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ORIGINAL PAPER

Development of a High Protein Beverage Based on Amaranth

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Abstract

The objective of this study was to formulate a beverage based on amaranth proteins, stable and nutritious. The process of obtaining the beverage was based on the existing knowledge about starch separation techniques and techno-functional properties of the amaranth proteins. Gums, gellan and xanthan were added to the protein extract and it was heat-treated at 80 °C during 20 min. A beverage with a composition similar to skim cow's milk was obtained (3.42 ± 0.08; 0.60 ± 0.06; 1.9 ± 0.4; 0.43 ± 0.01; 3 and 90.58 ± 0.01% for proteins, lipids, fiber, ashes, carbohydrates and water, respectively). Thermal treatment caused the denaturation and aggregation of the proteins, while the addition of gums induced a decrease in the sensitivity to heat treatment of the proteins. Formation of protein aggregates and gum-protein complexes was characterized by electrophoresis, differential scanning calorimetry, and particle size distribution. Heat treatment and addition of gums generated macrocomplexes with enhanced absolute value of ζ-potential, which contributed to the high colloidal stability of amaranth-based beverage. This beverage is suitable for vegans, celiac patients, and lactose intolerants.

Keywords Amaranth · Beverage · Physicochemical properties

Introduction

Both the growth of world's population and the rising of standard of living have driven a transition from animal towards plant proteins. Novel products that mimic traditional foodstuff are developed to satisfy those demands, while trying to generate healthy foods whose production is environment friendly. New food products have been designed at the same time as old food products have been adapted and revalued. In this way, plant-based beverages represent environment friendly and cheaper dairy substitutes [1]. Plant-based cow's milk substitutes have a reputation of "health foods" but the products on the market vary remarkably in their nutritional profiles, some having very low protein and mineral contents [2]. An interesting challenge in plant-based milks is to reach a protein content equivalent to cow's milk while maintaining acceptable rheological properties. There are soy milks with similar protein content to cow's milk, but other vegetable-based beverages, such as those prepared from almond, cashew, coconut and rice, at most reach 1% (w/v) of protein [3]. A threat to reach this goal is the starch content of many seeds, since after thermal treatments it gelatinizes, greatly increasing the viscosity. To prevent this, in the further processing steps starch content should be reduced using enzymes or other strategies [2]. However, this step can be economical and technologically adverse and also it may compromise the mouthfeel for the final product as occur in rice- and other plant-based milks [2]. Amaranth seed contain 13.6 ± 0.8% (w/w, w.b.) protein, 7.3 ± 0.3% (w/w, w.b.) lipids and 69.0 ± 0.2% (w/w, w.b.) carbohydrates, of which 84% corresponds to starch and 16% to dietary fiber [4]. Plant proteins are generally of a lower nutritional quality compared to animal ones due to limiting amino acids. However, amaranth proteins present a high biological (86%) and nutritional value since the proportion of their essential amino acids is close to that recommended by the World Health Organization [5]. The temperatures of

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gelatinization of amaranth starch are in the 55–82 °C range depending on variety and processing conditions [6]. Thus, avoiding gelatinization when starch is present is difficult in most of the processes required to make a safe food. Starch isolation can be carried out by several techniques. Some authors described methods that involve a strong alkaline soaking at which starch and other seed components remain insoluble whereas proteins are solubilized [7]. These methods could be adjusted to separate starch while preserving the functional and nutritional properties of the proteins, which would serve as the basis for the formulation of an amaranth-based beverage (ABB). Given its nutritional, physicochemical and functional properties, amaranth could be a good alternative for the development of plant-based beverages, however there are very few studies in this regard. Milán-Carrillo et al. [8] reported the preparation of an ABB, since no separation of starch was carried out, protein and carbohydrate content were 1.6 and 7.2% (w/v), respectively. The authors stated that this beverage was highly nutritious and antioxidant. de Meo et al. [9] analyzed the possible use of some pseudo cereals - buckwheat, quinoa, and amaranth - for making beer suitable for celiac patients, while recently Pivovarov et al. [10] developed a drink based on an extract obtained from amaranth leaves. An ABB would be also a vehicle for health promoting compounds such as bioactive peptides and antioxidants [5]. Our hypothesis considers that a safe and physically stable beverage with high protein and low starch contents can be obtained from amaranth seeds by combining knowledges from starch-isolating techniques and the physicochemical and functional properties of amaranth proteins. The aim of this work was to obtain processing conditions and a formulation to prepare an ABB with good physical properties, stability and microbiologically safe.

