ± 1.5; boiled, 59.2 ± 1.9; soak-boiled, 50.5 ± 3.2. Although the soak-boiling procedure decreased Fe concentration in beans, it increased Fe dialyzability (21.9%) when compared with boiled (9.1%) and raw beans (not detectable). Cooking decreased the content of soluble Fe-binding phenolic groups in the digests. Interestingly, soak-boiling increased the soluble phytate content in the *in vitro* supernatants. When comparing the different fortificants, $FeSO_4$ produced the lowest dialyzability, which values were similar in all of the samples (3.1-4.1%). Ferrous fumarate showed highest dialyzability in raw (12.4%) relative to boiled (2.3%) and soak-boiled (3.9%) beans. Sprinkles™ produced the highest Fe dialyzability values in all the samples following the gradation: raw, 24.8% < boiled, 27.9% < soak-boiled 45.0%. Sprinkles™ contain encapsulated Fe within a lipid layer. This encapsulation may have prevented the iron from interacting with soluble phenols and phytate, thus, preventing the inhibition of dialysis observed in the other treatments. Regarding cell Fe uptake, similar patterns emerged in all assayed samples; Sprinkles™ produced the highest ferritin concentrations in raw and cooked beans, followed by $FeSO_4$ and ferrous fumarate.

From these data, we can conclude that cooking enhanced Fe bioavailability in non-fortified beans. Fe bioavailability from beans fortified with Sprinkles™ was higher than either $FeSO_4$ or ferrous fumarate. Thus, Sprinkles™ supplementation can be helpful in improving Fe bioavailability by preventing interactions with well-known inhibitors of Fe uptake.

I. Introduction

Many efforts have been made in the treatment and prevention of iron (Fe) deficiency and anemia. In spite of these efforts, iron deficiency is one of the most prevalent nutritional deficiencies worldwide affecting an estimated 20-50% of the world's population (Beard and Stoltzfus, 2001). WHO estimated that 39% of children younger than 5 years, 48% of children between 5 and 14 years, 42% of all women, and 52% of pregnant women in developing countries are anemic (WHO, 2001). Fe is an essential micronutrient for many physiological processes. Its deficiency has a negative effect on the motor and mental development of young children (Hurrell and Zimmermann, 2007). Furthermore, the high prevalence of Fe deficiency in the developing world has substantial health and economic costs, including poor pregnancy outcome, impaired school performance, and decreased productivity (Hurrell and Zimmermann, 2007). The RDA (Recommended Daily Allowance) of Fe varies between 8 and 18 mg/day for different groups, i.e. age and gender, except in pregnancy when it is 30 mg/day (Institute of Medicine, 2001).

Diet composition has a major effect on Fe bioavailability. Absorption can vary from less than 1% to more than 50%. However, other important aspects to be taken into account are the type of Fe compound in the diet and the body's physiological need for Fe. One of the key causes of Fe deficiency is poor bioavailability in the diet. It has been widely reported that Fe absorption is negatively affected by the presence of phytates and polyphenols (found in most plant foods) (Welch et al., 2000; Hu et al., 2005). The lack of dietary promoters of Fe uptake such as ascorbic acid and meat is also considered to limit dietary Fe bioavailability.

Bioavailability can be defined as the fraction of an element that is solubilized and finally absorbed from the gastrointestinal tract into the systemic circulation of humans and animals (Eckmekcioglu, 2002). Bioavailability is the result of the integral sum of a four-part process: 1) ingestion, 2) bioaccessibility, 3) absorption and 4) first-pass effect. Bioaccessibility is defined as the fraction of an element that dissolves in the stomach and is available for absorption during transit through the small intestine (Eckmekcioglu, 2002). It depends, among other things, on the ability of digestive enzymes to release the element in the intestinal lumen, as well as on its solubility and behaviour in the gastrointestinal tract, which in turn is a function of the chemical form released from the food (Hocquellet and L'Hotelier, 1997, Eckmekcioglu, 2002).

In the developing countries, the source of Fe is mainly through vegetable-based diets; in the form of unfortified cereal grains, which mainly consist of blends of processed cereals and legumes. Economic factors often limit the intake of meat and ascorbic acid rich foods that would enhance the absorption of iron. In addition, parasitic infection resulting from poor sanitary habits, poverty and inadequate health facilities also contribute in increasing the risk of Fe deficiency anemia. (Hardjojoewono, 1990).

Legumes constitute one of the richest and cheapest sources of energy and are an important part of people's diets in many parts of the world. In Southeast Asian countries, mung beans (*Vigna radiata*) constitute the main source of protein and several minerals for large groups of the population (Barakoti and Bains, 2007). Thus, improvement of Fe bioavailability in mung beans could significantly contribute toward meeting RDA for Fe.

In addition, other strategies for combating Fe deficiency include supplementation, dietary diversification, and food fortification (Hurrell, 2002). Nonetheless, according to the latter author, the main barriers for successful Fe fortification of foods are the following: 1) finding an Fe compound that is adequately absorbed but causes no sensory changes to the food vehicle and, 2) overcoming the inhibitory effect on dietary Fe absorption caused by other food-derived components. The foods most often used for mass fortification are the staple cereal flours. A recent study proposed the strategy of "home fortification" where multiple micronutrient supplements (Sprinkles^{TM}), in a powdered form, are mixed into complementary weaning foods (Zlotkin et al., 2001, 2005) prepared in the household. In Sprinkles^{TM}, the Fe (ferrous fumarate) which is encapsulated within a thin lipid layer may prevent Fe from interacting with inhibitors of Fe absorption.

Although human studies are the definitive tool to evaluate Fe bioavailability, *in vitro* methods can be helpful in providing a valuable insight to factors affecting Fe absorption *in vivo*. In this sense, a Caco-2 cell culture model has demonstrated strong correlations with human studies in predicting the intestinal response to enhancers and inhibitors of Fe absorption (Glahn et al., 1998). The *in vitro* gastrointestinal models offer a simple and inexpensive approach to obtain information about bioavailability. However, the results of these models must be taken as relative indexes for bioavailability, which means that the methods provide a good basis for establishing tendencies, making comparisons and determining effects caused by different factors. *In vitro* studies in conjunction with human trials are a valuable tool to improve understanding of the occurring processes at the intestinal level.

II. Objective.

The objective of this study was to compare Fe bioavailabilities from mung beans prepared by different cooking procedures and fortified with selected Fe fortificants.

III. Material and Methods

All glassware used in the sample preparation and analyses was soaked in 10% (v/v) HCl concentrated (37%) for 24h – final concentration of 1.2 mol/L in the acid solution –, and then rinsed with 18 MΩ deionized water before being used to remove any contaminant Fe.

