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Article

Effect of Traditional Household Processes on Iron, Zinc and Copper Bioaccessibility in Black Bean (*Phaseolus Vulgaris* L.)

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Abstract: Micronutrient deficiencies are a major public health problem. Beans are an important plant-based source of iron, zinc and copper, but their absorption is reduced in the presence of anti-nutrients such as phytates, polyphenols and tannins. Soaking and discarding the soaking water before cooking is unanimously recommended, but this can result in mineral loss. Data on the consequences for mineral bioaccessibility is still limited. This study aimed to evaluate iron, zinc and copper bioaccessibility in black beans cooked (regular pan, pressure cooker) with and without the soaking water. Minerals were quantified by ICP-MS, *myo*-inositol phosphates ($InsP_5$, $InsP_6$) by HPLC ion-pair chromatography, total polyphenols using Folin-Denis reagent and condensed tannins using Vanillin assay. Mineral bioaccessibility was determined by *in vitro* digestion and dialysis. All treatments resulted in a statistically significant reduction of total polyphenols (30%) and condensed tannins (20%). Only when discarding the soaking water a loss of iron (6%) and copper (30%) was observed, and $InsP_6$ was slightly decreased (7%) in one treatment. Bioaccessibility of iron and zinc were low (about 0.2% iron and 35% zinc), but copper presented high bioaccessibility (about 70%). Cooking beans under pressure without discarding the soaking water resulted in the highest bioaccessibility levels among all household procedures. Discarding the soaking water before cooking did not improve the nutritional quality of the beans.

Keywords: beans; iron, zinc and copper bioaccessibility; *myo*-inositol phosphates; anti-nutrients; polyphenols; household processing.

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1. Introduction

Deficiencies of micronutrients are a major public health problem, in which iron and zinc malnutrition affects more than half of the population worldwide [1,2]. Iron deficiency anemia reaches more than 30% of world's population, approximately 20% in European Union and up to 40% in developing countries [1,3]. It contributes to 20% of maternal deaths besides being related to

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adult's low productivity at work [3,4]. Outcomes of zinc deficiency are depressed growth, immune dysfunction, lower respiratory tract infections, diarrhea, altered cognition and other clinical conditions [4,5]. Copper deficiency may also lead to anemia, but features of human copper deficiency mechanisms are still unknown [6], while most copper research is focused on soil, fruits and nuts [e.g. 7,8].

The major reason for iron deficiency is a poor availability of iron from the diet. Mineral deficiencies, however, are not only caused by low dietary intake. Many other factors affect the absorption such as the total content of the minerals and anti-nutrients, the processing applied and mineral interactions [9,10]. Beans are highly nutritious leguminous grains consumed worldwide, and an important plant-based source of iron, zinc and copper. They are part of many traditional diets, playing a major role in vegetarian diet in all countries [11,12], and common beans are a staple food in Latin America and Eastern Africa [13,14]. A portion per meal of cooked beans (100 g) [11] contains 6.6-10.0 mg iron, 0.93-1.21 mg copper and 3.18-3.60 mg zinc, which equals the daily requirements for healthy adults for iron and copper and half of that of zinc (8 mg/day, 0.9 mg/day and 8-11 mg/day, respectively) [15]. Therefore, a regular intake of beans could contribute to minimize deficiencies of micronutrients [11]. The nutritional quality of beans however, is usually reduced by the presence of anti-nutrients, such as phytates, polyphenols and tannins [9,14]. Those compounds bind to minerals such as iron, zinc, copper, calcium and magnesium, thus reducing bioavailability due to the formation of extremely insoluble salts or very poorly dissociated chelates.

