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Seed Coat Removal Improves Iron Bioavailability in Cooked Lentils: Studies Using an in Vitro Digestion/Caco-2 Cell Culture Model

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ABSTRACT: In this study we examined the range of Fe concentration and relative Fe bioavailability of 24 varieties of cooked lentils, as well as the impact of seed coat removal on Fe nutritional as well as antinutrient properties. Relative Fe bioavailability was assessed by the in vitro/Caco-2 cell culture method. While the Fe concentration of the whole lentil was moderately high (72.8 ± 10.8 μg/g, $n = 24$), the relative Fe bioavailability was moderate (2.4 ± 1.0 ng of ferritin/mg of protein). Although removing the seed coat reduced the Fe concentration by an average of 16.4 ± 9.4 μg/g, the bioavailability was significantly improved (+5.3 ± 2.2 ng of ferritin/mg of protein; $p < 0.001$), and the phytic acid concentration was reduced by 7% ($p = 0.04$). Like most legume seeds, the lentil seed coat contains a range of polyphenols known to inhibit Fe bioavailability. Thus, along with breeding for high Fe concentration and bioavailability (i.e., biofortification), seed coat removal appears to be a practical way to improve Fe bioavailability of the lentil.

KEYWORDS: *lentil, dehulling, iron, bioavailability, Caco-2 cell culture, polyphenols, phytic acid*

INTRODUCTION

Fe deficiency is the most prevalent nutrient deficiency worldwide, with women and preschool-aged children being the most vulnerable.[1] Fe deficiency is most prevalent among populations relying on micronutrient-poor staple food crops, which may provide dietary Fe, but its bioavailability is limited due to processing and the presence of inhibitors of Fe absorption, such as phytic acid (PA), polyphenolic compounds, oxalates, and fiber.[2]

Lentils (*Lens culinaris* L.) are high in complex carbohydrates and rich in protein and are an excellent source of micronutrients, including Fe.[3] Like other pulses, lentils also contain antinutrients such as polyphenols and PA that impair micronutrient bioavailability. Lentils are generally cooked in water prior to consumption, which decreases antinutritional factors, as well as the total concentration of micronutrients such as Fe.[4,5] In many parts of the world, the seed coat of the lentil (~10% seed weight) is removed through a process known as decortication or dehulling prior to cooking and consumption.[6] In addition to improving palatability and decreasing cooking time, mechanical processing (e.g., bran, germ, or seed coat removal) has been shown to decrease not only fiber and some minerals (e.g., ~20% Fe), but also antinutrients, including polyphenols, of many varieties of grains and legumes, increasing the Fe bioavailability.[4,7,8]

Several methodologies have been used to assess the Fe bioavailability of foods, including both in vitro and in vivo models, as well as human feeding trials. The in vitro digestion/Caco-2 cell model is a well-validated method that simulates gastrointestinal digestion and absorption of Fe (measured as ferritin formation by Caco-2 cells) in the food matrix.[9] This method is inexpensive, capable of high throughput, and appropriate for screening a variety of foods and their combinations and as a first step in examining the level of biofortification or the genetic potential for biofortification of a target food crop.[9,10]

The objectives of the current study were (1) to determine the Fe concentration, relative Fe bioavailability, and PA concentration of several lentil varieties from a current commercial harvest in Saskatchewan (SK), Canada, using the in vitro digestion/Caco-2 cell culture model and (2) to assess the impact of seed coat removal (dehulling) on the Fe concentration, relative Fe bioavailability, and PA concentration in these varieties of cooked lentils. To our knowledge, this is the first study to assess the effect of dehulling on Fe bioavailability in cooked lentils.

MATERIALS AND METHODS

Lentil Varieties. Twenty-eight commercial lentil samples were received from Simpson Seeds Inc., Moose Jaw, SK, Canada, in July 2011. A general description of each of the 28 varieties used in the study is presented in Table 1. Twenty-four of the samples were whole seeds, and four were red split lentils (RSLs, seed coat removed, and no whole seed condition). A total of 58% ($n = 14$) of the whole seed samples received were the yellow cotyledon type with a green seed coat (referred to as green lentils), 33% ($n = 8$) were the red cotyledon type with a brown-gray seed coat (referred to as red lentils), and two samples were the yellow cotyledon type with a light brown seed coat, commercially known as the Spanish brown type (referred to as brown lentils). Eston (E), Richlea (R), and Laird (L) were the original green lentil varieties, and though no longer grown, the trade still refers to these as the small, medium, and large green size classes, respectively.