III.1.- Sample preparation: household processing methods.

a.- Mung Bean. Commercial raw mung-bean samples were obtained from specialty food stores in Ithaca, New York (USA). Mung beans in distilled water (1:6 w/v), were boiled (15 minutes) or soak-boiled (24h-15minutes) (Ndossi et al 2003). A portion of

each boiled and soak-boiled bean sample was freeze-dried. Raw and cooked bean samples were milled to obtain fine flour, and then stored in the dark at 4ºC until analysis.

b.- Fe fortificants. $FeSO_4$, ferrous fumarate and Sprinkles^{TM} (**Table 1**), were solubilized in Milli-Quartz water and 0.1% (v/v) of 6N HCl. The final concentration of fortificants was 11.1 ± 1.2 µg Fe/mL. Sprinkles^{TM} solution was heated (<60ºC) further to solubilize the powder in the deionized water in a container covered with a watch glass to create a reflux system and minimize loss of water.

III.2.- Fe determination in mung beans.

Total Fe content was determined with an inductively coupled argon plasma emission spectrometer (ICP-ES, Model 61E Trace Analyzer, Thermo Jarrell Ash Corporation, Franklin, MA, USA) after wet-ashing (Laparra et al., 2008). Aliquots (0.3 g) of raw and cooked mung beans were acid digested (120ºC to dryness) in 1.0 mL concentrated HNO_3 until white ashes were obtained. Then, the samples were additionally digested with 1.5 mL $HClO_4$ at 180°C for 1 h and then at 220°C until $HClO_4$ fumes were observed. The samples were diluted with 5% HNO_3 to 10 mL. The instrument was calibrated with 10% $HClO_4$ as the low standard and 1 µg mL^{-1} Fe in a multi-element standard as the high standard. The Fe was determined using the 238.2-nm line.

III.3.- Cell culture.

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 50,000 cells/cm^2 in collagen-treated 6-well plates (6-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; the medium was changed every 2 days. The cells were used in the iron uptake experiments at 13 days postseeding. Under these conditions, the amount of cell protein measured in each well was highly consistent from well to well within each culture plate.

III.4.- In vitro digestion.

Porcine pepsin (800-2500 units/mg protein), pancreatin (activity, 4×USP specifications) and bile extract (glycine and taurine conjugates of hyodeoxycholic and other bile salts) were purchased from Sigma Chemical. Further preparation of the pepsin, pancreatin and bile extract was performed as follows. Shortly before use, 0.32 g pepsin was dissolved in 15 mL of 0.1 mol/L HCl, added to 3.5 g of Chelex-100 (Bio-Rad Laboratories, Hercules, CA) and shaken on a tabletop shaker for 30 min. The pepsin solution with Chelex was then poured into a 1.6-cm diameter filtration column to filter out the Chelex from the pepsin solution. An additional 15 mL of 0.1 mol/L HCl was added to the column and the filtrate collected into the pepsin solution.

For the intestinal digestion, 0.06 g pancreatin and 0.37 g bile extract were dissolved in 15 mL of 0.1 mol/L $NaHCO_3$. Chelex-100 (3.75 g) was added and the resulting mixture was shaken for 30 min on a tabletop shaker. The mixture was then poured into a 1.6-cm diameter filtration column to filter out the Chelex. An additional 6 mL of 0.1 mol/L $NaHCO_3$ was added to the column and the filtrate collected into the pancreatin/bile

solution. Treatment of the pepsin and pancreatin-bile solutions via the methods described above did not affect the activity of the enzymes.

Peptic and intestinal digestions were conducted on a rocking platform shaker (Reliable Scientific, Hernando, MS) in an incubator at 37°C with a 5% CO_2/95% air atmosphere maintained at constant humidity. The intestinal digestion was carried out in the upper chamber of a two-chamber system in 6-well plates, with the cell monolayer attached to the bottom surface of the lower chamber (**Figure 1**). The upper chamber was formed by fitting the bottom of an appropriately sized Transwell insert ring (gift from Costar) with a 15,000 molecular weight cut-off dialysis membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA). The membranes were soaked in deionized water before use. The dialysis membrane was held in place with a silicone ring (Web Seal, Rochester, NY).

To start the peptic digestion, the pH of each sample was adjusted to pH 2.0 with 5.0 mol/L HCl. The sample was transferred to a 50-mL screw-cap culture tube, and 0.96 mL of the pepsin solution was added. The tube was capped, placed horizontally and incubated for 60 min on the rocking shaker at rocking speed #7 (55 oscillations/min). For the intestinal digestion step, the pH of the sample (also referred to as the "digest") was raised to pH 5.5 by dropwise addition of 1 mol/L $NaHCO_3$. Then 1.19 mL of pancreatin-bile extract mixture was added. The pH was adjusted to pH 7.0 with NaOH, and the volume was brought to 17 mL with 120 mmol/L NaCl and 5 mmol/L KCl.

III.5.- Preparation of the 6-well culture plates with cell monolayers.

Immediately before the intestinal digestion period, the growth medium was removed from each culture well and the cell layer was washed twice with 37°C Minimum Essential Medium (MEM, GIBCO) at pH 7. This MEM was chosen because it contained no added Fe; upon formulation with the following ingredients, it was always found to contain <8 μg Fe/L. The MEM was supplemented with 10 mmol/L PIPES (piperazine-*N*,*N*'-bis-[2-ethanesulfonic acid]), 1% antibiotic-antimycotic solution (Sigma), hydrocortisone (4 mg/L), insulin (5 mg/L), selenium (5 μg/L), triiodothyronine (34 μg/L) and epidermal growth factor (20 μg/L). A fresh 1.0-mL aliquot of MEM covered the cells during the experiment. A sterilized insert ring (incubation overnight immersed in 0.5M HCl), fitted with a dialysis membrane, was then inserted into the well, thus creating the two-chamber system. Then, a 1.5-mL aliquot of the intestinal digest was pipetted into the upper chamber. The plate was covered and incubated on the rocking shaker at rocking speed #2 (6 oscillations/min) for 120 min.

When the intestinal digestion was terminated, the insert ring and digest were removed. The solution in the bottom chamber was allowed to remain on the cell monolayer and an additional 1mL of MEM was added to each well. The cell culture plate was then returned to the incubator for an additional 22 h, after which the cells were harvested for analysis.

III.6.- Harvesting of Caco-2 cell monolayers for ferritin analysis.