Materials and Methods

Experimental Design Amaranth slurries were prepared under different proportions between seeds and water (1:5; 1:4; 1:3) and pH (8, 9, 10). These slurries were characterized in terms of protein content. Once the proportion and pH were chosen, different thermal treatments were assayed (80 or 90 °C). Once the thermal treatment was chosen based on its efficiency to inactivate microorganisms, two concentrations of stabilizing-hydrocolloids (gellan gum (GG) and xanthan gum (XG)) were assayed. Finally, a comprehensive characterization was carried out on the final product.

Colloidal Milling of Amaranth Seeds Amaranth seeds (*Amaranthus hypochondriacus* var. Antorcha) were harvested in Río Cuarto, Córdoba, Argentina. Amaranth slurries were prepared from untreated seeds, which were ground with deionized water or with GG and XG dispersions (0.015 and 0.020 or 0.035 and 0.045 g of GG and XG, respectively/100 g of dispersion) using different seeds:water mass ratios. Amaranth slurries were obtained as follow: seeds were ground for 1 min at full speed using a colloidal mill (model AD 35-R, ColMil, Argentina) at 20 °C. The pH was accurately adjusted to 8, 9 or 10 with 10 mol/L NaOH and kept under magnetic stirring for 2 h at 25 °C. Then, samples were centrifuged at 6000 *g* for 20 min at 20 °C (Beckman Coulter Avanti J-25 (Brea, California, USA)). The supernatant was filtered through two layers of cheese cloth. The filtrate was collected, and the pH was adjusted to 7.30 with 5 mol L^{-1} HCl. Amaranth beverages were heated in a water bath Vicking model Masson 1002 (Buenos Aires, Argentina) until the temperature in the center of the sample reached 80 or 90 °C. The temperature was maintained at those values for 20 min.

Amaranth Beverage Characterization

Amaranth Beverages Proximal Composition Moisture, ash, fat and protein contents were determined in triplicate using AOAC procedures [11]. N to protein conversion factor was 5.85. Megazyme (Megazyme International Ltd., Wicklow, Ireland) was used for determining total, soluble and insoluble dietary fiber. Carbohydrate content was calculated by difference. Ca, P, K and Fe content of the beverage with and without gums was carried out by microwave-assisted digestion of the sample in concentrated HNO_3 until total dissolution (EPA 3052 Norm) and subsequent analysis by plasma emission spectroscopy by inductive coupling and optical detection (EPA Standard 6010D).

Confocal Microscopy Two hundred μL of fresh amaranth beverage were mixed with 50 μL of different fluorescent probes: BODIPY® 493/503 for lipids and Rhodamine b 553/627 for proteins. Five μL were expanded onto a glass slide and covered with a glass coverslip. The simultaneous labelling of two components of foods with probes, specific for each component, permitted a more detailed analysis of amaranth beverage structure. A Leica SP5 (Leica Microsystems Heidelberg GMBH) confocal microscope with LAS X Leica Application Suite X 2017 software was used. Objectives of 20 and 63 X were used, fitted with blue (496 nm), green (543 nm) and red (633) lasers.

Viscosity Samples of amaranth beverages without and with GG and XG (0.015 and 0.020%, w/v, or 0.035 and 0.045%, w/v) treated at 80 °C were used to determine viscosity at 20 °C. Two commercially beverages based on soy and almond were also analyzed. A Haake rotative Viscotester VT550 (Thermo Electron Corporation) was used. Rough concentric cylinders, model MV1P, were used as sensor.