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Table 1. Description of the Lentil Varieties Studied

variety or sample name	market class	seed coat color	cotyledon color
CDC Red Rider	small red	gray	red
CDC Maxim	small red	gray	red
CDC Impact	small red	gray	red
MJ-7369-Crimson	small red	brown	red
CDC Rouleau	small red	gray	red
CDC Redcoat	small red	gray	red
CDC Redbow	extrasmall red	gray	red
CDC Imax	small red	gray	red
MJ-7337-RSL	red split	none	red
MJ-7325-RSL	red split	none	red
MJ-7341-RSL	red split	none	red
MJ-7370-RSL	red split	none	red
CDC Greenland	Laird (large green)	green	yellow
MJ-9774-R	Richlea (medium green)	green	yellow
MJ-7372-L	Laird	green	yellow
CDC Imvincible	Eston (small green)	green	yellow
CDC LeMay	French green	marbled/dark green	yellow
CDC Viceroy	Eston	green	yellow
CDC Impress	Richlea	green	yellow
MJ-9759-L	Laird	green	yellow
CDC Imigreen	Richlea	green	yellow
MJ-9763-E	Eston	green	yellow
MJ-7347-L	Laird	green	yellow
MJ-9765-L	Laird	green	yellow
MJ-9766-L	Laird	green	yellow
MJ-7321-L	Laird	green	yellow
CDC SB-1	Spanish brown	brown	yellow
Nicole MJ-9753-MB	Spanish brown	brown	yellow

The commercial lentil samples were harvested in fall 2010 and originated from several regional municipalities (RMs) within the brown and dark brown soil zones of Saskatchewan. A total of 50% of the lentil samples originated solely from Moose Jaw, RM 161 (n = 1 brown, 6 green, and 7 red lentils). The other 50% of the samples (n = 1 brown, 8 green, and 5 red) ranged from RM 128 (east) to RM 228 (west) and from RM 254 (north) to RM 45 (south). The majority of these samples originated from two to five locations, and five included Moose Jaw as one of the locations (n = 3 green, 1 brown, and 1 red). The yield, environmental, and soil data for each variety and at each of the growing locations were not examined in this study.

For whole seed samples, a 50 kg bag from commercial shipments was set aside after cleaning. A 250 g subsample from each bag was withdrawn and set aside for dehulling. For split red lentil samples, a 50 kg bag from commercial shipments was set aside for analysis. Those samples identified specifically by variety name originated from commercial seed samples. All others were considered blended commercial samples of unknown varietal origin, described only on the basis of seed size, seed coat color, and cotyledon color parameters commonly used in international lentil trade.

Lentil Seed Preparation and Processing. Prior to analysis, the whole lentil seed samples were dehulled at the University of Saskatchewan's Crop Development Centre (Saskatoon, SK, Canada) in a Satake TM-05 grain-testing mill (Satake Engineering Co. Ltd., Japan) and then separated into seed coat and unsplit cotyledon fractions. A portion (30 g) of the unsplit cotyledon fraction of each variety was shipped to the USDA-ARS (Agricultural Research Service, U.S. Department of Agriculture) Robert Holley Center for Agriculture and Health (Ithaca, NY) and used for analyses of the Fe concentration and relative Fe bioavailability.

Whole and dehulled lentils were rinsed with 18 MΩ deionized water, soaked for 15 min, and cooked in boiling 18 MΩ deionized water on an electric hot plate until tender, approximately 5−10 min. After being cooled to room temperature, the samples were frozen, freeze-dried, finely ground, and stored at room temperature (22 °C) prior to experimentation. The results presented here pertain to only cooked samples.