Especially phytates ($InsP_6$), have been reported to affect iron and zinc absorption negatively even at low concentrations [9,16-18]. Condensed tannins are able to form tannin-protein complexes, which can chelate iron and calcium [9,19,20]. A reduction of mineral bioavailability was observed when condensed tannins concentration was higher than 10% of the total dry weight of the samples or ranging from 2.5 to 4.7 mg eq. CE g^{-1} [20,21]. With regard to polyphenolic compounds, it has been reported that they reduce bioavailability of some minerals. Although there is no consensus on the quantity needed to decrease iron absorption in beans, a reduction in iron bioavailability was observed above 50 mg of polyphenols [20,22]. Furthermore, the polyphenols in legumes have been extensively correlated with health benefits in humans due to their potent anti-oxidant activities [23,24]. In common beans those bioactive compounds mostly comprise phenolic acids and condensed tannins which are found in the cotyledons, and exhibit anti-diabetic, anti-obesity, anti-inflammatory, anti-mutagenic and anti-carcinogenic effects [23-25].

In a recent study [26], polyphenols of black beans were individually examined for their effect on iron uptake by Caco-2 cells. Half of the polyphenols studied were shown to inhibit iron absorption, but the other half were found to clearly promote iron absorption. So far, many studies [16,17,20,27] reported the link between a reduction of the total content of anti-nutrients in food grains with a higher availability of iron and zinc. Food processing and food preparation techniques like soaking, germination, hydrothermal treatment and fermentation can reduce the content of anti-nutrients [9,18,27]. Soaking and discarding the soaking water before cooking beans has been unanimously recommended due to a higher reduction of the anti-nutrients. An average reduction of 20% to 30% of condensed tannins and total polyphenols can be obtained in legumes by applying household processes [20,27,28]. The effect on mineral bioavailability was assessed in those studies mainly by molar ratios and statistical correlations between the content of anti-nutrients and the mineral content [20,27,28]. In general, digestibility and not bioavailability assays were applied in those studies. Bioavailability and bioaccessibility are often used indistinctly [29].

Only direct feeding trials can fully determine biological efficacy and mineral interactions, but they are long-lasting, cost intensive, and nonetheless the results need to be extrapolated to the human organism. A simple method to estimate the effect of for example food processing on mineral bioavailability is the use of bioaccessibility assays [13,17]. Although there is a substantial amount of information about binding of iron and zinc, and anti-nutrients reduction by food processing, data on the consequences for mineral absorption are still limited. Discarding the soaking water before cooking beans can result in loss of minerals and anti-oxidants and thus the nutritional quality of cooked beans is not necessarily improved. Thus, this study aimed to evaluate iron, zinc and copper bioaccessibility in black beans cooked with and without the soaking water using traditional household processes in order to expand the knowledge about the nutritional value of this basic and accessible food and the options to use beans in combating micronutrients deficiencies.

2. Materials and Methods

All glassware used in sample preparation and analyses was washed in distilled water and for mineral analysis also immersed in a 5% nitric acid solution for more than 1 hour and rinsed with ultrapure water (Milli-Q, Millipore).

2.1. Samples

Three different batches of common beans (*Phaseolus vulgaris* L., black bean variety) from markets in Rio de Janeiro, Brazil (of geographic origin: São Paulo, – 23° 10' 45 S, 45° 53' 12 W) were used in this study. All batches were harvested in 2015. The black bean samples were sent to Germany (Max Rubner-Institut, Karlsruhe) for processing, and the experiments were performed in a period of one year. The samples were stored at 4 °C with an extra vacuum-packaging. The raw grains were cleaned before use. All dirt was removed manually and then the beans were washed with deionized water. For analysis they were freeze-dried and finely ground in a stainless steel grinder. The cooked bean samples were analyzed with the broth and a quarter technique was applied to obtain two final fractions properly homogenized for each analytical determination.

