Phenolic Acid, Flavonoids and Antioxidant Activity of Common Brown Beans (*Phaseolus vulgaris* L.) Before and After Cooking

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Abstract

This study aimed to evaluate the impact of thermal processing and soaking on the profiles and contents of phenolic compounds in brown beans, as well as their antioxidant activity. We evaluated the antioxidant activity of extracts and the contents of phenolic acids and flavonoids by high-performance liquid chromatography (HPLC). With the exception of chlorogenic acid, the contents of all of the other phenolic acids were increased by cooking. Kaempferol was only detected in samples treated by soaking followed by cooking. Catechin and kaempferol-3-glucoside were found in all of the brown bean extracts. Cooking, with or without soaking, caused significant increases in the concentrations of quercetin and quercetin-3-glucoside only. The effect of the heat treatment increased the antioxidant activity and the concentrations of the phenolic compounds evaluated.

Keywords: 2,2-diphenyl-1-picrylhydrazyl (DPPH); 2,2′-azino-bis (ABTS); Polyphenols; Bioactive compounds; Thermal processing

Introduction

Similar to other legumes, beans possess large contents of bioactive compounds such as polyphenols [1]. These compounds are secondary metabolites in plants and are widely known for their antioxidant capacities. Polyphenols therefore play an important role in reducing the risk of cardiovascular disease, diabetes, some cancers, and Alzheimer and Parkinson’s diseases [2]. The antioxidant activity of beans is due mainly to the reducing properties of polyphenols, which play an important role in the neutralisation or sequestration of free radicals and in the chelation of transition metals, acting against both the initiation and propagation of oxidative processes. The intermediates formed by the action of phenolic antioxidants are relatively stable due to the resonance of the aromatic rings in the structures of these substances [3].

Common beans possess antioxidant activity due to the presence of phenolic acids and flavonoids, mainly tannins [4].

It is known that the phenolic acids most commonly found in raw and cooked beans are gallic acid, vanillic, p-coumaric, ferulic, sinapic and chlorogenic acids and that they have great importance as precursors for the synthesis of other phenolic compounds in plants [5,6].

Flavonoids share a common structure consisting of two aromatic rings that are linked through three carbons, forming an oxygenated heterocycle. They are divided into six subclasses, depending on the type of heterocycle formed: Flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols (catechin and proanthocyanidin). The major flavonoid representatives are quercetin and kaempferol in glycosylated forms. In raw and cooked beans, the main flavonoid representatives are catechin, kaempferol, quercetin, myricetin and procyanidins [3,5].

To improve the nutritional quality of beans, peeling, soaking, cooking and germination methods are used. Therefore, the present study aimed to evaluate the impact of cooking preceded by soaking and cooking without soaking on the profiles and contents of phenolic acids and flavonoids, as well as the antioxidant activity of brown beans.

Materials and Methods

Samples of the common bean (*Phaseolus vulgaris* L.) BRS9435-cometa cultivar (brown) were analysed raw and after thermal processing.

The beans were stored at refrigerator temperature (4°C) before the measurements and then they were ground and sieved. The beans intended for use as cooked samples were divided into two treatments: Treatment 1 involved soaking the beans for 10 h in distilled water, followed by an exchange of the water and then cooking in an autoclave at 121°C for 10 min; Treatment 2 involved cooking the beans in an autoclave at 121°C for 10 min. After cooking, the samples were lyophilised and stored at ~26°C. Milling of the cooked beans was performed at the time of the analyses and under the same conditions as the raw samples (Treatment 3).

The polyphenol extraction was performed according to Cardador-Martínez et al. [6]. Five extractions were performed for each treatment.