Assessment of the Fe Concentration. The iron concentration (μg/g) and other minerals were quantified using an inductively coupled argon-plasma emission spectrometer (ICP-ES, ICAP model 61E Thermal Jarrell Ash Trace Analyzer, Jarrell Ash Co., Franklin, MA). To prevent possible Fe contamination from laboratory utensils, all equipment used in the assessment of mineral and ferritin quantification was soaked in 10% HCl and rinsed with 18 MΩ deionized water before use.

Analysis of the PA Concentration. A Dionex liquid chromatography system (AS50 auto sampler, Dionex Corp., Sunnyvale, CA), equipped with a conductivity detector, model ED50, and a gradient pump, GS50, was used along with an IonPac AG11 guard column and IonPac AS11 column (4 × 250 mm) to quantify PA. PeakNet 6.40 software was used for data processing. The mobile phases were (A) 200 mmol/L NaOH (carbonate-free) and (B) deionized water, using a flow rate of 1 mL/min. PA was extracted from 0.25 g of a dry, lyophilized cooked lentil sample in 10 mL of a 1.25% H_2SO_4 solution. After a 2 h extraction, the samples were centrifuged at 3660g for 10 min. Subsamples were diluted 1:10 with deionized water, and 10 μL was injected; all samples were analyzed in duplicate.

In Vitro/Caco-2 Cell Cultures. An in vitro digestion/Caco-2 cell culture model was used to assess the Fe bioavailability of the cooked lentil samples.[9] Iron uptake by cell monolayers was assessed by measuring Caco-2 cell ferritin formation. The cooked lentil samples, both whole and dehulled, were subjected to simulated gastric and intestinal digestions. Briefly, gastrointestinal digestion is carried out in cylindrical inserts closed on the bottom by a semipermeable membrane and placed in wells of a six-well plate (Costar Corp., Cambridge, MA) containing Caco-2 cell monolayers (American Type Culture Collection, Rockville, MD) immersed in culture medium (minimum essential medium, MEM; Gibco, Rockville, MD). The upper chamber was formed by fitting the bottom of a Transwell insert ring (Corning Inc. Life Sciences, Tewksbury, MA) with a 1500 Da molecular weight cutoff membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA). The dialysis membrane was held in place using a silicone O-ring (Web Seal, Rochester, NY). For the experiment, 1.5 mL of the digested sample was added to the upper chamber of the insert and incubated for 2 h. The inserts were then removed, and 1 mL of MEM was added. Cell cultures were then incubated for 22 h at 37 °C. After incubation, growth medium was aspirated from the cell culture wells and the cells were washed twice with buffer solution (140 mmol/L NaCl, 5 mmol/L KCl, and 10 mmol/L PIPES at pH 7.0). The cells were then harvested by adding an aliquot of deionized water and placing them in a sonicator (Lab-line Instruments, Melrose Park, IL).

Assessment of Fe Bioavailability. The protocols used in the analysis of concentrations of ferritin (fer) and total protein (pro) of the Caco-2 cells were similar to those previously described.[11] Ferritin and total protein concentrations were determined on an aliquot of the harvested cell suspension with a one-stage sandwich immunoradiometric assay (FER-IRON II ferritin assay, Ramco Laboratories, Houston, TX) and a colorimetric assay (Bio-Rad DC protein assay, Bio-Rad, Hercules, CA), respectively. We used the ratio of ferritin to total protein (ng of fer/mg of pro) as an index of the cellular Fe uptake.

Statistical Analysis. Each outcome variable presented in this study (Fe concentration, relative Fe bioavailability) represents three replicates of the experimental method. Descriptive statistics are presented as means ± standard deviations (SDs). To compare means between whole and dehulled lentils for all outcome variables, one-way analysis of variance (ANOVA) in SPSS (version 19.0, IBM, Armonk, NY) was completed using post hoc tests adjusted for multiple comparisons (Bonferroni method). Pearson's correlations were used to examine associations between variables. A p value of <0.05 indicated statistical significance.