2.2. Household treatments

In order to simulate traditional household processes for cooking beans, an overnight soaking (12 hours) at room temperature was performed, followed by two cooking methods (boiling and pressure cooking) in tap water. Three different batches of black beans were used. A proportion of 100 g of the black beans and 400 mL of water were used for soaking. The following cooking strategies were performed: 1) with the soaking water in a pressure cooker; 2) without the soaking water in a pressure cooker; 3) with the soaking water in a regular pan; and 4) without the soaking water in a regular pan. The regular pan had a capacity of 3 L and the beans were cooked for 35 minutes. 200 mL of tap water were added during cooking to replenish the loss of evaporated water. The pressure cooker had a capacity of 3 L and the beans were cooked for 5 minutes. No water was added during the cooking process. The cooking times were chosen according to the results of a test cooking simulation. Before cooking the black beans, either tap water was added to the soaking water to give a final volume of 600 mL or the soaking water was discarded and replaced by tap water to give a final volume of 600 mL. All treatments were performed in duplicate. The same cooking methods were also performed without bean samples to quantify the concentrations of the minerals in the water after the cooking process, either present in the tap water or released from the pressure cooker or pan (Appendix A).

2.3. Myo-inositol phosphates

Quantification of *myo*-inositol phosphates was performed by extracting 1 g of a freeze-dried sample with 20 mL of 2.4% HCl for 3 h with constant shaking at room temperature. The resulting suspensions were centrifuged (30 min, 15000 rpm). The supernatant was collected and used for *myo*-inositol phosphate quantification [30]. 2 mL of the supernatant were diluted with ultrapure water to give a final volume of 60 mL. The entire solution was applied to a column (0.7 × 15 cm) containing 0.5 g of AG 1-X4 200–400-mesh resin. The column was washed with 25 mL of ultrapure water and 25 mL of 25 mM HCl. Then *myo*-inositol phosphates were eluted with 25 mL of 2 M HCl. The eluates obtained were concentrated in a vacuum evaporator (at 40 °C) and dissolved in 1 mL of

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ultrapure water. Then 20 μL of the samples were chromatographed on Ultrasep ES 100 RP18 (2 × 250 mm). The column was run at 40 °C and 0.2 mL min^{-1} of an eluent consisting of formic acid/methanol/water/tetrabutylammonium hydroxide (44:56:5:1.5 v/v), pH 4.25. A mixture of the individual *myo*-inositol phosphate esters ($InsP_3$–$InsP_6$) was used as a standard [31].

2.4. Total polyphenols

Total phenols were extracted with water. An internal standard curve was prepared by adding 10 mL of 0–0.01% tannic acid to the flasks. The flasks were heated for 30 min at 70 °C with constant shaking. Clear supernatants were collected after centrifugation at 2500 *g* for 15 min followed by filtration. Polyphenols were determined using the Folin–Denis reagent [32].

2.5. Condensed tannins

Condensed tannins were extracted with HCl:methanol (1:100 v/v) for 2 h with mechanical shaking at 25 °C and centrifuged at 5000 *g* at 15 °C for 15 min. Aliquots were immediately analyzed for tannins using the 0.5 % vanillin assay [33].

2.6. Minerals

Iron (Fe), zinc (Zn), copper (Cu) and calcium (Ca) concentrations were measured. Therefore, 150 mg of each ground sample was microwave-digested in a MWS-1 (Berghof Products + Instruments GmbH, Eningen, Germany) with 3 mL of concentrated HNO_3 (65% v/v) and 0.75 mL of H_2O_2 (30% v/v). Heating was performed in four successive steps: linear temperature increased up to 150 °C in 5 min (80 W); 5 min at 150 °C (70 W); linear temperature increased up to 180 °C in 40 min (80 W); 10 min at 180 °C (80 W). All samples were analyzed in triplicate and a set of digestion blanks were prepared with each sample batch. The data was expressed as mean ± standard deviation on dry matter (DM) basis.

Element analysis was performed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS), iCAP Q (Thermo Scientific, Waltham, Massachusetts, United States). The ICP-MS operating conditions and measurement parameters are given in Table 1. Standard addition was used for calibration. The limit of quantification (LOQ) was calculated based on the measured values of the blanks (n = 152), where LOQ = mean + 10 x standard deviation. The Extreme Studentized Deviate Test was used to remove outliers from the data set. Fresh Kidney beans NCS ZC73019 (GSB-12) was used as reference material (n = 84) to determine precision and accuracy of the method (Table 2). The relative standard deviations were less than 3% for all investigated elements, and at a 95% confidence level showed that there was no significant difference between the means of the certified and determined values for the analytes under investigation.