The extracted material was separated into six fractions (A, B, C, D, E and F) using open column chromatography under vacuum according to the methodology proposed by Aparicio-Fernandez, Manzo-Bonilla, and Loarca-Piña [7]. Lyophilised extract in 0.5 g quantities was diluted in 1 mL of methanol and poured into an open column over silica gel (Sigma Aldrich, 13% CaSO₄). The reagents were added as shown in Figure 1 and the fractions were collected after each passage.
Figure 1: Polyphenol separation into six fractions (A-F) using open column chromatography under vacuum.

After the recovery of each fraction (except for fraction A which was discarded as a lipid-removal and column-cleaning fraction), the mixtures were placed in a flask to evaporate the solvents in a rotary evaporator. The fractions FB, FC, FD and FF were lyophilised for further analysis; however, Fraction FE did not generate sufficient phenolic compounds for analysis.

To measure antioxidant activity via 1,1-diphenyl-2-pycrylhydrazyl (DPPH) Sigma Chemical Co., we used the methodology proposed by Re et al. [9], Van Den Berg et al. [10] and Arts et al. [11], with modifications to determine the antioxidant activity. The results were expressed in mg of Trolox-equivalent antioxidant capacity (TEAC) g\(^{-1}\) of extract.

To identify and quantify the phenolic acids, free phenolic acids were separated by high-performance liquid chromatography (HPLC) according to Xu and Chang [12], with modifications. A Shimadzu model 20 A, equipped with a UV detector (270 and 325 nm) was used. A Zorbax ODS Stablebond-C18 (Agilent Technology), 4.6 × 250 mm, 5 mm analytical column was used for the separation, at 40°C. The mobile phases used were A, 0.1% trifluoroacetic acid solution in water; B, 100% methanol and an isocratic gradient that include 20% phase A and 80% phase B with flow rates of 1.0 mL min\(^{-1}\).

To identify the HPLC peaks, one stock solution (1 mg mL\(^{-1}\)) of each phenolic acid profile was individually prepared and diluted. These dilute solutions were injected (20 µL) separately, duplicating the conditions described above. The peak areas and their retention times were used to compare, identify and quantify these phenolic acids in the samples injected afterwards.

To prepare the stock solutions, 10 mg of each phenolic acid was dissolved in 10 mL of 80% methanol and then diluted in 80% methanol in the following solutions: 0.5, 1, 2.5, 5, 10 and 25 µg mL\(^{-1}\) of vanillic acid; 10, 25, 50 and 100 µg mL\(^{-1}\) of chlorogenic acid; 1, 2.5, 5, 10, 25, 50 and 100 µg mL\(^{-1}\) of sinapic acid and 1, 5, 10, 25 and 50 µg mL\(^{-1}\) of gallic acid. The content of each phenolic acid was expressed in micrograms per gram of extract (µg g\(^{-1}\)).

The chromatographic system and methodology used for the separation of flavonoids were the same as those used for the phenolic acids analysis. The oven temperature was 34°C. The mobile phases used were A, 0.1% acetic acid solution in water and B, 0.1% acetic acid in acetonitrile in the following concentration gradients and flow rates: 1.0 mL min\(^{-1}\) with 15% phase B and 85% phase A during the first 5 min; 1.5 mL min\(^{-1}\) with an increase in phase B to 29% and a reduction in phase A to 71% from 5 to 23 min; 1.0 mL min\(^{-1}\) with an increase in phase B to 35% and a reduction in phase A to 65% from 23 to 44 min; 1.0 mL min\(^{-1}\) with an increase in phase B to 50% and a reduction in phase A to 50% from 44 to 46 min and 1.0 mL min\(^{-1}\) with a reduction in phase B to 15% and an increase in phase A to 85% from 46 to 48 min.

To identify the HPLC peaks, a stock solution (1 mg mL\(^{-1}\)) of each phenolic acid profile was individually prepared and then diluted. These dilute solutions were injected (20 µL) separately, duplicating the conditions described above. The peak areas and their retention times were used to compare, identify and quantify these phenolic compounds in the subsequently injected extracts.

