

Biofortification potential in common bean (*Phaseolus vulgaris* L.): bioactive compounds, antioxidant capacity and physicochemical properties of 155 varieties grown in México

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Abstract

Common bean has been classified as an almost perfect food due to its high content of fiber, protein and bioactive compounds and its high antioxidant capacity, which has been attributed to having prevent diabetes, oxidative stress, bowel inflammation and cardiovascular diseases. The objective of this research work was to characterize the physicochemical properties and bioactive compounds of 155 varieties of common bean (*Phaseolus vulgaris* L.) produced in Mexico with potential to be biofortified. The contents of protein, fiber, fat, carbohydrates and energy were analyzed by the methods established by the AOAC, in addition the antioxidant capacity (DPPH), the content of total phenols, flavonoids, anthocyanins and phytic acid were obtained. Cluster, correlation, and principal component analysis were conducted. A total of 14 outstanding varieties was identified, where four varieties stood out about phenol and flavonoid content. A second group comprised by 10 varieties had high protein and anthocyanin levels and showed the second highest content of phenols and flavonoids. The diversity of beans obtained in outstanding varieties provides options for biofortification programs based on the consumption or place of origin of each variety.

Keywords: biodiversity; biofortification; nutritional quality; *Phaseolus* sp.

Introduction

At present, changes in demand of food and trends to consume products containing beneficial compounds for health, in addition to a promotion towards plant based products, has forced the industry to find new dietary attributes in its products, so they may have the capacity of attracting new market preferences without neglecting the nutritional quality of the crops, being one of the most important parameters that must

Received: 20 Oct 2020. Received in revised form: 21 Dec 2020. Accepted: 22 Mar 2021. Published online: 24 Mar 2021.

From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal will use article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

be taken into account when talking about food, since they determine their functionality in the diet (Sida-Arreola *et al.*, 2015; Gutiérrez, 2012).

Legumes, such as beans, lentils or chickpeas, have been considered as staple foods, due to their nutritional quality and because they have shown the ability to improve human health. Within this group, beans are the most consumed legume in the world, having an average per capita of 6.6 Kg. Around 150 species of beans are known, of which approximately 70 varieties have their center of origin in Mexico with a great variety of sizes and colors (FIRA, 2016; Silva-Cristobal *et al.*, 2010).

Among the nutritional properties of beans, one of the most notable is its high protein content and to a lesser extent its contribution of carbohydrates, vitamins and a high contribution of fibers and minerals such as calcium, iron and zinc, beans are also rich in antioxidants due to its high phenol content (Korus *et al.*, 2007; Ulloa *et al.*, 2011). The protein content in beans ranges between 15% and 30%, a 90 g portion of beans provides from 7 to 8 g of protein, which constitutes 15% of the recommended daily intake, they also have enough content to meet the needs daily basic amino acids lysine (6.4-7.6 g, 100 g⁻¹ of protein) and phenylalanine plus tyrosine (5.3-8.2 g, 100 g⁻¹ of protein) (Mederos, 2006; Suárez-Martínez *et al.*, 2016). Beans are also a good source of fiber with values that vary from 14 g to 19 g per 100 g of raw food, of which it can reach 50% soluble fiber, which has been related to a reduction in cholesterol levels (Messina, 2014).

The common bean has been classified as an almost perfect food, due to its high content of fiber, protein, its high antioxidant capacity and the large number of bioactive compounds it has, to which qualities have been attributed in the prevention of diseases such as diabetes, oxidative stress, intestinal inflammation and cardiovascular diseases (Xu and Chang, 2009; Câmara *et al.*, 2013). These effects are related to the high antioxidant capacity, granted by high concentrations of phenols, flavonoids and to a lesser extent anthocyanin and phytic acid, which in turn generate antimutagenic and antigenotoxic effects (Akond *et al.*, 2011¹). However, Suárez-Martínez *et al.* (2016), suggest that it is necessary to make efforts to increase bean consumption, making the population aware of the benefits attributed to it. Based on the above, the objective of this research work was to characterize the physicochemical properties and bioactive compounds of 155 varieties of beans produced in Mexico with potential to be biofortified.