Table 1. ICP-MS operating conditions and measurement parameters.

Parameter	**Value**
RF power	1550 W
Argon flow rates	
Cooling	13.8 L min^{-1}
Auxiliary	0.65 L min^{-1}
Nebulizer	1.05 L min^{-1}
Sample cone	Ni
Skimmer cone	Ni
Analyte	43Ca, 56Fe, 65Cu, 66Zn
Internal standard	103Rh (Fe, Cu, Zn), 45Sc (Ca, Fe), 89Y (Fe, Cu), 72Ge (Ca, Zn), 115In (Cu, Zn)
Aquisition/scanning mode	STD (Ca), KED (Fe, Cu, Zn)
Sweeps per reading	100

Dwell time	10 ms (Ca, Cu, Zn); 40 ms (Fe)
No. of runs	5
Replicate time	21 s
Sample uptake rate	0.2 mL min^{-1}
Wash time between samples (2% HNO_3)	30 s
Sample delay	50 s
Stabilization time	5 s

Table 2. ICP-MS precision and accuracy of the method.

Element	LOQ (µg kg^{-1})	Reference material measured value (mg kg^{-1})	Reference material certificate value (mg kg^{-1})
Ca	29.8	0.66 ± 0.06	0.67 ± 0.04
Fe	1.8	306 ± 29	330 ± 20
Cu	7.2	8.4 ± 1.5	8.7 ± 0.5
Zn	6.6	34 ± 4	32 ± 2

2.7. Iron, zinc and copper bioaccessibility

Iron, zinc and copper bioaccessibilities in black bean samples were determined based on *in vitro* digestion and dialysis method described by [34] with modifications. For gastric digestion, 10 g of ground sample were suspended in 60 mL of 20 mM glycine-HCl buffer, pH 2.0. After, adjusting pH to 2.0 by with 2 M HCl, 1.3 mL of pepsin (porcine, Fluka) solution (1.6 g pepsin in 10 mL 20 mM glycine-HCl buffer, pH 2.0) were added. The suspension was incubated at 37°C for 2 h under agitation. To simulate intestinal digestion, the pH of the gastric digestion was adjusted to 7.2 with 1 M $NaHCO_3$. 13 mL of a pancreatin (porcine, P1750, Sigma) solution (0.4 g pancreatin in 100 mL of ultrapure water) were added and a dialysis bag (cut of 10,000 Da; Carl Roth GmbH + Co., Germany containing 2 mL of ultrapure water) was placed in the digestion system. The system was incubated at 37 °C for 2 h, under agitation. Thereafter, the dialysis bag was removed and iron, zinc and copper in the dialysate were analyzed by ICP-MS. Bioaccessibility (%) was calculated as 100 x Y/Z whereby Y represents the dialyzable amount of the mineral per 100 g DM of cooked beans and Z the total of the same mineral per 100 g DM of the cooked beans.

2.8. Statistical analysis

All the analyses were conducted in triplicate and expressed as mean ± standard deviation of three separate determinations. The data generated was subjected to one-way analysis of variance (ANOVA) using the software Sigma Plot version 13.0. A Tukey's paired comparison test was used to determine statistically significant differences ($p < 0.05$) among the batches and in between raw and treated samples mean values, at a 95% confidence level.

3. Results and Discussion

3.1. Mineral contents of raw and cooked beans

The mean iron, zinc, copper and calcium contents of the three black bean batches are presented in Figure 1. All batches are not significantly different ($p > 0.05$) among each other.

Black beans were confirmed to be a good source of iron, zinc and copper. A portion per meal of cooked black beans (100 g) contains an average of 6.5 mg iron, 4 mg zinc and 1 mg copper. Those contents are in good agreement with data published by the Food and Agriculture Organization of

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the United Nations database [11] for common beans of the same origin. Thus, a black bean portion meets the daily requirement for copper (0.9 mg/day), and partially that for iron (8 mg/day) and zinc (8-11 mg/day) [15].