Exactly 24 h after the start of the intestinal digestion period, the cell monolayers were harvested for various analyses. To harvest the cells, the medium covering the cells was removed and the cells washed once with a 2 mL volume of a "rinse" solution containing 140 mmol/L NaCl, 5 mmol/L KCl and 10 mmol PIPES, at pH 7. The rinse solution was

then aspirated and 2 mL of deionized water was placed on each monolayer. The plates were then placed on a rack such that the bottom of each plate was in contact with the water of a benchtop sonicator, which was kept in a cold room at 4°C. The cells were sonicated for 15 min, scraped from the plate surface, harvested along with the 2 mL volume of water in each well and then stored at −20°C.

III.7.- Ferritin analysis and total protein determination.

A one-stage, two-site immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-IRON II Ferritin Assay, RAMCO Laboratories, Houston, TX). A 10 µL sample of the sonicated Caco-2 cell monolayer, harvested in 2 mL of water, was used for each ferritin measurement. Caco-2 cell protein was measured on samples that had been solubilized in 0.5 mol/L NaOH, using a semimicro adaptation of the Bio-Rad DC protein assay kit (Bio-Rad Laboratories).

III.8.- Fe quantification in dialyzable fraction.

The ferrozine assay was used to determine the total amount of dialyzable Fe (Kapsokefalou and Miller, 1991) during *in vitro* digestion (*see below*). This method is based in the measurement of a complex of Fe^{+2} with ferrozine (Stookey, 1970). For quantification, a standard curve ranged 0-3.0 µg Fe/mL was used. The source of Fe was an Iron Atomic Absorption Standard solution (1000 µg Fe/mL in 1% HCl) (Sigma I9011). The reactions for ferrozine assay were done in 1.5 mL micro centrifuge tubes to facilitate complete reaction and mixing of the reagents. A total of 1 mL of the dialyzate from digested samples was centrifuged (4000 xg/10 min) and aliquots of 0.8 mL were taken from the supernatant. Afterwards, 0.1 mL of a reducing solution [10% HCl containing 5% hydroxylamine hydrochloride (Sigma 55469)] was added and the mixture was allowed to stand at room temperature for 1h. Then, 0.1 mL of a ferrozine (3-2-pyridyl-5,6-bis 4 phenyl-sulfonic acid 1,2,4-triazine) solution (5 mg/mL) was added (Sigma p9762). After 15 minutes the absorbance was measured at 562-nm.

Dialyzable total Fe was expressed as percentages of the calculated total amount of Fe in the treatment at the beginning of the digestion by using the following equation: Fe dialyzability (%) = (dialysate Fe/initial Fe amount) x 100

III.9.- Total Phenolics determination.

The amount of total phenolics was determined using a modified Folin-Ciocalteu colorimetric method (Dewanto et al 2002). The assay is based in the presence of the molybdenum-tungsten blue compounds as a result of the Folin-Ciocalteu reagent (FCR) reduction. An aliquot of 125 µL from the supernatant (5000 xg/4ºC/15 min) of the gastrointestinal digests of raw and cooked mung beans was obtained, and mixed with 500 µL of deionized water and 125 µL of the FCR. After 6 min, the mixture was mixed with 125 µL 7% aqueous Na_2CO_3 solution. The final volume of the mixture was adjusted to 3 mL with deionized water and was allowed to stand for 15 min at room temperature. The intensity of the blue colour was measured at 760 nm against the prepared blank in comparison with standards with known gallic acid concentrations (2.0-8.0 µg/mL). The amount of total phenolics was expressed as gallic acid equivalents (G7384, Sigma) (mg/g of mung bean).

III.10.- Fe-binding phenolic groups determination.

The total galloyl and catechol complexes were determined spectrophotometrically (Brune et al., 1991). In 10 mL test tube, 0.5 mL from the supernatant (5000 xg/4ºC/15 min) of the gastrointestinal digests of raw and cooked mung beans was mixed with 2 mL of dimethylformamide (DMF)-0.1M acetate buffer (pH 4.4) mixture (50% v/v).To 0.5 mL of the previous mixture solution was added 4 mL of fresh FAS-reagent (89 parts of 50% w/v urea-0.1M acetate buffer, 10 parts of 1% gum arabic solution, and 1 part of 5% ferric ammonium sulfate dissolved in 1M HCl). After 15 min, the absorbances at 578 nm (galloyl groups) and 680 nm (catechol groups) were measured versus a reagent blank containing of 0.5 mL DMF-acetate and 2 mL FAS-reagent. The content of galloyl and catechol groups in the sample was calculated using linear regression equations for the standard curves prepared using catechin (C-1251, Sigma) (catechol groups) and tannic acid (T8406, Sigma) (galloyl groups).

III.11.- Phytate determination.

The phytate was determined based on the reaction between ferric chloride ($FeCl_3$) and sulfosalicylic acid (Latta and Eskin 1980). An aliquot of 1.5 mL from the supernatant (5000 xg/4ºC/15 min) of the gastrointestinal digests of raw and cooked mung beans was mixed with 0.5 mL Wade reagent (0.03% $FeCl_3$ and 0.3% sulfosalycylic acid in distilled water). The mixture was allowed to stand for 5 min at room temperature. The amount of phytate was measured at 500 nm against the prepared blank in comparison with a series of standard solutions containing 5-40 µg/mL phytic acid (sodium form) (P8810, Sigma) in distilled water.

III.12.- Statistical analysis.

Each treatment was performed in triplicate. A one-factor analysis of variance (ANOVA) and the Tukey test (Box et al., 1978) were applied to determine statistical differences. All experiments were conducted in triplicate in two different days. A significance level of $p<0.05$ was adopted for all comparisons. Statgraphics Plus version 5.1 (Rockville, MA, USA) was used for the statistical analysis.

Micronutrient	Amount
Vitamin A	300 µg RE
Vitamin C	30 mg
Vitamin D	5.0 µg
Vitamin E	6 mg a-TE
Vitamin B1	0.5 mg
Vitamin B2	0.5 mg
Vitamin B6	0.5 mg
Vitamin B12	0.9 µg
Folic Acid	160 µg
Niacin	6 mg
Iron	12.5 mg
Zinc	5 mg
Copper	0.3 mg
Iodine	90 µg

Table 1. Standard composition of multi-micronutrient Sprinkles™.