ζ-Potential ζ-potential of diluted samples (1 mL beverage in 3 mL deionised water) was evaluated in triplicate in a dynamic light scattering (Zetasizer Nano Zs, Malvern Instruments Ltd., Malvern, UK). The ζ-potential was evaluated from the electrophoretic mobility of the particles present in whole samples. The conversion of the measured electrophoretic mobility data into ζ-potential was obtained using Henry's equation: $U_e = 2\ \varepsilon\ \zeta\ f(Ka)\ /\ 3\ \eta$ where U_e ($m^2\ V^{-1}\ s^{-1}$) is the electrophoretic mobility, ε ($C^2\ N^{-1}\ m^{-2}$) is the dielectric constant, ζ (mV) is the ζ-potential, η (Pa s) is sample viscosity, and f(Ka) (dimensionless) is Henry's function.

Particle Size Distribution Particle size distribution of diluted samples (1 mL beverage in 3 mL deionized water) were determined by dynamic light scattering (DLS) using a Zetasizer Nano Zs (Malvern Instruments Ltd., Malvern, UK). Data analysis was performed with Zetasizer Nano software according to the Stokes-Einstein equation: $d(H) = k\ T\ /\ 3\ \pi\ \eta\ D$ where d (nm) is the hydrodynamic diameter of the particle, k ($J\ K^{-1}$) is the Boltzmann's constant, T (K) is the absolute temperature, η (Pa s) is sample viscosity, and D ($m^2\ s^{-1}$) is the diffusion coefficient.

Molecular Characterization of Amaranth Slurries and ABB Using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) The composition of the whole untreated and heat-treated slurries and ABB was analyzed by SDS-PAGE under reducing and non-reducing conditions according to Laemmli [12]. All gels were run in minislabs (Bio-Rad Mini Protean II Model). The electrophoretic runs were conducted for 1 h at a constant voltage of 200 V. The molecular weights of polypeptides were calculated by using low molecular weight standards (GE-Healthcare, USA).

Differential Scanning Calorimetry (DSC) Thermal properties were determined in a TA-Q100 calorimeter (TA-Instruments, New Castle, DE, USA). Hermetically sealed aluminum pans were prepared containing 10–15 mg of suspended lyophilized ABB (ca. 3.5 mg protein/pan) equilibrated at 20 °C for 2 min, heated from 20 to 130 °C at a rate of 10 °C min^{-1} and finally equilibrated at 130 °C for 2 min. Thermograms were analyzed with the Software Plus V5.41 Universal Analysis 2000. Peak temperature (Td) and enthalpy change (ΔH) were determined.

Activity of Trypsin Inhibitors Total trypsin inhibitors activity was assessed using the enzymatic method described by González and Carrillo [13] with some modifications. This method is based on the hydrolysis of hemoglobin by trypsin. If trypsin is active, a blue color is formed due to the reaction between the peptides released from hemoglobin and Folin-Ciocalteu reagent (Sigma, St Louis, USA). Trypsin inhibitors were extracted in triplicate from untreated and treated ABB [14].

Physical and Microbiological Stability of Amaranth Beverage

Microbiological Analyses Ten g of amaranth beverages (control and heat treated) were taken aseptically and homogenized with 90 mL of sterile 0.1 g 100 mL^{-1} peptone water (Britania, Argentina). Decimal dilutions were prepared with sterile 0.1 g 100 mL^{-1} peptone water and plated in: Plate Count Agar (PCA, Britania, Argentina) for mesophilic aerobic bacteria (MAB) count, or Yeast extract glucose chloramphenicol agar (YGC, BioKartell, Milan, Italy) for molds and yeast counts. PCA plates were incubated at 37 °C for 24–48 h and YGC plates at 28 °C during 48–72 h [15]. The results were expressed as colony forming units per g of amaranth beverage (CFU g^{-1}).

Colloidal Stability The colloidal stability of amaranth slurries (without or with GG and XG) was assessed by evaluating phase separation during storage. Samples of 20 mL were poured into transparent glass tubes (internal diameter 15 mm) and stored at 20 °C. Observations were made after 7, 14, 30 and 60 days of storage. Physical stability was estimated indirectly as the ratio between the length of the upper clarified phase and the total length of the sample.