Materials and Methods

Selection of material

One hundred and fifty-three varieties of beans (*Phaseolus vulgaris* L.) were used, representative of all Mexico, based on the consumption preference of the States of Chihuahua, Durango, Zacatecas, Sinaloa, Tabasco, Yucatán, Oaxaca, Veracruz, Puebla, Jalisco, Morelos, Guanajuato, Michoacán and State of Mexico, in addition to two more samples from Cuba selected for their nutritional quality.

Reagents used

The following reagents were used: potassium sulphate (99.4%), sulfuric acid (97%), sodium hydroxide (97%), boric acid (100%), hydrochloric acid (38%), petroleum ether (35-60 °C), isoamyl alcohol (99.7%), methanol (99.97 %), sodium carbonate (100%), potassium chloride (99%) and sodium acetate (99.3%), all of them obtained from JT Baker (USA), DPPH, sodium nitrite (99%), Folin-Ciocalteu, aluminum chloride (99%) and catechin (98%) acquired from Sigma-Aldrich (USA) and gallic acid (99.5%) from CTR (MEX) were used too.

Assessed variables

Once the bean materials were obtained, they were cleaned, selected and milled to perform the analysis of physicochemical properties and bioactive compounds, which were determined in accordance with the

Association of Official Analytical Chemists (AOAC 2000) and in accordance with the provisions of the Official Standards Current Mexicans. The parameters evaluated were the following:

Determination of physicochemical properties

Protein

Protein determination was performed using the Kjeldahl method of the Association of Official Analytical Chemists (AOAC, 2000). 1 g of homogenized sample was weighed into a Kjeldahl digestion flask, 3 boiling beads, 10 g of potassium sulfate and 20 ml of concentrated sulfuric acid were added for digestion. When the mixture became clear, it was allowed to cool, and 200 ml of tri-distilled water was added. It was connected to the still while adding 100 ml of 30% sodium hydroxide, distilled and at least 150 ml was collected in a flask containing 50 ml of 3% boric acid and titrated with 0.1 N hydrochloric acid. The results obtained were multiplied by the factor 6.25 and expressed as a percentage.

Total fat

The determination of the fat of the bean samples was carried out using the Goldfish method (AOAC, 2000). Goldfish flasks were prepared by oven drying to constant weight. The LABCONCO equipment was assembled and the sample was placed inside filter papers and covered with cotton and introduced into the equipment. Solvent (petroleum ether) was added and refluxed for 2.5 hours. After the extraction was complete, the solvent was recovered by distillation, leaving only the fat in the flask. Finally, the flask with the residue was weighed and the fat percentage was determined according to the Mexican Standard NMX-F-427-1982 published in the official gazette of the federation by the government secretary.

Crude fiber

Crude fiber was determined by the Mexican Standard NMX-F-90-S-1978 published in the official gazette of the federation by the government secretary. From the sample that was previously degreased. The weight of the sample was recorded and transferred to the fiber cups, then 200 ml of 1.25% sulfuric acid with 1 ml of isoamyl alcohol as defoamer was added to each beaker. The mixture was left to boil for 30 minutes, at the end, rinses were carried out to eliminate the residues of the reagents and at the same time neutralize the mixture, subsequently, 200 ml of 1.25% sodium hydroxide was added to the sample and it was allowed to boil for another 30 minutes, at the end, fiberglass rinses were performed until neutrality was reached. Subsequently, the fiberglass with the sample was placed in the capsule and they were introduced into the oven, it was left to dry for 12 hours to ensure that the sample was perfectly dry. After drying, the capsule was weighed with the glass fiber and the sample. By difference in weight, the percentage of fiber contained in each of the samples was determined.

Total carbohydrates

Determination of total carbohydrates was carried out by difference of the protein, fiber, fat, moisture and ash content and was reported in percentage.

Metabolizable energy

Energy contained in each of the samples was measured by the sum of the calories contained in carbohydrates, fat and protein, calculated using the Atwater system, reported by Acheson *et al.* (1980) Energy was expressed in Kcal.