Discarding the soaking water before cooking the beans resulted in a lower content ($p < 0.001$) of iron (6%) and copper (30%) compared to the raw beans (Figure 1.a and 1.b). According to Raes et al. [35], the differences in leaching of micronutrients can be attributed to the fact that these minerals are bound by different food constituents with different binding strength. Furthermore, their location within the food matrix might be different. Zinc and calcium contents was found to be increased irrespective the household procedure applied ($p < 0.001$). The highest contents (Zn: 132-150%, Ca: 191%) were found in black bean cooked in a regular pan (Figure 1.c and 1.d).

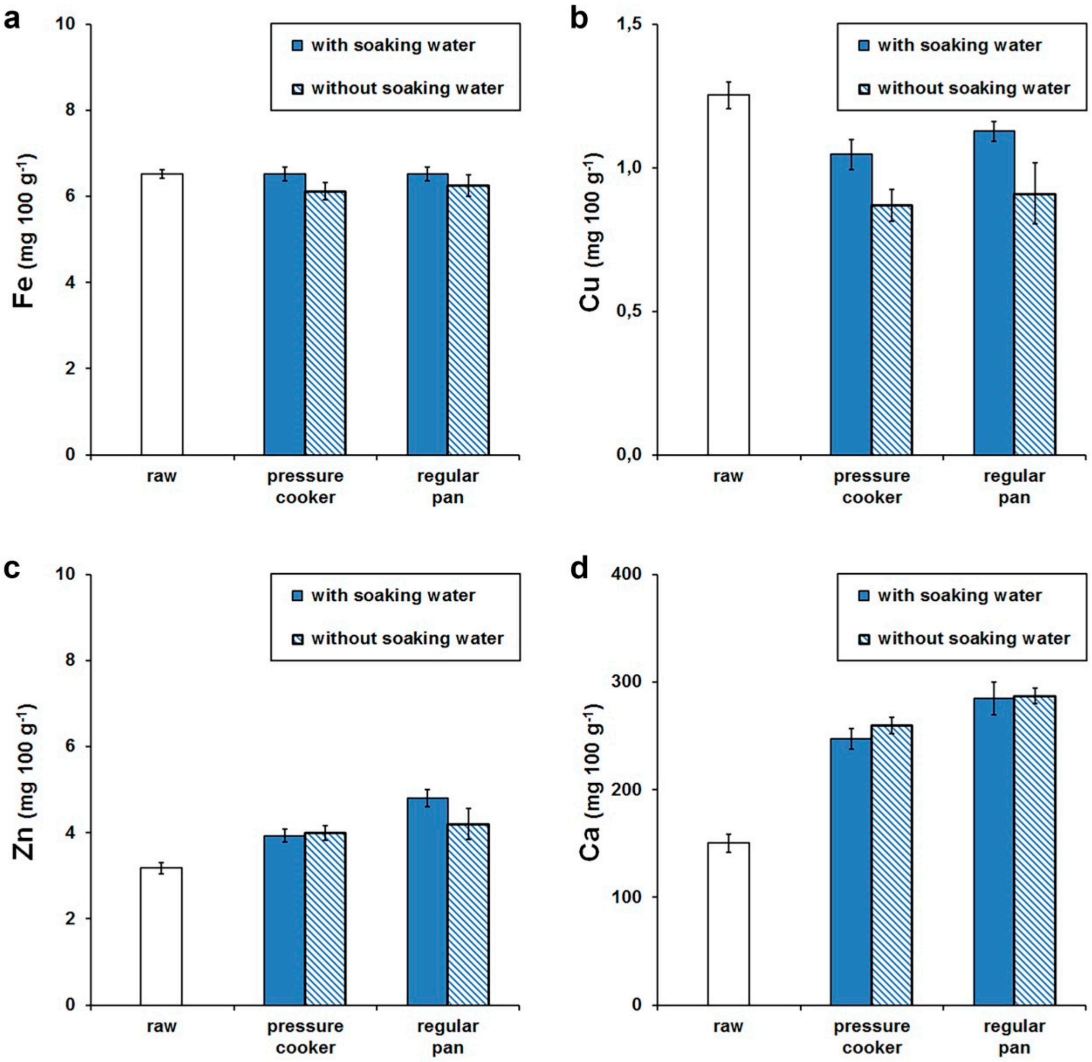

Figure 1. Black bean contents of iron (**a**), copper (**b**), zinc (**c**) and calcium (**d**). Data expressed as mean ± standard deviation (dry matter).