Statistical Analysis Three different batches of slurries and ABB were prepared. For each determination, triplicate samples were analyzed. One-way ANOVA was applied on data, differences between means were analyzed by Tukey test ($p < 0.05$) using the OriginPro 8.5 software.

Results and Discussion

Effect of Processing Conditions

Effect of pH of Protein Extraction and Seed:Water Ratio on Protein Content of Amaranth Seed Extracts Protein content in supernatants after the centrifugation step in the procedure for obtaining the beverage was function of extraction ratio seed:water and pH. The seed:water ratio that yielded the highest protein content in supernatants was 1:3 (Table 1), protein extraction can be facilitated by a great water volume, but it would lead to a lower protein concentration. This ratio allowed a relatively high protein concentration in the slurry while the viscosity of the dispersion was compatible with the milling. These results are in accordance with those reported by Diarra et al. [16] for peanut milk. The highest contents of proteins and yield were obtained at pH 10 (Table 1). Amaranth proteins were more soluble as the pH increased, due to the increase in negative charges by moving away from the isoelectric point (ca. 5 [17]). Higher pH, such as 11 or 12, as it as used by Perez et al. [7] could improve protein

Table 1 Protein content of different amaranth slurries obtained from different seed:water mass ratio

Seed:water	pH	Protein content (g/100 g sample)	Yield (g protein in slurry/g protein in seeds)
1:5	10	1.60 ± 0.05^{d}	0.71
1:4	10	1.85 ± 0.01^{c}	0.75
1:3	10	3.42 ± 0.08^{a}	0.77
1:3	9	3.5 ± 0.1^{a}	0.73
1:3	8	2.77 ± 0.05^{b}	0.66

Different superscript letters indicate significant differences ($p < 0.05$). Results are expressed as mean ± SD

extraction, but they are not recommendable because lysinoalanine formation is favored at those pHs and was reported as a toxic amino acid [18]. Since our aim was to obtain a beverage with the highest protein content, and the yield was not greatly affected by the seed:water ratio, as it was by the pH (Table 1), we decided to choose the 1:3 ratio and pH 10 to continue the work. The protein extraction at 25 °C minimized the possible adverse effects of the alkaline treatment. At this temperature and considering the treatment time, the level of amino acid racemization and lysinoalanine formation is very low, particularly in vegetable proteins such as those from alfalfa and soybeans [18].

Thermal Treatment Amaranth slurries without thermal treatment contained microorganisms that grew in PCA and YGC with counts that were 10^5 and 10^2 CFU. 100 mL^{-1}, respectively. After both treatments assayed, counts of bacteria, fungus and yeast were undetectable. Treatment at 90 °C during 20 min produced coagulation of amaranth components and rapid sedimentation of insoluble macroscopic particles. Konishi and Yoshimoto [19] studied solubility of amaranth globulins at pH 7.5 after thermal treatments of 15 min and reported that solubility was high (ca. 85%) up to 80 °C and it diminished drastically after treatments at 90 and 100 °C. Since in our samples insolubilization was not observed when samples were heated at 80 °C, this temperature was chosen to inactivate microorganisms.

Effect of Addition of Gellan and Xanthan Gums on Viscosity

Viscosity of amaranth slurries was increased by the addition of GG and XG (Table 2). This effect could be due to their ability to form networks among themselves and also to interactions with amaranth components. The highest gums concentration led to the highest viscosity. The values achieved in gum-added samples were higher than those of commercially plant-based milks (7.9 ± 1.3 and 6.8 ± 1.2 cp were detected for soy- and almond-milks, respectively). Since our aim was to obtained a stable beverage with no excessive viscosity, the lowest level of hydrocolloids was chosen to formulate the ABB.