Extraction of bioactive compounds

1 g of raw beans was macerated with 10 ml of 80% methanol and centrifuged at 2560 RCF for 10 minutes at 4 °C, then the aliquot was extracted to measure the following four parameters.

Antioxidant capacity

For the determination of the antioxidant activity, the DPPH method described by Brand-Williams *et al.* (1996) was used, for which 500 µl of each sample was taken and 2.5 ml of DPPH 0.1 mM prepared in 80% methanol was added to each one. All the reactions were carried out in one hour and at room temperature and in the absence of light; after the time the absorbance was measured at 517 nm. Antioxidant activity is expressed as percentage of inhibition corresponding to the amount of DPPH radical neutralized by the extract at a certain concentration according to the following equation.

$$\%I = \frac{A - A_1}{A} * 100$$

Where: A is the absorbance of the blank, A₁ is the absorbance of the sample and %I is the percentage of inhibition

Total phenols

The total phenols were determined by colorimetry against a gallic acid standard curve using the Folin-Ciocalteu method (Peyrat-Maillard *et al.*, 2000); 250 µl of sample added with 750 µl of sodium carbonate 2 %; subsequently, 250 µl of reagent Folin-Ciocalteu 50% were added and filled to the mark with distilled water to make up the volume of 2.625 ml, finally, it was vortexed and incubated at room temperature for 60 min; absorbance was measured at 725 nm. Results were expressed in milligrams of gallic acid per gram (mg GA g⁻¹).

Flavonoids

Flavonoid analysis was performed following the method of Zhishen *et al.* (1999), 250 µl of the sample was taken and 75 µl of sodium nitrite 5% was added, it was vortexed, and the reaction was allowed to settle. for 5 minutes, once the time had elapsed, 150 µL of 10% aluminum chloride were added together with 500 µL of sodium hydroxide, finally, it was added with distilled water to make a volume of 3 ml. The samples were incubated for 45 minutes and then absorbance at 510 nm was measured; flavonoids were quantified based on a standard catechin curve and the results were expressed in mg of catechin per gram of dry weight (MgCE.g⁻¹).

Monomeric anthocyanins

To determine the content of monomeric anthocyanins, the differential method by pH described by Wrolstad (1976) was used. The reaction was divided in two phases.

The first consisted of taking 0.5 ml of sample and adding 2 ml of 0.25 M potassium chloride adjusted to a pH of 1.0, then it was vortexed and the absorbance was measured at 460 nm. For the second phase, 0.5 ml of sample was taken and 2 ml of 0.4 M sodium acetate adjusted to a pH of 4.5 were added, and the same process was carried out as in phase one, with the difference that it was measured at a wavelength of 710 nm. The monomeric anthocyanin content was calculated using the following formula:

$$\frac{(A - B) * (449.2) * (0.2) * (1000)}{26900}$$

Where A-B is the difference between absorbances between phase 1 and phase 2, 449.2 is the molecular weight of cyanidine-3-glucoside, 0.2 the dilution factor, 1000 is a constant and 26900 is the coefficient of molecular extinction of cyanidine-3-glucoside.

The results were reported as mg of cyanidin-3 glucoside per gram of sample (mg C3G g⁻¹).

Phytic acid

For the determination of phytic acid, the method proposed by McKie and McCleary (2016) was used, for which the phosphorous total was obtained by the ammonium metavanadate method in an absorption range of 430 nm against a calibration curve of potassium phosphate. Once the results were obtained, the following formula was applied:

$$\text{Phytic Acid} = \frac{\text{Total phosphorous}}{0.282}$$

Where 0.282 is a conversion factor from total phosphorous to phytic acid. Results were expressed in grams of phytic acid per each 100 g of dry weight of sample.

Statistical analysis

A Pearson's correlation analysis, as well as a cluster analysis of K means and a principal component analysis using the SPSS statistical package (SPSS, 2011).