To prepare the stock solution, 10 mg of each flavonoid was dissolved in 10 mL of 80% methanol and then diluted in 80% methanol in the following concentrations: 5, 10, 25 and 50 µg mL\(^{-1}\) of catechin; 25, 50, 100 and 250 µg mL\(^{-1}\) of quercetin; 2.5, 5, 10, 25 and 50 µg mL\(^{-1}\) of kaempferol-3-glucoside; 10, 25, 50 and 100 µg mL\(^{-1}\) of kaempferol 3-O-rutinoside; 10, 25, 50 and 100 µg mL\(^{-1}\) of kaempferol and 10, 25, 50 and 100 µg mL\(^{-1}\) of quercetin-3-glucoside. The content of each flavonoid was expressed as micrograms per gram of extract (µg g\(^{-1}\)).

The statistical design was completely randomised. Analyses of the antioxidant activity were performed in duplicate, with two replicates for each extract and fraction and the identification and quantification analyses of the phenolic acids and flavonoids were performed in duplicate, with two replicates only for crude extracts of polyphenols. The results were analysed by the Tukey test at 5% probability.

**Results**

Some differences between the two methods can be observed. According to the DPPH method, both cooking processes resulted in higher antioxidant activity in the cooked beans compared to the raw beans. For crude extracts of the beans, the results of DPPH ranged from 2.96 to 14.04 mg TEAC g\(^{-1}\) extract.
Table 1: Antioxidant activity (mg TEAC g\(^{-1}\) of extract) of crude extracts and fractions of polyphenols from brown raw, cooked and cooked and soaked beans as measured by the DPPH and ABTS methods.

In the ABTS assays, the extracts ranged from 13.70 and 29.51 mg TEAC g\(^{-1}\) extract. Table 1 shows the antioxidant activity values according to the DPPH and ABTS methods.

The levels of phenolic acids present in the crude extract are shown in Table 2. It was found for phenolic acids that all extracts evaluated have the gallic acid ranged from 2203.55 to 3693.39 µg g\(^{-1}\) extracts of the beans, chlorogenic from 1272.04 to 4430,26 µg g\(^{-1}\) extracts of the beans, vanillic from 377.49 to 1404.45 µg g\(^{-1}\) extracts of the beans and sinapic from 88.52 to 382.37 µg g\(^{-1}\) extracts of the beans, and the first two were found at higher levels.

Table 2: Phenolic acids (µg g\(^{-1}\) extract) in crude extracts from raw, cooked and cooked-and-soaked brown beans.

Table 3: Phenolic acid (µg g\(^{-1}\) of extract) fractions of polyphenols from raw, cooked and cooked-and-soaked brown beans.

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The results for the flavonoid fractions in crude beans extracts are presented in Table 5. The highest level were for soaked and cooked beans.

### Discussion

Some differences between the two methods can be observed. According to the DPPH method, both cooking processes resulted in higher antioxidant activity in the cooked beans compared to the extracts of the raw beans. Similar results were also reported in the study of Rocha-Guzmán et al. [4], who concluded that cooked beans removed of free radicals at a higher rate compared to raw beans.

In the ABTS assays, the extracts and fractions from the raw beans did not have the lowest results. In the FC and FD fractions, cooking promoted a decrease in antioxidant activity, with or without soaking, compared to the extract of the same raw bean. Xu and Chang [12] attributed this decrease in antioxidant activity after thermal treatment to the possibility that chemical transformations, decomposition of phenolic compounds, formation of complexes between polyphenols and proteins and solubilisation of water-soluble antioxidants in the discarded soaking water occurred.

Although both results of the ABTS and the DPPH that the evaluated antioxidant has the ability to donate hydrogen, a comparative study of the FRAP, ORAC, DPPH and ABTS methodologies by Fernandez-Panchon et al. [13] indicated that the sensitivity of the ABTS radicals was lower compared to the DPPH radical following heat processing.