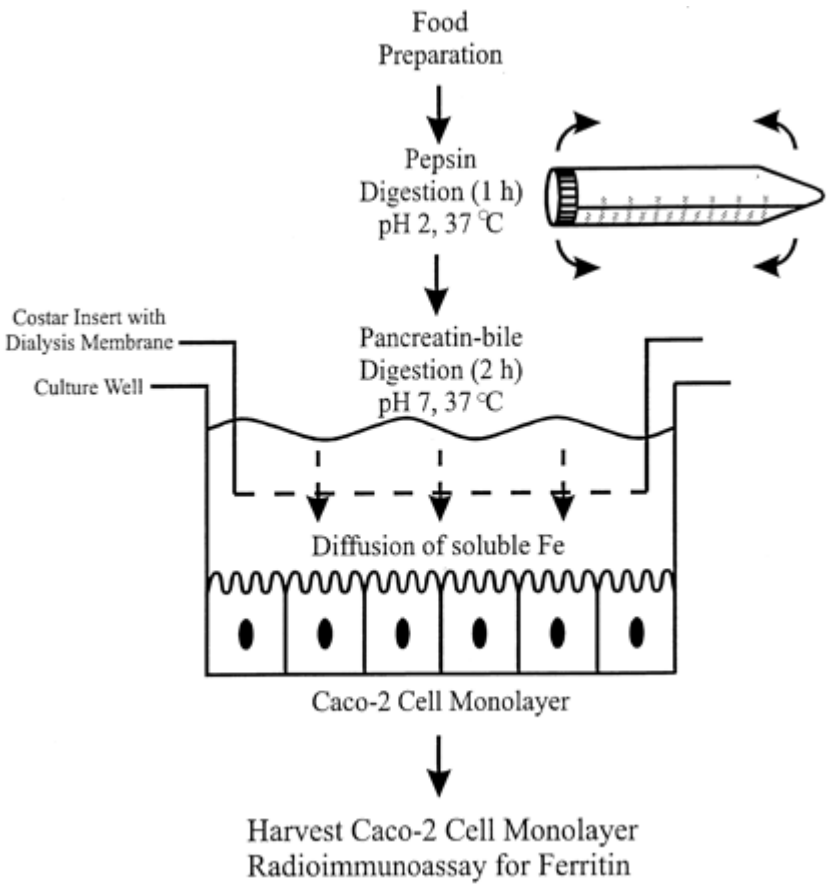

Figure 1. Diagram of in vitro digestion/Caco-2 cell culture model (Glahn et al., 1998).

IV. Results and discussion

IV.1.- Fe content in raw and cooked mung beans.

Legumes constitute a good source of protein for large groups of people around the world, and are also a good source of essential minerals such as Fe. The raw, boiled and soak-boiled mung beans used in the experiments had the following total Fe contents (µg/g bean sample), 60.1 ± 1.5, 59.2 ± 1.9, and 50.5 ± 3.2 respectively. These contents of Fe are in accordance with the data previously reported for raw mung beans (67.4 µg/g beans) and mung beans boiled without salt (51.2 µg/g beans) (USDA).

Analysis of the water used for boiling and soak-boiling mung beans revealed that Fe contents solubilized in the water were 0.1 and 0.5 µg/mL, respectively. A mass balance was performed after the cooking procedure to analyze the Fe content in beans and water used for cooking purposes. Recovery of 99.3 and 88.3% Fe were obtained from boiling and soak-boiling procedures respectively. This result supports the quality assurance in the Fe determination.

It is also important to say that the Fe contents found in mung beans are similar to those previously reported in other beans such as white beans (58.0-66.3 µg/g) (Viadel et al., 2006; Hu et al., 2006) and red beans (76.0 µg/g dry basis) (USDA).

IV.2.- Quality assurance-quality control in Fe determination by using the ferrozine assay.

A rapid and simple spectrophotometric method was the analytical tool used to determine the total dialyzable Fe from digested mung beans; raw, boiled and soak-boiled. As stated in the previous section, this method is based in the formation of a complex of Fe^{+2} with ferrozine (Stookey, 1970). It is well known that mung beans contain some antinutritional factors such as polyphenolic compounds and phytate, which impair Fe absorption (Welch et al., 2000). Thus, potential food matrix interference in quantifying Fe in the dialysate by using the ferrozine assay was evaluated by the method of standards addition. This method is based on the addition of appropiate volumes of the dialysate (50:50 v/v) from the respective digested samples (raw, boiled or soak-boiled mung beans) to the Fe standard solutions for further statistical comparison of the slope of the curve relative to the aqueous (non-added) curve. The typical calibration curve of aqueous (non-added) standard and those with added volume of dialysate from raw, boiled, and soak-boiled beans are shown in **Figure 2**.

As can be observed, there were statistically significant ($p<0.05$) decreased values for the slope of the addition curves relative to the non-added standard curve. These results indicate that there is matrix interference in quantifying Fe by using this method, which did not produce the expected response in quantifying the ferrozine complex and subsequently underestimating the dialyzable Fe content. Mung beans have high contents of phenolic compounds and phytate which can be solubilized during in vitro digestion and chelate iron (Hu et al., 2006), and these might be responsible for the matrix interference effect.

To further characterize these addition curves, the theoretical detection limits were calculated as: (3a/b) x 1 √n, where "a" is the independent term of the curve, "b" is the slope and "n" is the number of replicates used to prepare the calibration curves (Tormo

and Izco, 2004). The detection limit of the aqueous (non-added) curve was estimated to be 0.2 μg/mL, while the addition curves showed theoretical detection limits of 0.5-0.6 μg/mL. These results also supported the presence of matrix interference effects. Thus, the addition curves were used in further analysis to quantify Fe in the dialysate throughout the experiments.

Ferrozine method is being used to quantify Fe in food matrices such as fish (Roos et al., 2007), or wines under the conditions of in vitro digestion (Argyri et al., 2006). To the best of our knowledge, there is no report that monitors potential negative food-matrix interferences in quantifying Fe by using the ferrozine method.

IV.3.- Effect of household processing methods on Fe bioavailability.

In the present study, all values are expressed as percentages to allow the comparison of dialyzability values from both unfortified and fortified bean samples. The Fe content in the dialyzable fraction from raw and cooked mung beans, and ferritin concentration values in Caco-2 cultures exposed to gastrointestinal digests of raw, boiled and soak-boiled beans are shown in **Figure 3**.