Results and Discussion

Physicochemical properties

The selected bean varieties showed a large diversity size, color and probably due to the differences in geographical origin (Table 1). Mean protein content was 23.16%, which is over the one reported by the U.S. Department of Agriculture (21-22%) and with 11.61% of the varieties evaluated over the range (15-26.35%) found for Mexican varieties of common beans (Chávez-Mendoza and Sánchez, 2017; USDA, 2018). In addition, very similar data were recorded for raw bean flour, to those reported in previous works for the variables of metabolizable energy, fat and carbohydrates, while for the fiber values the range found (1.22-7.82%) is very much below the general fiber average for bean varieties worldwide (Campos-Vega *et al.*, 2009; Suárez-Martínez *et al.*, 2016).

Table 1. Mean value of bioactive compounds and physicochemical properties of 155 bean genotypes

Variable	Mean	Max.	Min.	SD	CV
Fat (%)	1.38	2.97	0.41	0.52	0.38
Fiber (%)	3.31	7.82	1.22	1.18	0.36
Carbohydrates (%)	57.49	67.70	43.95	4.07	0.07
Protein (%)	23.16	29.79	17.48	2.81	0.12
Metabolizable energy (Kcal)	335.05	355.15	306.10	10.07	0.03
Antioxidant capacity (%)	64.02	96.76	0.54	29.16	0.46
Phenols (mgGA/g)	0.77	2.50	0.06	0.45	0.58
Flavonoids (mgCE/g)	1.60	8.83	0.20	1.33	0.83
Anthocyanins (mgC3g/g)	1.25	2.58	0.21	0.55	0.44
Phytic acid (mg/g)	0.51	1.45	0.05	0.21	0.41

Bioactive compounds

Regarding the bioactive compounds (Table 1), the antioxidant capacity range varied from 0.54 to 96.76% with a mean value of 64.02% exceeding 8.23% to the reported by Boateng *et al.* (2008), who found a mean value of 55.97% in varieties of red, black and pinto from Alabama, US. In turn, Garretson *et al.* (2018), reported a maximum value of 94.3% for purple beans of 'Koronis' variety; those studies show that our varieties are found within the range described for antioxidant capacity worldwide. However, it may be observed that, 62 % of varieties exceeded the higher value found by Chávez-Mendoza and Sánchez (2017) for Mexican varieties, which found a 'Flor de Junio' variety with an inhibition percentage of 29%.

In respect of phenols content, results were between 0.06 y 2.5 mgGA.g⁻¹, which are found below the maximum values published by Garretson *et al.* (2018), Akond *et al.* (2011₁) and Gan *et al.* (2017); however, 8

varieties registered values above the range for Mexican varieties (0.9-2.11 mgGA.g⁻¹) reported by Espinoza-Alonso *et al.* (2006). On the other hand, the range obtained for flavonoid content (0.20-8.83 mgCE.g⁻¹) is found within the information reported by several researchers (Gan *et al.*, 2017; Garretson *et al.*, 2018). While for the anthocyanin and phytic acid parameters, maximum values were registered above those reported by Akond *et al.* (2011_{1,2}) varieties from Colombia, US, Brazil and India. Regarding the previous results in Mexican varieties, anthocyanin exceeded the range from 0.01-1.85 mgC3g.g⁻¹ published by Espinoza-Alonso *et al.* (2006) for 62 varieties of common bean. On the contrary, the content of phytic acid obtained an average of 1.45 mg.g⁻¹, which places it 0.08 mg.g⁻¹ below the average published by Iniestra-González *et al.* (2005) in 16 bean varieties of different seed coat colors.

Correlation analysis

In Pearson's correlation analysis, protein and fiber content parameters showed a negative correlation with carbohydrate content and with metabolizable energy (Table 2), which could be explained by the carbohydrate calculation method, which in turn is the parameter that most contributes to the calculation of metabolizable energy. A positive correlation was also found between said parameters with antioxidant capacity, while for bioactive compounds a high correlation was found between the content of phenols with flavonoids and anthocyanins, in addition to both being correlated with antioxidant capacity. In the case of beans, this results from the fact that antioxidant activity is related to the phenolic compound content they possess (Huber *et al.*, 2016; Gan *et al.*, 2017; García-Díaz *et al.*, 2018). Finally, phytic acid showed a negative correlation with carbohydrates.