With the DPPH method, the antioxidant activities of the extracts were higher than those found for fractions. This is possibly due to synergy between all of the antioxidant compounds that were present in the crude extract, while no such synergy was possible in the isolated fractions. However, in the ABTS assays, the extracts did not did not contain higher fractions.

### Table 4: Flavonoids (μg g⁻¹ of extract) in crude extracts of polyphenols from raw, cooked and cooked-and-soaked brown beans.

| Flavonoid               | Raw         | Cooked       | Cooked and soaked |
|-------------------------|-------------|--------------|-------------------|
| Kaempferol              | 0.00 ± 0.00B | 0.00 ± 0.00B | 127.41 ± 10.29A   |
| Kaempferol-3-glucoside  | 1096.62 ± 37.57B | 1690.77 ± 16.71A | 1136.59 ± 24.50B |
| Kaempferol-3-rutinoside | 13.62 ± 0.83C | 69.78 ± 13.41B | 176.81 ± 1.36A   |
| Catechin                | 1105.77 ± 112.37B | 1886.61 ± 73.03A | 1428.83 ± 36.50B |
| Quercetin               | 9.58 ± 2.03A | 10.67 ± 4.03A | 12.14 ± 1.22A    |
| Quercetin-3-glucoside   | 23.07 ± 5.54A | 33.70 ± 9.58A | 12.81 ± 5.44A    |

*Means of two replicates ± standard deviation; **Means with uppercase different letters(s) in the horizontal direction(s) differ significantly (p ≤ 0.05).

### Table 5: Flavonoid (μg g⁻¹ of extract) fractions of polyphenols from raw, cooked, and cooked-and-soaked brown beans.

![Table 5: Flavonoid (μg g⁻¹ of extract) fractions of polyphenols from raw, cooked, and cooked-and-soaked brown beans.](image-url)
Overall, the crude extracts showed similar antioxidant activities in relation to their respective fractions. The same finding was reported in the study by Beninger and Hosfield [14], who explained their results by noting that the flavonoids present in the crude extract were further concentrated in the extracts; this concentration may have been primarily responsible for the observed antioxidant activity.

In all of the samples analysed, gallic and chlorogenic acids were found to have the highest contents. These findings regarding beans have also been reported in the literature. The contents measured in this study are higher than those in other studies due to the units in which these data are expressed (per gram of extract in this study vs. per gram of food in others). Xu and Chang [5] reported contents of 89.64 and 32.93 μg of gallic acid g⁻¹ in black bean that were raw and cooked under pressure for 10 min, respectively, and 226.1 and 89.9 μg of chlorogenic acid g⁻¹ in black bean that were raw and cooked under pressure for 10 min, respectively. However, Luthria and Pastor-Corrales [15] evaluated 15 types of beans and detected only p-coumaric, sinapic and ferulic acids, the last showing the highest concentration. Ranilla et al. [16] detected chlorogenic acid in only three of twenty-eight cultivars analysed (between 2.8 and 5.6 mg 100 g⁻¹).

It was observed that cooking significantly increases the concentration of vanillic acid. By contrast, Diaz-Batalla et al. [1] found lower levels of vanillic acid in cooked beans than in raw beans for the 14 analysed cultivars. Aguiler a et al. [17] found no vanillic acid in cooked beans, with or without soaking and found 10.71 μg g⁻¹ in raw beans. This phenolic acid has the following desirable activities: antihelmintic activity, the prevention of sickle erythrocytes and the suppression of liver fibrosis in chronic liver diseases [5,18].

Similarly, higher concentrations of gallic acid were found in the extracts of cooked beans. Xu and Chang [5], however, reported contents of 83.17 μg g⁻¹ in raw beans and 38.16 μg g⁻¹ in cooked brown beans; both of these values are well below those reported here and indicate an effect of cooking on the content of this specific phenolic acid that is the opposite of that reported here. We note that recent studies report that gallic acid, in addition to its antioxidant properties, has the following biological effects: antineoplastic and bacteriostatic activity, inhibition of brain tumours, antitumor properties, induction of apoptosis in prostate carcinoma cells, antiangiogenic activity, inhibition of disaccharidases in the intestinal brush border of mammals and induction of apoptosis or necrosis of cancer cells [18]. Therefore, the consumption of beans is highly beneficial, as high contents of gallic were measured in the extracts that were analysed.