The increase in the Zn contents was found to be due to a leaching of zinc ions from the pan surface (Appendix A). A smaller increase ($p < 0.05$) in Zn content (123-125%) was also observed during pressure cooking (Fig. 1.c). Katzenberg et al. [36] also reported higher zinc concentrations in beef as an effect of the cooking method and Quintaes et al. [37] have shown the migration of metal ions from cookware into foods. The increase in Ca contents was due to the addition of tap water

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during cooking (Appendix A). The mean Ca concentration in the tap water was determined to be 16.83 ± 1.38 mg 100mL^{-1}. Thus, the theoretical amount of calcium added was 100.98 mg 100 g^{-1} for pressure cooking and 134.64 mg 100 g^{-1} in the regular pan. The observed increases in calcium were found to be 97.00–109.27 mg 100 g^{-1} for pressure cooking and 134.66–136.95 mg 100 g^{-1} using the regular pan.

3.2. Anti-nutrients

The mean contents of InsP$_6$, InsP$_5$, total polyphenols and condensed tannins are presented in Figure 2. All traditional household processes applied resulted in a statistically significant reduction in total polyphenols (about 30%) and condensed tannins (about 20%) compared to raw black bean (Figure 2.c and 2.d). Discarding the soaking water before cooking the beans resulted in a greater reduction of polyphenols and tannins, which is in good accordance with the majority of studies [20,27]. Since polyphenols of legumes have been extensively correlated with health benefits in humans due to their potent anti-oxidant activities [23-25], their reduction during processing does not necessarily improve the nutritional quality of beans. With regard to *myo*-inositol phosphates, only with beans cooked without the soaking water in a pressure cooker a slightly decrease (7%) in InsP$_6$ content was observed. InsP$_5$ contents, however, increased with all cooking procedures applied. Furthermore, no statistical difference was observed among the three batches of the black bean samples regarding the contents of anti-nutrients.