Table 2 Apparent viscosity (η_{app} at 400 s^{-1}) of amaranth beverages

Beverage	η_{app} (cP)
Non-added beverage	8.8 ± 0.7^{c}
Lowest GG-XG	18.9 ± 2.9^{b}
Highest GG-XG	28.2 ± 0.9^{a}

GG: Gellan gum; XG: Xanthan gum. Different superscript letters indicate significant differences ($p < 0.05$). Results are expressed as mean ± SD

Characterization of Amaranth-Based Beverage

Proximate Composition The composition of macrocomponents (Table 3) corresponds to an ABB with protein content similar to that of cow's milk, but with a lower energy contribution from lipids and carbohydrates. Thus, this product could be classified as "low calories", while with a protein content higher than that of the most of commercially available plant-based beverages, which have protein contents between 0.3 and 2.2% (w/v) [20]. Fat content of ABB was low because the moderate lipid content of the seeds (6.9–9.1% w/w d.w. [4]) and because the ABB was prepared from the supernatant of protein aqueous extraction. Lipids in the seeds are present as lipid bodies in the embryo and endosperm cells [21]. Probably, during homogenization cells

Table 3 Centesimal composition of amaranth-based beverages

	g 100 g^{-1} sample
Proteins	3.42 ± 0.08
Carbohydrates*	3.07
Lipids	0.60 ± 0.06
Fibres	1.9 ± 0.4
Ashes	0.43 ± 0.01
Water	90.58 ± 0.01
	mg 100 g^{-1} sample
P	32.70 ± 3.60
Ca	14.48 ± 0
K	5.00 ± 0.69
Fe	0.74 ± 0.09

* Carbohydrates content was obtained by difference

were not broken enough to allow the release of lipid bodies, thus a high proportion of lipids remained in the pellet of centrifugation. Lipid extraction is favored at high temperature, but in our case, the step of extraction at pH 10 was carried out at 20 °C, so fat release from seed matrix was not favored. The carbohydrate content of the beverage was 3% (w/w), which indicates that the main component of the amaranth seed carbohydrates, the starch, was discarded in the step of centrifugation after extraction at alkaline pH. According to Chalupa-Krebzdak et al. [20] the plant-based milks contain a wide range of carbohydrate, 0.42–11.05% (w/v), depending on the milk origin and formulation. ABB contained 1.9% w/w of soluble dietary fiber constituted fundamentally by xyloglucans branched with disaccharides and trisaccharides and pectins constituted by homogalacturonans and arabinans [4, 22]. The presence of insoluble dietary fiber was not detected.

Water content was in the expected range for this type of beverages (Table 3). Chalupa-Krebzdak et al. [20] reported water contents between 88 and 97% for plant-based beverages from different sources; the highest water content corresponded to almond-based samples which in turn had the lowest protein content. The dietary energy provided by 100 g of the ABB obtained was 31.44 Kcal, which is equivalent to the contribution of the same amount of skim cow's milk.

Although the Ca content was not equivalent to those of cow's milk (Table 3), it was higher than that found in other non-calcium-added plant-based milks [20, 23]. The addition of Ca to plant-based beverages is an important topic from the nutritional and colloidal stability points of view [1]. Starting from a relatively high value can simplify issues related to colloidal stability. The Fe content of ABB was slightly higher than that of soy milk, while the P content was slightly lower and the K content was very poor (Table 3).

Protein Profile Different samples, with or without heat treatment and in the absence or presence of gums, were analyzed by using SDS-PAGE. In non-reducing conditions (Fig. 1panel a) the profiles corresponding to unheated samples (lanes 2 and 2′) showed typical species of the albumin fraction of amaranth seeds (Molecular mass (MM) species less than 30 kDa and between 30 and 60 kDa), 11S globulins (2 bands ~ at 55 kDa, ~. 40 kDa, and 30 and 20 kDa), P-globulin (characteristic band at ~. 55–60 kDa) and glutenins (bands shared with 11S globulin and P globulin). Furthermore, species with high MM equal to or greater than 97 kDa were detected. These results are consistent with previous ones obtained by Martinez and Añón [17] for purified fractions and isolates obtained at different extraction and precipitation pH. Although no precipitation step was included in the production of ABB, the polypeptide species present in the isolates extracted at pH 9 and precipitated at pH 7 [17] should be present in ABB, in both soluble and dispersed aggregates of high MM. In isolates precipitated at pH 7 these authors detected a significant amount of aggregates in which 7S, 11S and P globulins, and glutenins participated. The heated samples showed similar profiles (lanes 1 and 1′), with a smaller amount of aggregates of high MM, higher intensity of the band corresponding to 97 kDa and aggregates that did not enter into the gel. These results suggest that heat treatment induces a redistribution of polypeptides in different aggregates, basically by destabilization of non-covalent interactions initiated by the weakening of hydrogen bonds followed by the formation of new bonds. Some, which do not enter the gel without heating, could break and increase the proportion of 97 kDa aggregates while others would form very high MM aggregates which would precipitate during centrifugation before loading the sample on the SDS-PAGE gel. In the presence of gums (lanes 3, 3′, 4 and 4′) the profiles were similar to those indicated above, although a decrease in the intensity of the bands between 30 and 66 KDa can be observed. It could be assumed that these protein species could be interacting between them and with the gums, forming high molecular mass complexes that did not penetrate the gel.