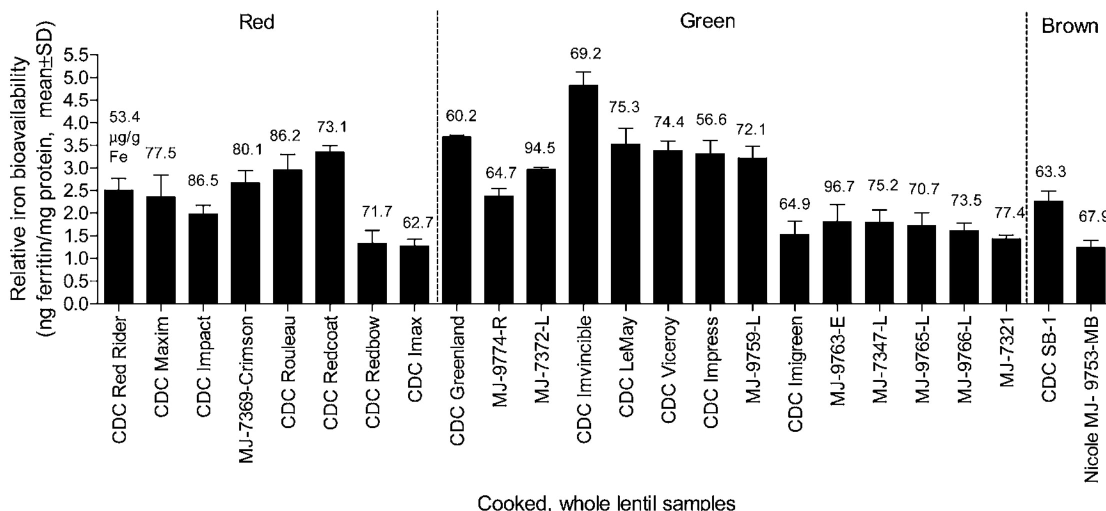

Figure 1. Relative Fe bioavailability (ng of ferritin/mg of protein) and Fe concentration (μg/g, above each bar) of cooked, whole lentil varieties (n = 24) assessed using the in vitro/Caco-2 cell model.