Large amounts of chlorogenic acid were measured in the extracts of raw beans. The action of this compound in the prevention of Alzheimer’s disease via a reduction in apoptosis induced by amyloid-β cells. Additionally, chlorogenic acid displays anticholinesterase, anti-inflammatory and antioxidant activities. The phenolic compound can be easily oxidised via polyphenol oxidases leading to its interaction with NH₃ groups of proteins and amino acids, resulting in reducing the nutritional value of foods. This acid in question increases homocysteine levels in human plasma, which constitutes a risk factor for the onset of cardiovascular disease [18].

Regarding sinapic acid, the highest levels were found in extracts of cooked beans without soaking. In a study by Espinosa-Alonso et al. [19] sinapic acid as found at a concentration equal to 22.4 mg Kg⁻¹ of different coloured beans. Campos-Vega et al. [2] reported that antioxidant activity is the main biological activity provided by sinapic acid.

We analysed the two fractions that had the highest antioxidant activity according to the DPPH and ABTS methods and these fractions varied according to the beans and treatment. Therefore, tests of the averages of duplicates that were injected into the chromatographs were performed.

In general, heat treatment increased the antioxidant activities and the concentrations of phenolic compounds evaluated. This finding is important because raw beans have antinutritional factors and should therefore be cooked before they are consumed. Soaking, on the other hand, had variable effects in each of the analyses.

Table 3 shows the results regarding the phenolic acid fractions of the crude extracts. We analysed the two fractions that had the highest antioxidant activity according to the DPPH and ABTS methods and these fractions varied according to the beans and treatment. Therefore, tests of the averages of duplicates that were injected into the chromatographs were performed.

In general, heat treatment increased the antioxidant activities and the concentrations of phenolic compounds evaluated. This finding is important because raw beans have antinutritional factors and should therefore be cooked before they are consumed. Soaking, on the other hand, had variable effects in each of the analyses.

It is observed that some phenolic acids were not present in either fraction evaluated. It must then be the case that the significant antioxidant activity of these fractions was due to other, unidentified materials present in the extracts evaluated.

The phenolic acids that were found in the highest concentrations were gallic and chlorogenic acid. Among the various phenolic compound fractions that were evaluated, the FB and FC fractions had higher scores compared to the FF, although many of the fractions did not differ significantly. This finding corroborates the results from the DPPH and ABTS methods, which indicated that the FF fraction had lower antioxidant activity and the results regarding the polarity of acids that were evaluated and solvents used in the fractionation. It is known that the phenolic acids quantified herein are typically polar.

The literature reports that chlorogenic, gallic and sinapic acid have high antioxidant activities and that the activity of vanillic acid is lower [20]. Thus, it is concluded that, due to the large fractions of chlorogenic and gallic acid measured in the extracts of the beans evaluated here, brown beans have high potential as antioxidants, as confirmed by the results of the DPPH and ABTS assays.

Table 4 shows the results of the analyses of the flavonoids in the crude bean polyphenol extracts. Most of the flavonoids examined were detected in almost all of the samples, except for the kaempferol, which was only detected in the soaked and cooked samples. However, in a study by Diaz-Batalla et al. [1], kaempferol was present at higher concentrations in raw beans (average of 52.3 μg g⁻¹ of bean) compared to cooked beans (average of 27.2 μg g⁻¹ of bean). Likewise, in a work by Amarowicz and Pegg [3], cooking promoted a reduction of this flavonoid by 5-71%. Romani et al. [21], who assessed the levels of various flavonoids in Italian beans, did not detect even trace amounts of quercetin-3-glucoside and this flavonoid as not detected in the extracts evaluated in this study either.