Table 2. Pearson correlation analysis with bean varieties grown in Mexico

	Fat	Fiber	Carbohydrates	Protein	Metabolizable energy	Antioxidant capacity	Phenols	Flavonoids	Anthocyanins	Phytic acid
Fat	1	.063	-.056	-.123	.099	-.012	-.080	.021	.024	.047
		.438	.492	.127	.219	.880	.325	.795	.765	.560
Fiber	.063	1	-.476**	.086	-.503**	-.303**	.115	-.087	-.010	-.012
	.438		.000	.288	.000	.000	.153	.281	.899	.882
Carbohydrates	-.056	-.476**	1	-.542**	.864**	.307**	-.057	.207**	-.061	.176
	.492	.000		.000	.000	.000	.480	.010	.451	.028
Protein	-.123	.086	-.542**	1	-.094	-.066	-.030	-.123	.078	.152
	.127	.288	.000		.247	.412	.710	.128	.332	.059
Metabolizable energy	.099	-.503**	.864**	-.094	1	.325**	-.105	.180	-.021	-.111
	.219	.000	.000	.247		.000	.195	.025	.792	.171
Antioxidant capacity	-.012	-.303**	.307**	-.066	.325**	1	.021	.185*	.163*	.043
	.880	.000	.000	.412	.000		.791	.022	.043	.599
Phenols	-.080	.115	-.057	-.030	-.105	.021	1	.354**	.221**	.008
	.325	.153	.480	.710	.195	.791		.000	.006	.917
Flavonoids	.021	-.087	.207**	-.123	.180	.185*	.354**	1	.121	.145
	.795	.281	.010	.128	.025	.022	.000		.132	.072
Anthocyanins	.024	-.010	-.061	.078	-.021	.163*	.221**	.121	1	-.147
	.765	.899	.451	.332	.792	.043	.006	.132		.069
Phytic acid	.047	-.012	.176	.152	-.111	.043	.008	.145	-.147	1
	.560	.882	.028	.059	.171	.599	.917	.072	.069	

** The correlation is significant at the 0.01 level (bilateral).

* The correlation is significant at the 0.05 level (bilateral).

Cluster analysis

The cluster analysis (Table 3) was performed based on the physicochemical properties and the content of bioactive compounds, obtaining as a result five groupings based on their similarities, from which clusters 2 and 3 were selected because the varieties that the conglomerates presented the highest values for protein, fibre and anthocyanins (cluster 2) as well as the highest phenol and flavonoid values (cluster 3). Phytic acid content was not taken into account when selecting the outstanding groups, because its capacity as a beneficial or antinutritional compound does not depend on the content or the source that comes from Rather, it depends

on the diet followed by the consumer, since in a diet based on grains and cereals with a low mineral protein intake, phytic acid can act negatively, on the contrary, in a balanced diet with micronutrients availability, the phytic acid expresses its value as a bioactive compound (Díaz-Batalla *et al.*, 2006).

Table 3. Centre of final clusters for 155 bean varieties

	Cluster				
	1	2	3	4	5
Number of varieties	45	10	4	56	40
Protein (%)	20.16	28.13	19.89	23.18	25.25
Phenols (mgGA/g)	0.90	0.79	1.63	0.81	0.79
Flavonoids (mgCE/g)	1.44	1.11	5.78	1.36	1.63
Anthocyanins (mgC3g/g)	1.17	1.51	1.35	1.20	1.15
Phytic acid (mg/g)	0.49	0.53	0.59	0.51	0.58
Fat (%)	1.43	1.56	1.63	1.31	1.26
Fiber (%)	3.51	4.55	3.33	3.27	3.67
Carbohydrates (%)	62.36	49.26	59.01	58.26	54.90