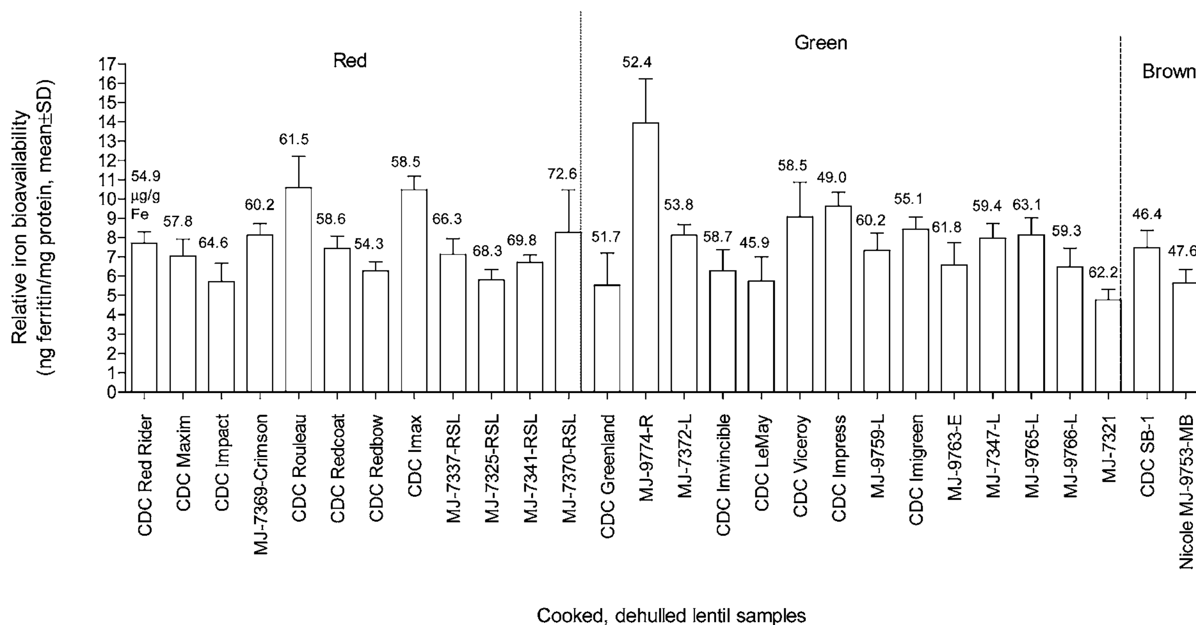

Figure 2. Relative Fe bioavailability (ng of ferritin/mg of protein) and Fe concentration (μg/g, above each bar) of cooked, dehulled lentil varieties (n = 28) assessed using the in vitro/Caco-2 cell model.

■ RESULTS AND DISCUSSION

Whole Lentils. The Fe concentration of the whole lentils was moderately high (mean 73 μg/g), indicating that some of the samples could be considered "biofortified" (>90 μg/g). The mean Fe concentration of the whole lentils was 72.8 ± 10.8 μg/g among the 24 samples tested and ranged from 53.4 to 96.7 μg/g. As shown in recent in vivo trials by our group, those lentil harvests that are high in Fe have the potential to provide a nutritionally significant increase in Fe absorption relative to lentils or other legumes that are lower in Fe.[10]

The relative Fe bioavailability (ng of fer/mg of pro) of the individual whole lentil samples is shown in Figure 1, along with each sample's Fe concentration above each bar. The relative Fe bioavailability of whole lentils ranged from 0.3 (CDC Redbow) to 4.8 ng of fer/mg of pro (CDC Imvincible), the mean of the samples being 2.4 ± 1 ng of fer/mg of pro. The PA concentration of the whole lentils ranged from 8.6 (Nicole MJ-9753 MB) to 19.2 (MJ-7347-L) μmol/g, the mean of the samples being 13.6 ± 2.9 μmol/g. When analyzed by lentil color group, there were no significant differences in Fe concentration, relative Fe bioavailability, or PA concentration among the three groups of cooked whole lentils.

Dehulled Lentils. The iron concentration of the dehulled lentils ranged from 45.9 μg/g (CDC LeMay) to 69.2 μg/g (MJ-7341-RSL), the mean of the samples being 57.7 ± 6.0 μg/g. The relative Fe bioavailability of the dehulled lentil samples is shown in Figure 2, along with each sample's Fe concentration above each bar. Differences in Fe bioavailability among the 24 commercial sample lines remained after dehulling. The relative Fe bioavailability of the dehulled lentils ranged from 4.76 (MJ-7321) to 13.94 (MJ-9774-R) ng of ferritin/mg of protein, the mean of the samples being 8.0 ± 2.2 ng of fer/mg of pro. The PA concentration of the dehulled lentils ranged from 8.5 (Nicole MJ-

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Table 2. Effect of Dehulling on the Fe Concentration and Relative Fe Bioavailability in Different Color Classes of Cooked Lentils[a]

	iron concn[b] (μg/g)		relative iron bioavailability[b] (ng of ferritin/mg of protein)		phytic acid concn[c] (μmol/g) (mg/g)		PA:FE[d]	
color class	whole	dehulled	whole	dehulled	whole	dehulled	whole	dehulled
red (n = 12)	73.9 ± 11.4	62.3 ± 6.0 a	2.2 ± 1.0	7.6 ± 1.6	12.3 ± 1.8 8.1 ± 1.2	13.6 ± 1.4 a 9.0 ± 0.9	9.4 ± 0.9	12.5 ± 1.6
green (n = 14)	73.2 ± 11.2	56.5 ± 5.3 b	2.7 ± 1.0	7.7 ± 2.3	14.9 ± 2.7 9.9 ± 1.8	12.6 ± 2.5 a 8.3 ± 1.7	11.7 ± 2.7	12.6 ± 3.1
brown (n = 2)	65.6 ± 3.3	47.0 ± 0.9 b	1.8 ± 0.7	6.6 ± 1.3	8.9 ± 0.5 5.9 ± 0.3	9.2 ± 1.0 b 6.1 ± 0.7	7.6 ± 0.8	10.9 ± 1.4
total (n = 28)	72.8 ± 10.8	57.7 ± 6.0	2.4 ± 1.0	8.0 ± 2.2	13.6 ± 2.9 8.9 ± 1.9	12.7 ± 2.3 8.4 ± 1.5	10.6 ± 2.6	12.5 ± 2.5

[a]Mean ± SD (n = 24 whole/dehulled lentil varieties + 4 RSLs). Mean scores followed by different Roman letters within columns are significantly different between color classes for that variable. [b]$p < 0.001$. [c]$p = 0.04$. [d]$p = 0.004$ between dehulled and whole lentils in the complete sample.