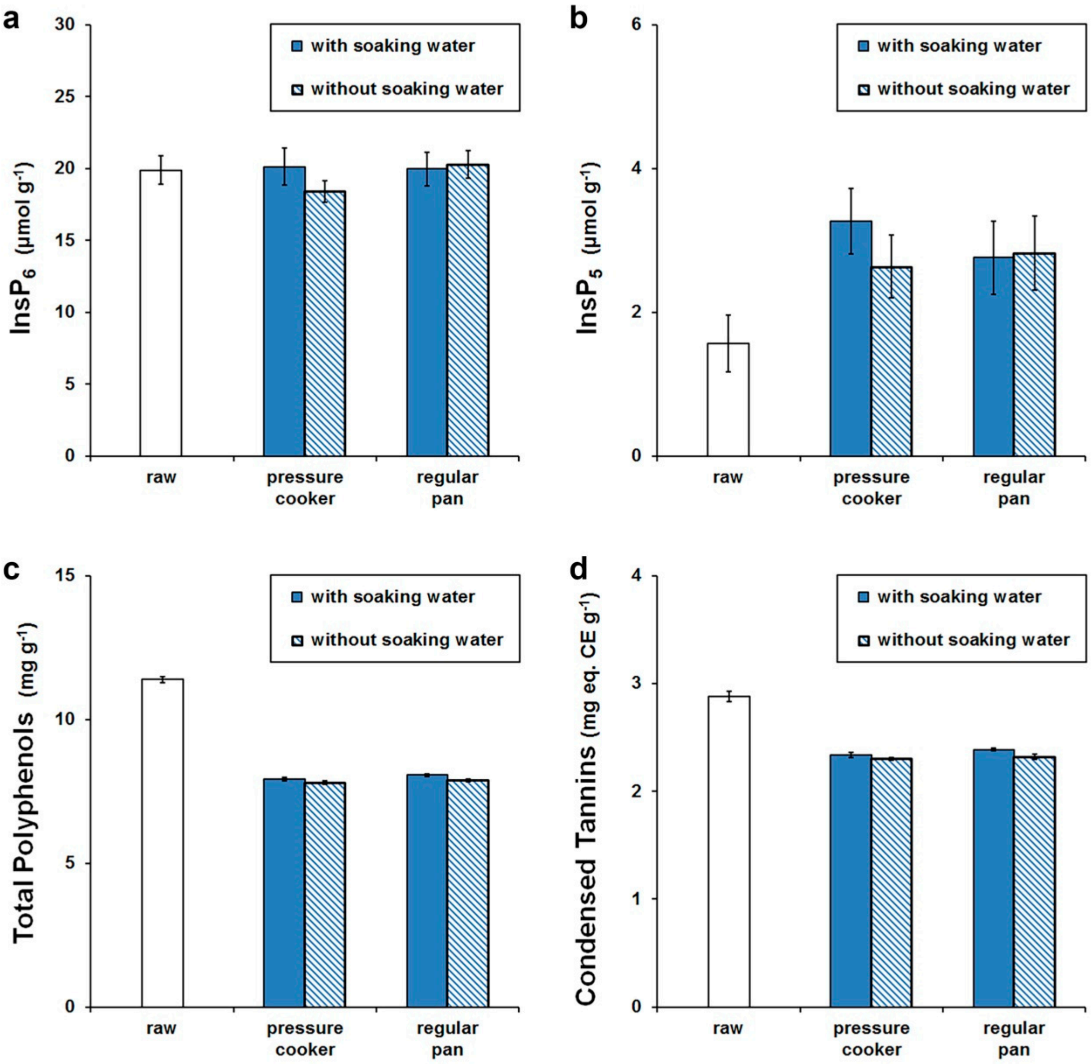

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Figure 2. Black bean anti-nutrients content of $InsP_6$ (**a**), $InsP_5$ (**b**), total polyphenols (**c**) and condensed tannins (**d**). Data expressed as mean ± standard deviation (dry matter).

3.3. Bioaccessibility of iron, zinc and copper

The mean levels (%) of iron, zinc and copper bioaccessibility in cooked black beans are shown in Table 3. The determination of the micronutrient bioaccessibility makes it possible to estimate the percentage of absorption of those minerals with a simple and affordable assay compared to bioavailability assessment. Beans are highly nutritious legumes that have been reported as one of the best plant-based sources of bioaccessible iron and zinc [12-14]. In this study howver, iron bioaccessibility levels were found to be low with all household processes (Table 3). On the other hand, copper showed high bioaccessibility, followed by zinc (Table 3).

Table 3. Bioaccessibility levels (%) of iron, zinc and copper in black bean cooked with traditional household processes.

Household processes	**Iron (%)**	**Zinc (%)**	**Copper (%)**
Regular pan with soaking water	0.18^a	33.94^a	71.53^a
Pressure cooker with soaking water	0.33^b	44.66^b	73.35^a
Regular pan without soaking water	0.17^a	31.55^a	66.42^b
Pressure cooker without soaking water	0.22^a	35.04^a	68.16^b

Within the same column all samples were significantly equal ($p > 0.05$). In each column, values marked by the same letter are not significantly different ($p > 0.05$), and values marked by different letters are a significantly different ($p < 0.05$).