The samples obtained before and after heat treatment, marked with Rhodamine were analyzed by confocal microscopy. Before heating small aggregates of proteins were observed scattered in the aqueous matrix, the heat treatment caused aggregation of the proteins that make up a network similar to that of a gel (additional data are given in Supplementary Material (SM), Fig. S1). These results coincided with the presence of high MM aggregates detected in the

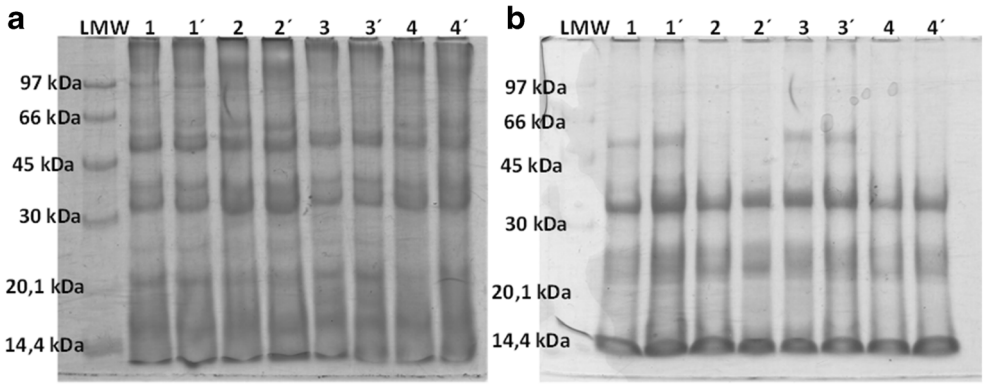

Fig. 1 SDS-PAGE profiles in absence (panel **a**) or presence (panel **b**) of reducing conditions. LMW: standards low molecular weight. 1, 1′, 2, 2′: non gum-added amaranth beverages treated at 80 °C 20 min (1, 1′) or without thermal treatment (2, 2′). 3, 3′, 4, 4′: gum-added amaranth beverages treated at 80 °C 20 min (3, 3′) or without thermal treatment (4, 4′)

electrophoretic runs. Possibly, the largest aggregates would remain insoluble and were not present in SDS-PAGE lanes. The samples were also labeled with BODIPY, but it was not possible to distinguish the precise location of lipids in the beverage matrix (additional data are given in SM, Fig. S1). The low ratio between lipids and proteins (0.17 g lipids/g proteins) and the colocalization of fluorescent markers suggest that lipids could be interacting with oil body associated proteins rather than under the form of droplets. Oil body associated proteins in amaranth seeds were previously described [24]. Moreover, the pH shifting during protein extraction could increase surface hydrophobicity, as it was reported for cowpea proteins [25], which in turn favored protein-lipid association.

Under reducing conditions (Fig. 1, panel b) the disappearance of protein species with MM higher than 35 kDa as an increase of the acid and basic polypeptides of 11S globulins were clearly observed both in the presence and absence of gums. The presence of a protein specie of ~55 kDa was detected in heated samples, both in the absence and presence of gums, which were not sensitive to the reducing conditions. This band would correspond to the globulin P subunit, which lacks a disulfide bond between acidic and basic polypeptides [26], it would indicate in principle that this specie would be forming part of the high MM aggregates present in unheated samples, which would be rearranged and released during heat treatment. Another alternative would be the appearance of an unidentified protein species as a consequence of the thermal treatment. The results obtained by electrophoresis suggest the presence of aggregates in the samples, of different molecular size depending on whether or not they were heated, aggregates that would be stabilized by non-covalent bonds sensitive to SDS and covalent, disulfide bridges, sensitive to mercaptoethanol treatment. These aggregates would be formed mainly by 11S globulins and P globulin, and the presence of glutenins cannot be ruled out.