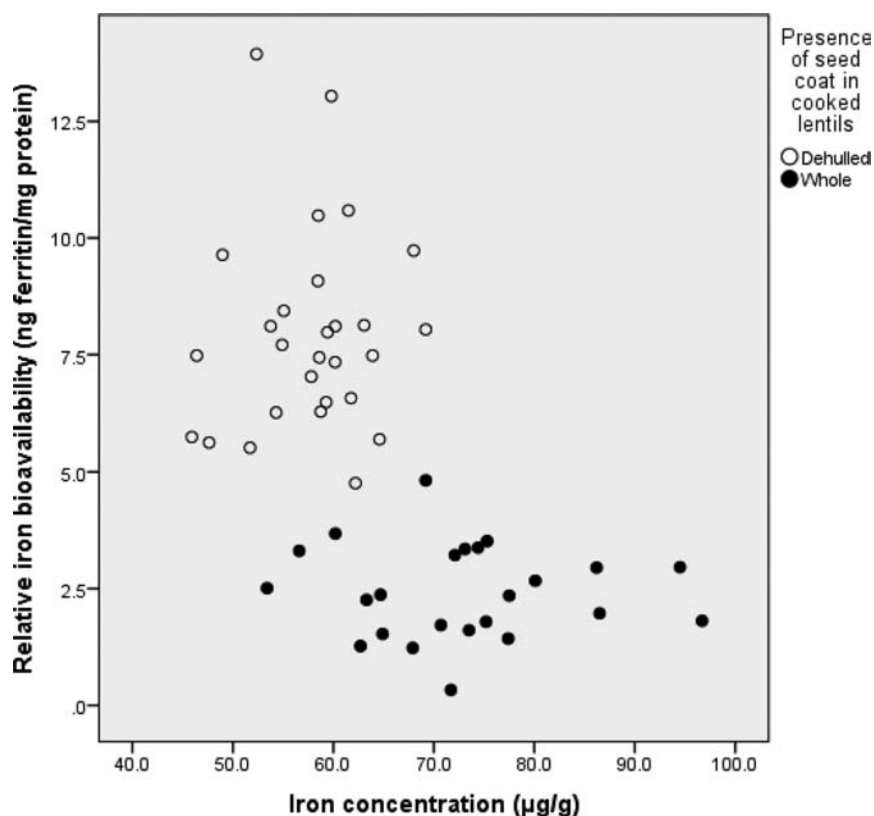

Figure 3. Relationship between Fe concentration and relative Fe bioavailability (ng of ferritin/mg of protein) in cooked lentil that have been analyzed with and without the seed coat.

9753 MB) to 16.23 (MJ-9765-L) μmol/g, the mean of the samples being 12.7 ± 2.3 μmol/g.

Effect of Dehulling on the Fe Concentration, Relative Fe Bioavailability, and PA Concentration. The Fe concentration, relative Fe bioavailability, and PA concentration of the whole and dehulled samples are shown in Table 2. Removing the seed coat significantly decreased the Fe concentration across the 24 varieties tested by an average of 16.4 ± 9.4 μg/g ($p < 0.001$). Differences in Fe concentration after dehulling ranged from −40.7 μg/g (MJ 7372-L) to +1.5 μg/g (CDC Red Rider). After dehulling, red lentils retained significantly more Fe than either green or brown lentils. While 26% of the Fe was lost during the dehulling process, we did have one sample that increased in Fe concentration after dehulling (CDC Red Rider) without any obvious signs of contamination during our processing (analysis of aluminum and titanium via ICP-ES). Although we take all precautions to minimize heavy metal contamination during each step of our analyses, the lack of a decrease in the Fe concentration in this sample suggests some type of Fe contamination occurred during or after dehulling.