The low dialyzable Fe contents from raw mung beans were below the theoretical detection limit estimated for the ferrozine assay, 0.6 μg/mL, in the experimental conditions used. When considering the cooked beans, the dialyzable Fe percentage in boiled beans was 9.1% which increased ($p<0.05$) up to 21.9% in soak-boiled beans. These results show that although the soak-boiling procedure decreased ($p<0.05$) total Fe content in beans (by 16.4%), it increased ($p<0.05$) Fe dialyzability. Thus, this observation suggests a higher ability of digestive enzymes to release Fe from the cooked than the raw food. In addition, from a physiological point of view, the increased dialyzability is important because increased Fe solubility in the gut lumen increases the amount of Fe that reaches the brush border membrane of enterocytes and in this way may improve the absorption (Ekmekcioglu, 2002). The hypothesis regarding the increased activity of the digestive enzymes was supported by the increasing ferritin concentration in Caco-2 cell cultures (Fig. 3) exposed to the digests from soak-boiled beans. Interestingly, the increased dialyzable Fe percentages in boiled mung beans had no effect on Fe uptake as concluded from the non statistically ($p>0.05$) significant differences in ferritin values between boiled and raw mung beans.

Our results indicate a positive effect of soak-boiling on Fe uptake, and are in good agreement with the enhanced Fe bioavailability (estimated by the predicted equation; $y = 0.4827 + 0.4707X$ where X is the percentage of ionizable Fe) in mung beans subjected to pressure cooking (Barakoti and Bains, 2007). The higher ($p<0.05$) dialyzable Fe content in soak-boiled mung beans can be explained by the fact that tissue softening during soaking could increase proteolysis, digestion and degradation of food components facilitating the release of Fe (Kapanidis and Lee, 1995).

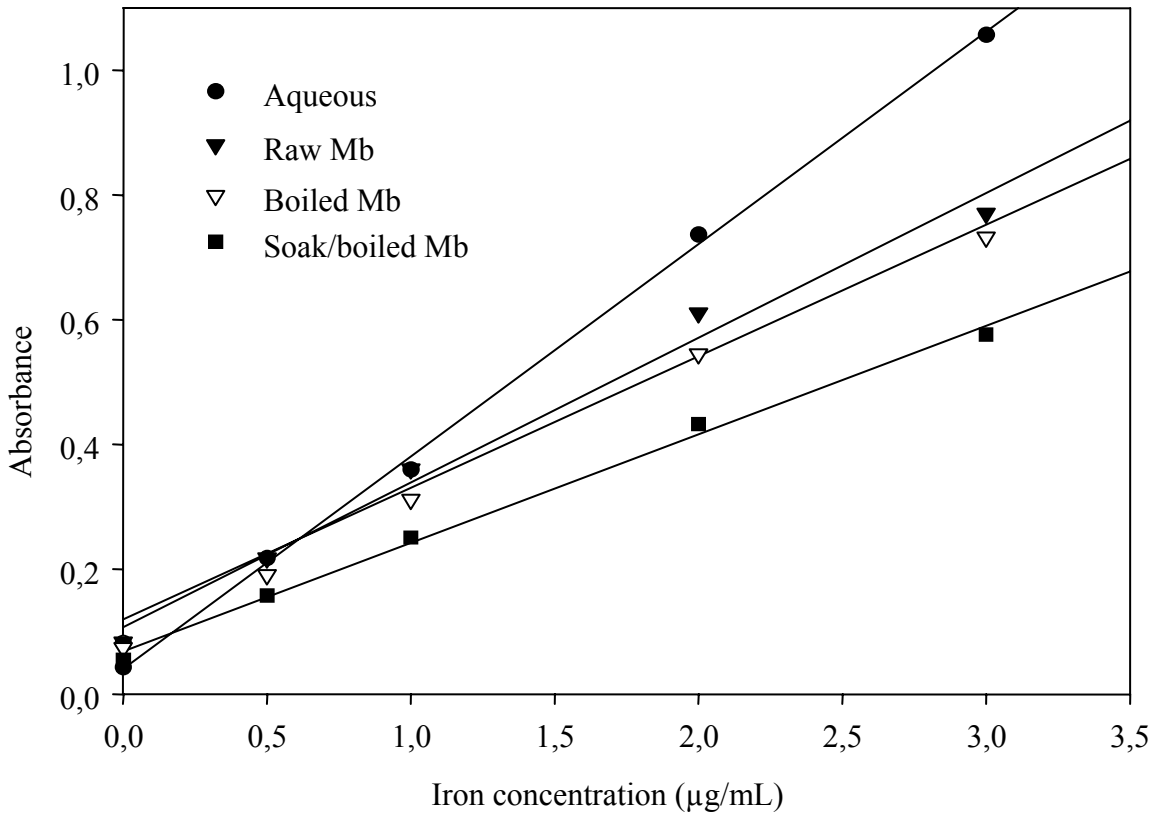

Figure 2. Typical regression from aqueous (non-added) curve and those added with dialysate (50%, v/v) from raw, boiled, and soak-boiled beans. The Fe concentrations were measured by using the ferrozine assay.