We mainly found catechin and kaempferol-3-glucoside flavonoids. Ranilla et al. [16] detected quercetin and kaempferol in brown beans and Lin et al. [22] detected kaempferol-3-glucoside. It is known that kaempferol is associated with a reduced risk of developing cancer and that kaempferol-3-glucoside possesses anticancer properties and...
neuroprotective properties against diseases such as Parkinson's and Alzheimer's [2,18].

It was expected that higher concentrations of catechin would be detected because catechin is a type of procyanidin and is the main flavonoid present in beans [23]. However, Xu and Chang [5] did not detect this compound in raw or cooked brown beans.

Based on the data in Table 4, it is observed that, for all flavonoids, when there was a significant difference among the extracts from raw and cooked beans, the former always exhibited inferior results. The effect of soaking varies for each compound evaluated; however, the effect of this process on the increases in the concentrations of kaempferol and kaempferol-3-rutinoside was clear. These results are contrary to published reports claiming that cooking and soaking have a negative impact on flavonoid concentrations [1,5,17].

It is also notable that quercetin and quercetin-3-glucoside were not influenced by cooking, with or without soaking. Amarowicz and Peggy [3], however, found that the concentration of quercetin was reduced by 12-65% after the beans were cooked and Díaz-Batalla et al. [1] found mean values of 10.9 µg of quercetina g⁻¹ in raw beans and 6.5 µg of quercetina g⁻¹ in cooked beans. It is reported in the literature that quercetin has various effects in the prevention and treatment of some types of cancer, in the reversal of cognitive deficits and the inhibition of H₂O₂ and histamine generation [2,18].

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The results for the flavonoid fractions in crude beans extracts are presented in Table 5. It is noted that many of the fractions did not contain one or more of the phenolic compounds analysed; additionally, the behaviour of the kaempferol was similar to that observed for other polyphenols and this compound was present only in extracts of cooked and soaked beans.

Aparicio-Fernandez et al. [7] performed fractionations similar to those in this study, but they used methanol extracts of black beans. They detected kaempferol-3-glucoside, quercetin-3-glucoside and myricetin glycosylated only in the FB fraction, at ratios of 5.8, 4.1 and 6.7 respectively. These authors note that the presence of glycosylated myricetin in these beans was unexpected and has been rarely documented for these beans.

Catechin was the only flavonoid that was found in all of the analysed extract fractions; the fact that this compound belongs to the procyanidin group, which is present in virtually all beans, further explains this observation. A study by Aguiera et al. [17] reported concentrations of catechin in macerated raw and cooked beans of 142.58 µg g⁻¹ and 76.25 µg g⁻¹, respectively; these results are very similar to those obtained in this study (Table 5). It is known that catechin assists in the reduction of cholesterol absorption in the intestine and in the inhibition of LDL cholesterol oxidation [2].

Virtually all of the flavonoids analysed occurred in lower concentrations in the FF fraction relative to the fraction analysed for the same treatment. This observation leads to the conclusion that most of these polyphenolic compounds were not extracted or were extracted in small amounts by the solvent used in each fraction.

Although Beninger and Hosflied [14] indicated that flavonoids exist mainly in the glycosylated form, the fractions evaluated in this study also contained the aglycone flavonoid.

The occurrence, in significant quantities, of almost every flavonoid analysed (especially after cooking, which reflects how beans are most commonly prepared for consumption) confirms the importance of including beans daily as part of a healthy diet that is rich in bioactive compounds because all of the analysed flavonoids demonstrate free radical scavenging activity and other biological effects, which were described above [2,18].

Brown beans contain all of the flavonoids and phenolic acids analysed in this study, making their consumption highly beneficial due to the rich variety of physiological activities that phenolic compounds exert.

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