Regardless of the color classification, seed coat removal significantly increased the relative Fe bioavailability across the 24 lentil samples tested by 5.3 ± 2.2 ng of ferritin/mg of protein ($p < 0.001$). Increases in the relative Fe bioavailability of the samples after dehulling ranged from 1.5 (CDC Imvincible) to 11.6 (MJ-9774-R) ng of fer/mg of pro. Although removal of the seed coat resulted in ∼26% loss of Fe, most of the polyphenols (inhibitors of Fe absorption) are localized to the seed coat[12] and include a large range of compounds such as catechins, procyanidins, and flavanols.[13,14] In the current study, the relative Fe bioavailability was improved by more than 2-fold after dehulling, which is even greater than that in previous studies of legumes (bioavailability

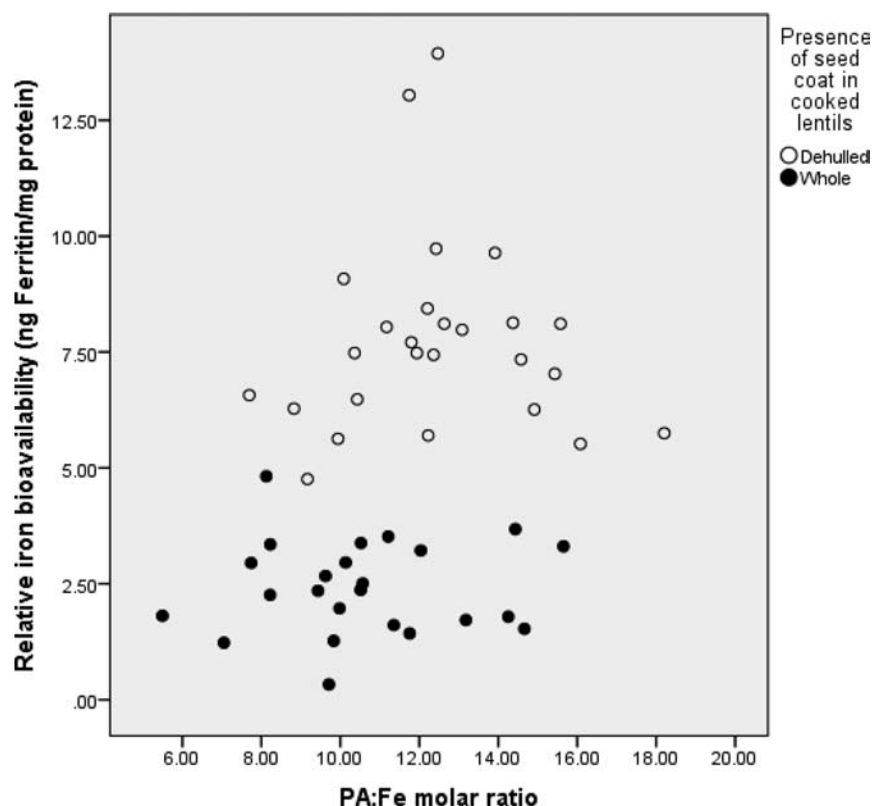

Figure 4. Relationship between the PA:Fe molar ratio and relative Fe bioavailability in cooked lentils that have been analyzed with and without the seed coat.

increases of 30−50%)[15] and overshadows the Fe lost to dehulling. While we did not profile the polyphenolic compounds in the lentils tested in the current study, we hypothesize that the observed improvement in the relative Fe bioavailability is likely attributable to a loss of those inhibitory compounds present in the seed coat.