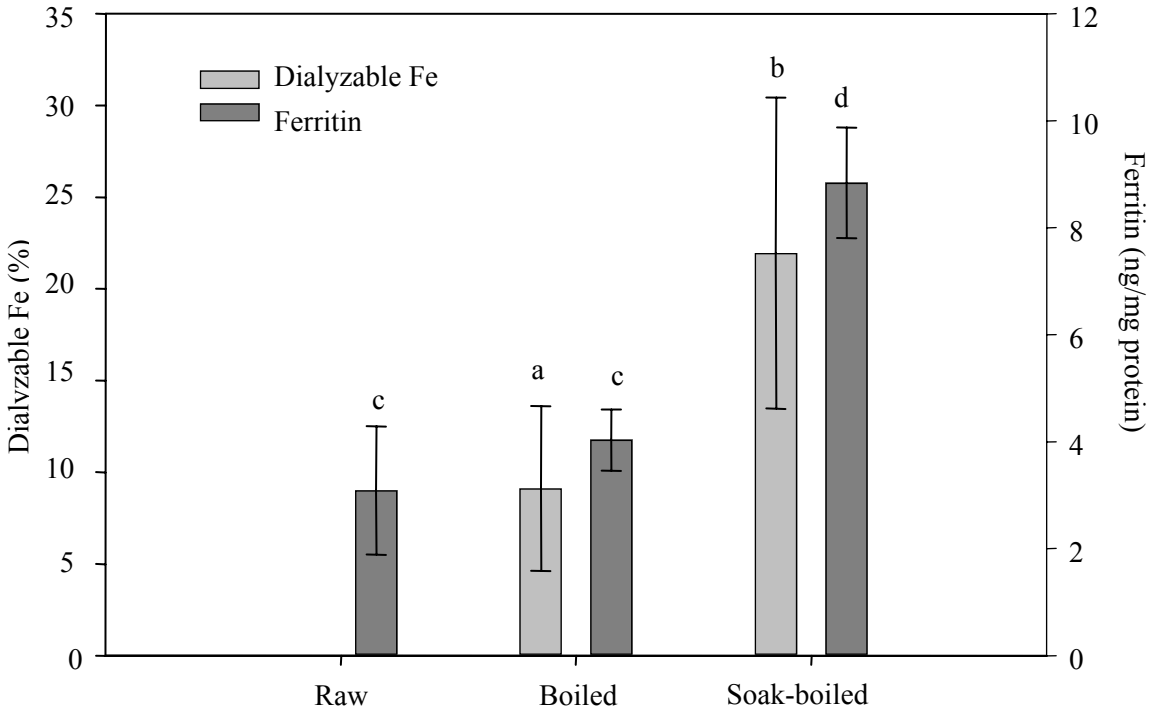

Figure 3. Iron (Fe) content in the dialyzable fraction from raw and cooked mung beans, and ferritin concentration values in Caco-2 cultures exposed to gastrointestinal digests of raw, boiled and soak-boiled beans. The cells grown in MEM alone had baseline ferritins of 2.1 ng/mg protein. Results are expressed as mean ± standard deviation (n=3). Different superscript letter indicate statistically ($p<0.05$) differences (gray bars compared to gray bars and dark gray bars compared to dark gray bars).

IV.4.- Effect of household processing methods in the removal of phenolics and phytate from mung beans.

Previous studies reported that polyphenols (Hu et al., 2006) and phytate (Welch et al., 2000) inhibit Fe absorption. The total polyphenol content, Fe-binding galloyl and catechol groups, and phytate content in the gastrointestinal digests from raw and cooked beans are shown in **Figure 4**.

Both of the cooking procedures – boiling and soak-boiling – caused increased total soluble phenolics content in the *in vitro* digest. From these results, we would expect lower Fe uptake values from cooked beans. However, we must bear in mind that different Fe-chelating ability has been reported for different polyphenols (Brune et al., 1991). The latter authors demonstrated that galloyl (trihydroxy-benzene) groups exhibit higher Fe-binding ability compared to the catechol (ortho dihydroxy) group (Brune et al., 1991). Thus, we further investigated the presence of these groups in the supernatant of the digested beans (Fig.3).

Cooking decreased the concentration of soluble catechol groups; the soak-boiled samples exhibited the lowest ($p<0.05$) soluble content of catechols in the in vitro digests. Although, the cooking procedures decreased the soluble galloyl content in the in vitro digests compared to the raw samples, there was no statistical ($p>0.05$) difference in the content of soluble galloyl between boiled and soak-boiled samples. Interestingly, the Fe uptake was significantly ($p<0.05$) higher only from the soak-boiled samples.

It is well known that Fe bioavailability from beans is also affected by other components found within the bean such as phytate, which is predominantly in the form of myo-inositol hexakisphosphate (IP6) (Welch et al., 2000). In the present study, differences on ferritin formation between raw and cooked beans cannot be attributed to the soluble phytic acid content quantified in the digests. As shown, phytic acid content in the digests from soak-boiled beans was 3.4- and 2.5-fold higher relative to the raw and boiled samples respectively. However, we cannot rule out the possibility of the breakdown of phytates as a consequence of the cooking effect (Barakoti and Bains, 2007). This observation may explain the high phytic acid content from the soak-boiled sample. We are assuming that all the phytic acid quantified is present as IP6, however, we can speculate that other degradation products formed during soak-boiling from IP6, for example inositol trisphosphate (IP3) and phosphate (PO_4) groups, might interact with the Wade (0.03% $FeCl_3$/ 0.3% sulfosalicylic acid) reagent, and subsequently overestimating the content of phytic acid. Further analysis using more accurate techniques in identifying specific degradation products from IP6, such as HPLC (High Performance Liquid Chromatography), would be helpful to confirm this hypothesis.

It has been reported that the breakdown of phytates and reduction of polyphenols content by household processing methods had a positive effect on enhancing mineral bioavailability (Barakoti and Bains, 2007), which is in accordance with the results presented in this study. However, not only removal of inhibitors could explain the obtained results, protein denaturation during cooking also renders proteins more susceptible to proteolytic enzymes and causes faster release of soluble digestion products that can increase Fe dialyzability by complexing Fe in low molecular weight complexes (Kapanidis and Lee, 1995).

IV.5.- Fe bioavailability from mung beans prepared by different cooking procedures and fortified with selected Fe fortificants.

Fe-containing solutions were prepared freshly on the day of the experiment, and there was no significant ($p<0.05$) difference in the Fe concentration in each of the solutions (11.1 ± 1.2 µg as Fe/mL, n=6). This concentration level was chosen by taking into account the low Fe dialyzability (%) previously reported (4%) from a $FeSO_4$ solution (0.7 µg as Fe/mL) (Laparra et al., 2008). Assuming this reported value for Fe dialyzability, we would need a solution with a concentration of Fe around 10 µg/mL to reach the detection limits estimated in the ferrozine assay (*see page 12*). In the present study the Fe dialyzability values estimated from the different fortificants used were: 15.68 ± 1.28% for $FeSO_4$ = 14.55 ± 0.33% for ferrous fumarate < 24.06 ± 0.95% for Sprinkles™. These results indicate that although the Fe concentration had been increased by more than 10 times compared to the study by the latter author, the Fe dialyzability did not increase linearly in the in vitro method.

The Fe dialyzability percentages from raw and cooked (boiled and soak-boiled) mung beans are shown in **Figure 5**. In raw beans, the Fe dialyzability percentages varied between 3.2 and 24.8% with significant ($p<0.05$) differences depending on the fortificant considered ($FeSO_4$ < ferrous fumarate < Sprinkles™). A similar pattern was observed for all fortificants in boiled and soak-boiled samples. There was no statistically significant ($p>0.05$) difference between $FeSO_4$ and ferrous fumarate in showing Fe dialyzability values, which are in the range of 3.1-4.1%. Meanwhile, Sprinkles™ produced the highest Fe dialyzability values in both samples; 27.9% from boiled and 45.0% from soak-boiled mung beans. These results suggest that in Sprinkles™, the encapsulated Fe within a lipid layer prevented its interaction with phenolics and phytate, which renders high dialyzable Fe.