We obtained a total of 14 outstanding varieties (Table 4), where 4 varieties stood out in terms of their content of phenols and flavonoids, a second group composed of 10 varieties with high levels of protein and anthocyanins. Moreover, this group showed a low percentage of carbohydrates and a high content of fibre and fat, which makes these varieties future foods with a low glycaemic index (American Diabetes Association, 2015). A great diversity can be observed within the outstanding group, where there are varieties from different regions of the country and different shapes, sizes and colours of the bean grain. The outstanding varieties, according to the cluster analysis, showed great diversity, 'FIRA (2016)', indicate that beans are located in three large consumption groups: highly preferred ('Azufrado', 'Mayocoba', 'Negro Jamapa', 'Peruano', 'Flor de Mayo' and 'Flor de Junio'), preferred ('Garbancillo', 'Manzano', 'Negro' and 'Pinto Americano') and non-preferred ('Alubia', 'Bayo', 'Negro Zacatecas', 'Ojo de Cabra' and 'Pinto Mexicano'). Therefore, within the results obtained, there is at least one variety of the most consumed beans within the national territory. Also, five varieties of black and pinto beans were obtained, which together contribute more than 60% of the national production (Lara-Flores, 2015; FIRA, 2016).

Main components analysis

On the other hand, the principal component analysis had information on 6 variables that included bioactive compounds, protein content and carbohydrate content (Table 5). The first three components explained 74.75% of the total variation: first component 31.09%, second component 24.48% and third component 19.18%. In the first component, the most significant variables were the content carbohydrate, phenols and flavonoids. In addition, there was a contrast in the protein content with carbohydrates. In the second component, there was a contrast between the bioactive compounds (phenols, flavonoids, anthocyanins and phytic acid) and the protein content with the carbohydrate content. While in the third component, he contrasted the concentration of phytic acid with that of anthocyanins.

Table 4. Outstanding bean varieties based on the cluster analysis






No	Variety	Place	Protein	Phenols	Flavonoids	Anthocyanins	Photo
43	'Pelón Oscuro'	Teapa, Tabasco	26.75	0.5682	1.3183	0.8617	
54	'Frijol Mejenives'	Dzutoh, Yucatán	29.08	0.4476	0.7689	0.9268	
57	'X-Pelón'	Dzutoh, Yucatán	29.37	2.4982	1.6795	2.5149	
63	'Pelón Rojo'	Teapa, Tabasco	28.72	1.1054	1.7473	1.3593	
67	'Higuera azufrado'	Culiacán, Sinaloa	28.86	0.3470	0.7652	1.1472	
74	'Alubia'	Guadalajara, Jalisco	25.98	0.3050	0.9533	0.8260	
82	'Ejotero Silvestre'	San Juanito, Chihuahua	29.79	0.2713	1.3748	1.6482	
84	'Negro guaca'	Tuxtepec, Oaxaca	28.13	1.0155	1.3371	1.8068	
87	'Negro'	Tuxtepec, Oaxaca	27.44	0.7868	0.6447	1.8480	
98	'Sangre de Toro'	Tlayacacan, Morelos	18.65	0.9497	3.1584	2.4002	
100	'Peruano'	Cuautla, Morelos	27.19	0.6062	0.5507	2.1475	
114	'Frijolón Amarillo'	Nochixtlán, Oaxaca	19.52	2.3486	8.8254	0.7125	
133	'Frijolón Negro'	Ocotlán, Oaxaca	21.00	1.2383	5.5441	0.9129	
138	'Frijolón 1'	Centro, Oaxaca	20.38	1.9739	5.5817	1.3649	

Table 5. Principal components of six quality variables observed in 155 bean varieties grown in Mexico

Variable	Principal components (PC)		
	1	2	3
Protein (%)	-0.927	.133	-0.041
Phenols (mgGA/g)	.210	.768	-0.009
Flavonoids (mgCE/g)	.220	.718	.333
Anthocyanins (mgC3g/g)	-0.064	.555	-0.602
Phytic acid (mg/g)	-0.242	.140	.795
Carbohydrates (%)	.922	-.138	.055

The spatial distribution of varieties was made based on the first 2 components (Figure 1), it can be observed in quadrant I (upper-right) a clustering trend based on the high concentrations of the bioactive compounds evaluated, in turn quadrant IV (upper-left) groups varieties with high concentrations of bioactive compounds and also adds varieties with high levels of protein. Finally, in quadrants II and III (Lower-right and lower-left) those varieties were found with a high content of carbohydrates, but which did not stand out in the levels of the other variables evaluated.