Recent reviews [20,27] reported a link between iron and zinc availability from common beans and cooking soaked beans without the soaking water. This was reported to be due to the reduction of the content in anti-nutrients during food processing [9]. In this present study, however, black bean cooked with the soaking water in a pressure cooker resulted in the highest bioaccessibility for all three minerals in spite of higher total anti-nutrients reduction in beans cooked without the soaking water. According to Hoppler et al. [38] 70%–85% of the iron in beans is present in the form of non-ferritin-bound iron and it is is possibly bound to myo-inositol phosphates. Phytate is abundant in legumes, cereals and nuts, being considered to be the most powerful anti-nutrient due to their high binding capacity for metals and also their ability to form large insoluble aggregates [16,18].

Discarding the soaking water was shown to have a negative effect on the bioaccessibility of all three minerals in regular pan. Assessing mineral bioavailability in those studies mainly by molar ratios and statistical correlations between the content of anti-nutrients and the mineral content might be responsible for the observed differences in the obtained results [20,27,28]. In addition, details on the cooking methods applied were not reported. Pereira et al. [13] studied the effect of household cooking methods on the bioaccessibility of iron and zinc in different beans cultivars. Iron bioaccessibility of beans cooked with the soaking water in a pressure cooker were higher (6.46-40.68%) compared to beans cooked in a regular pan (2.42-8.92%). In the present study, the same tendency was observed. Even if lower Fe bioaccessibilities were found in this study, Zn bioaccessibilities were observed to be always higher than Fe bioaccessibilities. Bioaccessibility studies are a useful method to estimate the general trend of a household procedure on mineral bioavailability, but the absolute data obtained in those studies do not represent the situtation in a human digestive tract. Neither active mineral uptake nor the interaction of minerals in respect to binding to food constituents or interaction with mineral transporters in the small intestine can be considered with bioaccessibility studies. The interactions concerning iron, zinc and copper appear to be of utmost importance in respect to their bioavailability [39,40]. Since micronutrient uptake has been successfully studied by Caco-2 cells models due to their exclusive ability to model human

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absorption characteristics [41,42]. Glahn et al. [43], the data obtained in this study should be confirmed using a Caco-2 cell model.

5. Conclusions

Black beans were confirmed to be a good source of iron, zinc and copper with a high bioaccessibility of copper (about 70%) from cooked beans. Bioaccessibility of iron and zinc however, were found to be lower (about 0.2% for iron and 35% for zinc). Cooking beans under pressure without discarding the soaking water resulted in the highest bioaccessibility levels among all household procedures applied. Although a reduction in anti-nutrients content was observed, the *myo*-inositol phosphate content did not change significantly. In addition, discarding the soaking water before cooking the beans did not improve their nutritional quality. This procedure resulted in a loss in iron, copper and bioactive compounds.

In spite of the amount of information about binding of iron and zinc, and the influence of food processing on anti-nutrients reduction, data on the consequences for iron, zinc and copper absorption are still limited. Thus, improving the knowledge about the influence of traditional household processes on the nutritional value of this basic and accessible food can help to combat micronutrients deficiencies and developing strategies for low-income groups to have access to food with higher available levels of micronutrients. Further work is necessary to increase especially iron availability in home cooked beans. Since phytate is the constituent with the highest impact on mineral bioavailability in common beans, applying the most efficient household procedures combined with phytase application might be a promising approach.

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Appendix A

All household procedures were also performed without addition of a bean samples in order to determine the concentrations of the minerals leaching from the surface of the cooker and the pan. Furthermore, mineral contents of the tap water used were quantified. Iron (Fe), zinc (Zn), cooper (Cu) and calcium (Ca) concentrations were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) as described under methods.

Iron and copper concentrations of the tap water as well as in the samples obtained after cooking were below the LOQ (mg 100 g^{-1}): Fe (0.14), Cu (0.56).

Zinc mean concentrations were determined to be below the LOQ (0.51 mg 100 mL^{-1}) in the tap water. In the boiled water samples it ranged from 0.75 ± 0.06 mg (pressure cooker) to 1.92 ± 0.32 mg (regular pan). Calcium mean concentrations in the tap water were 16.83 ± 1.38 mg 100 mL^{-1}. Thus, according to the total amount of tap water used, calcium amounts added with the tap water were 100.98 mg (pressure cooker) and 134.64 mg (regular pan).

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