Figure 3 shows the relationship between Fe concentration and relative Fe bioavailability in whole and dehulled lentils. In the complete sample, the Fe concentration and relative Fe bioavailability were significantly correlated ($r = -0.56$, $p < 0.001$), which is in agreement with our previous study of dehulled lentils.[16] However, these two variables were not significantly correlated in the whole ($r = -0.05$, $p = 0.80$) or the dehulled samples ($r = 0.08$, $p = 0.70$). In this study, we used the in vitro digestion/Caco-2 cell culture model to calculate the relative Fe bioavailability from cellular ferritin formation. These estimates of Fe bioavailability are independent of host effects (e.g., body Fe status), which greatly influence human Fe absorption.[17] Also, this model enables us to factor the effects of the food matrix, including inhibitors and enhancers of Fe absorption, into the estimates of Fe bioavailability. Given the relationship between Fe concentration and bioavailability observed in the current and previous studies and the fact that Fe bioavailability exerts a great influence on Fe absorption from meals, such a model is useful when planning human studies.

The PA concentration was significantly decreased after dehulling (−1.2 ± 2.5 μmol/g, $p = 0.04$), and differences ranged from −6.1 to +2.9 μmol/g. The PA:Fe molar ratio was significantly increased after dehulling (+1.9 ± 2.7, $p = 0.01$), and differences ranged from −2.6 to +7.0. Figure 4 shows the relationship between the PA:Fe molar ratio and relative Fe bioavailability in whole and dehulled lentils. The PA:Fe molar ratio was significantly correlated with both the relative Fe bioavailability ($r = 0.31$, $p = 0.03$) and the Fe concentration ($r = -0.53$, $p < 0.001$) in the complete sample and with the Fe concentration in the whole ($r = -0.45$, $p = 0.03$) and dehulled ($r = -0.38$, $p = 0.05$) samples. The relationship between the PA concentration and Fe bioavailability was recently reported in a study of sorghum processing, showing that significantly reducing PA via germination and fermentation increased the relative Fe bioavailability.[18] While dehulling lentils in the current study resulted in a 7% reduction in the PA concentration, it also resulted in a 15% increase in the PA:Fe molar ratio. The majority of PA resides in the seed;[19] thus, the increase in the PA:Fe molar ratio is likely due to the Fe concentration effect, as 26% of the Fe was lost during dehulling and was corrected for in the PA:Fe molar ratio calculation.

The PA concentration alone was not significantly correlated with either variable in the complete sample, nor were there any significant relationships between variables in the whole or dehulled subgroups. This finding is in agreement with previous studies, demonstrating that these traits are genetically independent of each other.[20] However, this is in contrast to our previous study of dehulled lentils, in which we found clear negative correlations between the PA concentration, Fe concentration, and Fe bioavailability.[16] A recent human trial suggested that the PA (3.0:1 vs 3.3:1 PA:Fe molar ratio per serving), and not the polyphenolic content (74 vs 158 mg of gallic acid equivalents/serving), was the main inhibitor of Fe absorption in white- and red-cowpea-based meals.[21] That study, however, used whole and not dehulled cowpeas of different colors. Human and Caco-2 studies have shown maximal inhibitory effects of PA at ratios of 10:1 or higher. In the present study, our ratios ranged from 5.5 to 18.2, which are comparable

to that of the common bean.[22] Lentils grown in cooler temperatures, such as those in Canada, are naturally lower in PA compared to other crops due to an environmental interaction during seed filling,[19,23] further increasing the biofortification potential of this crop.

In conclusion, these in vitro findings show the range of Fe concentration and relative Fe bioavailability in a wide range of commercial lentil samples, as well as the effect of dehulling on these nutritional traits. We now have a baseline level of the current Fe concentration and bioavailability for lentils grown in Saskatchewan, Canada. This information is important for the planned breeding program to biofortify lentils with Fe. Seed coat removal significantly enhanced the relative Fe bioavailability of lentils primarily through the removal of polyphenolic compounds in the seed coat, and not PA. Given the strong effect of the seed coat on Fe nutritional quality, it is therefore important to improve our knowledge of seed coat genetics to effectively breed and market lentils to consumers. Dehulling and other forms of food processing need to be factored into the discussion of the nutritional quality of lentils, as well as other pulses and legumes.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Fe, iron; PA, phytic acid; PIPES, piperazine-*N*,*N*′-bis[2-ethansulfonic acid] sodium salt; fer, ferritin; pro, protein

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