From a physiological point of view, the solubility in the gut (i.e., bioaccessibility) is a pre-requisite for absorption (Ekmekcioglu, 2002). Taking into account this observation, higher Fe bioavailability from Sprinkles™ would be expected due to highest dialyzability values. The Fe uptake values estimated by quantifying cell ferritin formation are shown in **Figure 6**. Ferritin is a well known protein which works as an intracellular store of Fe (Arredondo et al., 1997; Glahn et al., 1998). Previous reports demonstrated that ferritin can be a good estimator of Fe uptake (Alvarez-Hernandez et al., 1991). Fortification using $FeSO_4$ showed similar ($p>0.05$) Fe dialyzability values (Fig. 4) in raw and cooked mung beans, however, cell ferritin concentration was highest from the raw relative to boiled and soak-boiled beans. Ferrous fumarate produced lower ferritin concentration than $FeSO_4$ in raw and cooked beans. Fortification with Sprinkles™ produced highest ferritin concentration compared to other fortificants. These results indicated that Sprinkles™ had the highest Fe uptake values, but we must bear in mind that this supplement contains ascorbic acid (35 mg/sachet), a well known promotor of Fe uptake by Caco-2 cells (Glahn et al., 1998), which can contribute to increased Fe bioavailability.

A previous in vivo study reported higher Fe bioavailability from cereal-based diet fortified with ferrous fumarate relative to the dissociable ferric phyrophosphate (Davidsson et al., 2000). Although the authors did not include a direct comparison with $FeSO_4$ bioavailability, based on their previous studies, they indicated a similar Fe bioavailability from both sources of Fe. These results are concordant with the slightly higher Fe dialyzability values estimated for $FeSO_4$ in the present study (Fig. 5) for boiled

and soak-boiled beans. However, it is important to point out that in our study $FeSO_4$ and ferrous fumarate showed differences in Fe uptake by Caco-2 cells from raw and cooked beans (Fig. 6), which resulted up to 35% lower for ferrous fumarate in raw beans.

Recently, Zlotkin et al. (2001) proposed "home fortification" with multiple micronutrient supplements (Sprinkles^{TM}), in a powdered form, to complementary weaning foods. They compared the efficacy of Sprinkles^{TM} to $FeSO_4$ dosed as drops. After 2 months of treatment, a significant but similar ($p<0.001$) increase in the hemoglobin concentration in both treatment groups, $FeSO_4$ versus Sprinkles^{TM}, relative to the initial condition, was showing the efficacy (56.3-57.7%) of this strategy to surmount anemia. This observation was correlated with increased ferritin concentrations; however, the mean value was significantly ($p<0.001$) higher in the drops group than in the Sprinkles^{TM} group despite the presence of ascorbic acid in the supplement (Zlotkin et al., 2001). This in vivo study may suggest that some other factors such as the physiological status in the in vivo situation can determine the absorption of Fe from the fortificants. When comparing those literature data with the results obtained in this study using the Caco-2 cell model, Fe uptake from fortified beans with Sprinkles^{TM} was higher ($p<0.05$) than $FeSO_4$.

The results of this in vitro model must be taken as relative indexes for bioavailability, which means that the methods provide a good basis for establishing tendencies and determining effects caused by different factors in the gut. However, the definitive or absolute values should be obtained by using human trials.

VI. Conclusions.

From the data presented we can conclude that cooking enhanced Fe bioavailability in non fortified beans. Iron bioavailability from beans fortified with Sprinkles^{TM} was higher than from either $FeSO_4$ or ferrous fumarate, thus, Sprinkles^{TM} supplementation can be helpful in improving Fe bioavailability by preventing interactions with well known inhibitors of Fe uptake.

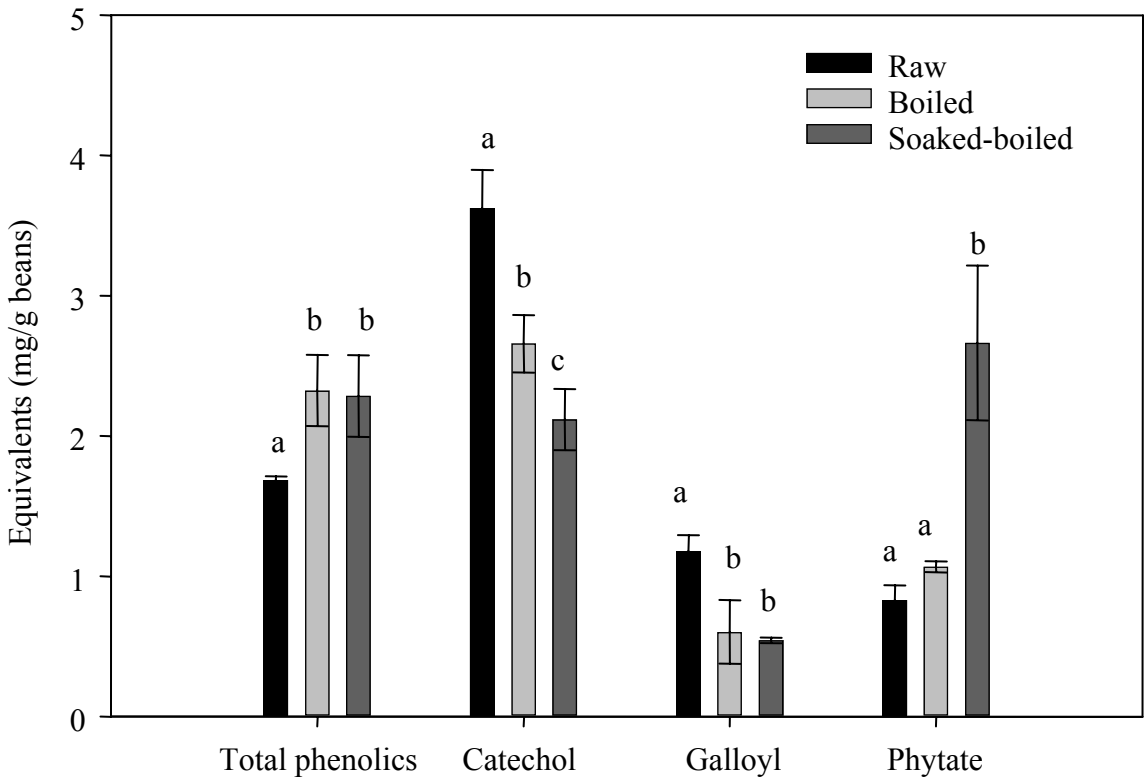

Figure 4. Total soluble polyphenols, Fe-binding galloyl and catechol groups, and phytate contents in the gastrointestinal digests from raw and cooked beans. Results are expressed as mean ± standard deviation (n=3). Different letters in the bars for each group indicate significant ($p<0.05$) differences.