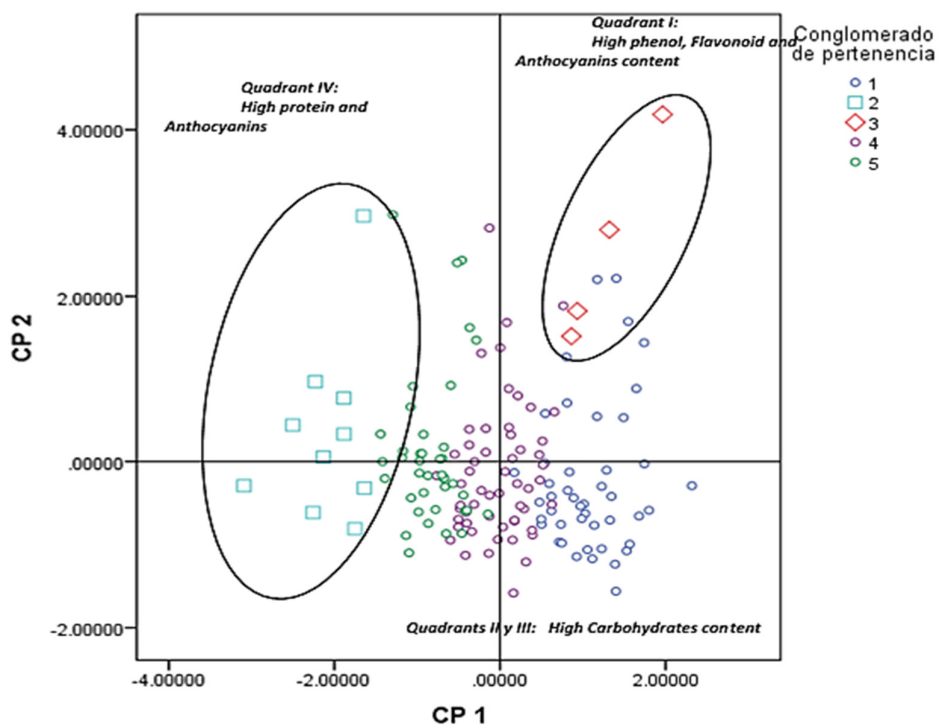


Figure 1. Spatial distribution of 155 bean varieties based on their first two components, grouped according to the cluster analysis

Conclusions

Fourteen outstanding varieties were obtained, based on the variables evaluated, where 4 stood out for their high levels of phenols and flavonoids; On the other hand, 10 more varieties stood out for a high level of protein, in addition to acceptable levels of anthocyanins, phytic acid and a low content of carbohydrates. Varieties such as 'Frijolón 1', 'Frijolón Amarillo', 'Frijolón negro' and 'Sangre de toro' were remarkable due to their high content of bioactive compounds, so it is recommended to incorporate these varieties to a biofortification program to improve the nutritional quality of beans. The other 10 remaining varieties, due to their high protein content, are recommended to be used as an alternative of vegetal protein with a good glycemic index. The diversity of beans obtained in the outstanding varieties generates options for biofortification programs based on the consumption of each variety or its place of origin.

Authors' Contributions

Conceptualization: ES; Methodology: APM, DOB; Validation: JJC, OAHR; Formal analysis: APM, JJC; Investigation: APM, DOB, OAHR; Data curation: ES, PPR; Funding acquisition: ES; Project administration: ES; Writing: APM, ES; Review and editing: APM, ES; All authors read and approved the final manuscript.

Acknowledgements

Alejandro Palacio Márquez acknowledges financial support given by Plant Physiology and Nutrition Laboratory of the Food and Development Research Center (CIAD Delicias) And the National Council of Science and Technology (CONACyT – Mexico) for the support provided by means of the Convocatoria Atención a Problemas Nacionales: Project #1529 "Biofortification of basic agricultural crops representing the key to combat malnutrition and ensure food security in Mexico"

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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