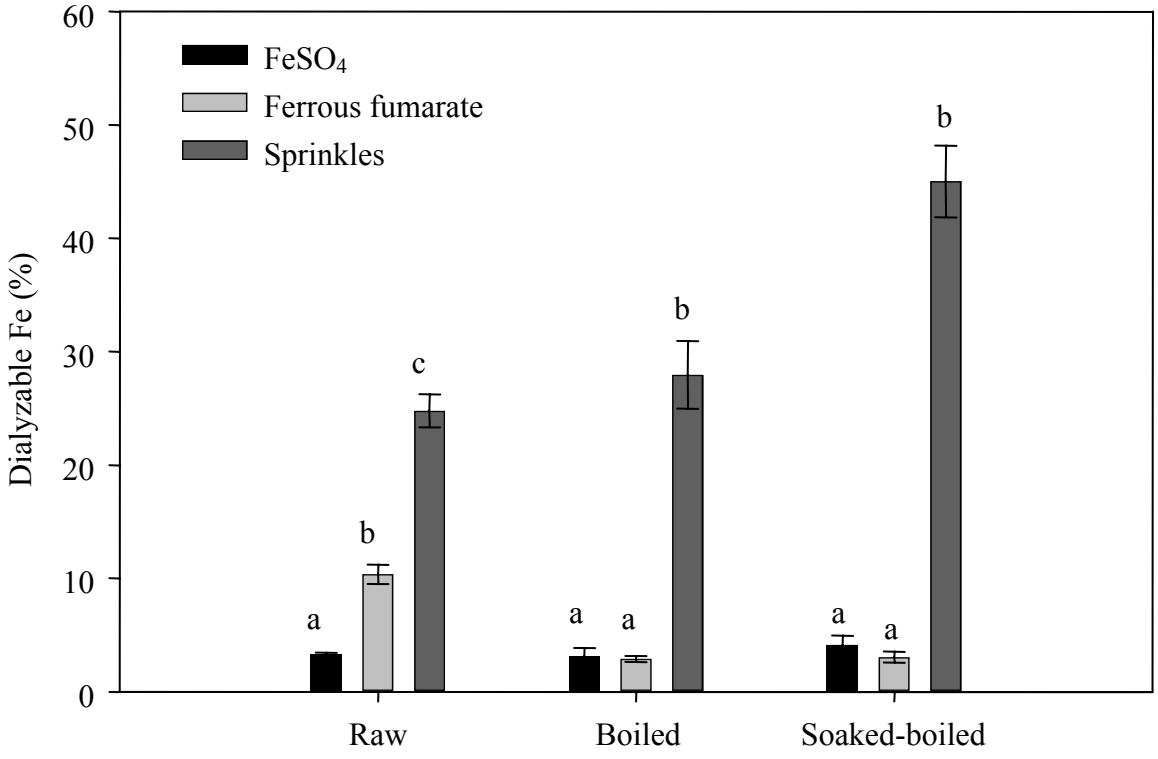

Figure 5. Fe dialyzability percentages from raw and cooked (boiled and soak-boiled) mung beans fortified with severeal fortificants. Results are expressed as mean ± standard deviation (n=3). Different letters in the bars within a group indicate statistical ($p<0.05$) differences.

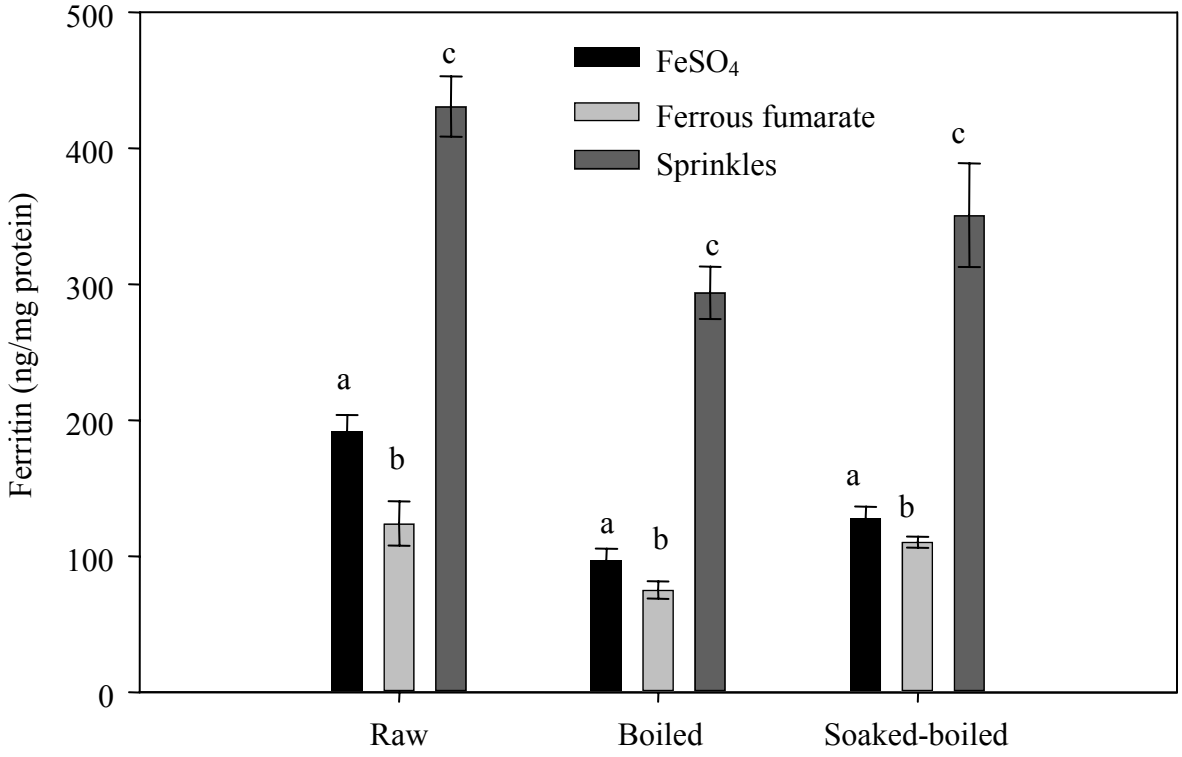

Figure 6. Caco-2 ferritin concentration values from raw, boiled and soak-boiled mung beans. The cells grown in MEM alone had baseline ferritins of 2.1 ng/mg protein. Results are expressed as mean ± standard deviation (n=3). Different letters in the bars within a group indicate statistical ($p<0.05$) differences.

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