Thermal Behavior of Proteins The slurries (samples without GG and XG) and the ABB were analyzed by DSC. Unheated slurries showed a single denaturation peak at 103.5 ± 1.6 °C and a ΔH of 11.1 ± 0.5 J g^{-1} protein, respectively. The extraction at pH 10 caused partial denaturation of the amaranth proteins which partially reversed when the pH was decreased to 7.3 (additional data are given in SM, Fig. S2). The protein fractions more affected in this condition are albumins and glutelins [17]. The addition of GG and XG to the slurries did not modify their calorimetric behavior. In this case, a thermogram with a single peak at 103.1 ± 1.8 °C and a ΔH of 10.3 ± 0.6 J g^{-1}. When the samples without GG and XG were heat-treated at 80 °C, the alkaline pH-heat treatment sequential combination induced a high degree of denaturation of the proteins (82%) (additional data are given in SM, Fig. S2). Noteworthy, the thermal treatment carried out at 80 °C was able to denature the protein species that had Td at 103 °C; considering that thermal treatment lasted 20 min and considering the heating and cooling times, this fact shows the kinetic character of the thermal denaturation. In ABB, the presence of GG and XG seemed to protect proteins against heat treatment. The thermograms corresponding to heated samples with GG and XG showed an endotherm at 106.9 ± 0.9 °C, with ΔH of 7.2 ± 1.9 J g^{-1} protein (additional data are given in SM, Fig. S2). The increase in Td and the smaller degree of denaturation (31%) suggest that the formation of protein-gum complexes led to a decreased sensitivity against heat treatment. Ibanoglu [27] stated that hydrocolloids associated with whey proteins, which resulted in increased Td.

Particle Size Distribution Samples without gums and before thermal treatment exhibited monomodal particle size distributions in both number and volume distributions, with modes of 0.7 and 1.4 μm, respectively (Fig. 2 panels a and b, solid lines). Since many plant globulins have sizes close to 10 nm, these values indicate that proteins were involved in aggregates. The addition of GG and XG (0.015 and 0.020% (w/v), respectively) increased the aggregation of amaranth proteins, which was reflected as the appearance of a second peak in the number distribution and of two peaks in the volume one (Fig. 2 panel c and d, solid lines). These observations reflect the different weighting in each distribution and indicate that there was a very small number of the largest aggregates. Gum-induced aggregation had also been suggested by the SDS-PAGE results. The association between GG and milk proteins was reported [28]. These authors stated that although GG is a polyanion at pH 7 (as are amaranth proteins), it has relatively low charge density, which would allow hydrogen bonds formation with proteins. Moreover, Dickinson and Pawlowsky [29] reported the association between bovine serum albumin and K-carrageenan through electrostatic attraction at pH 7 which became stronger at pH 6. The attraction of positively charged local patches on the protein and the negative charges on the polysaccharide result in the formation of soluble protein–polysaccharide complexes [27]. Thermal treatment promoted the formation of larger aggregates as shown in the previously described SDS-PAGE and confocal microscopy assays. In samples without gums, a slight increase in mode was detected in number distribution (from 0.7 to 0.8 μm), but a second peak with mode at 8.1 μm appeared in volume distribution (Fig. 2 panels a and b, dashed lines). For GG- and XG-added samples, heating resulted in the appearance of two new populations, thus the modes were 0.9, 19.9 and 239.5 μm in volume distribution (Fig. 2 panel d, dashed line). The largest particles were very few, as seen in number distribution where the highest population exhibited a mode of 0.7 μm and a shoulder at 2.5 μm (Fig. 2 panel c, dashed line). XG promoted the aggregation of soybean proteins at pH 7, the aggregates were stabilized by electrostatic

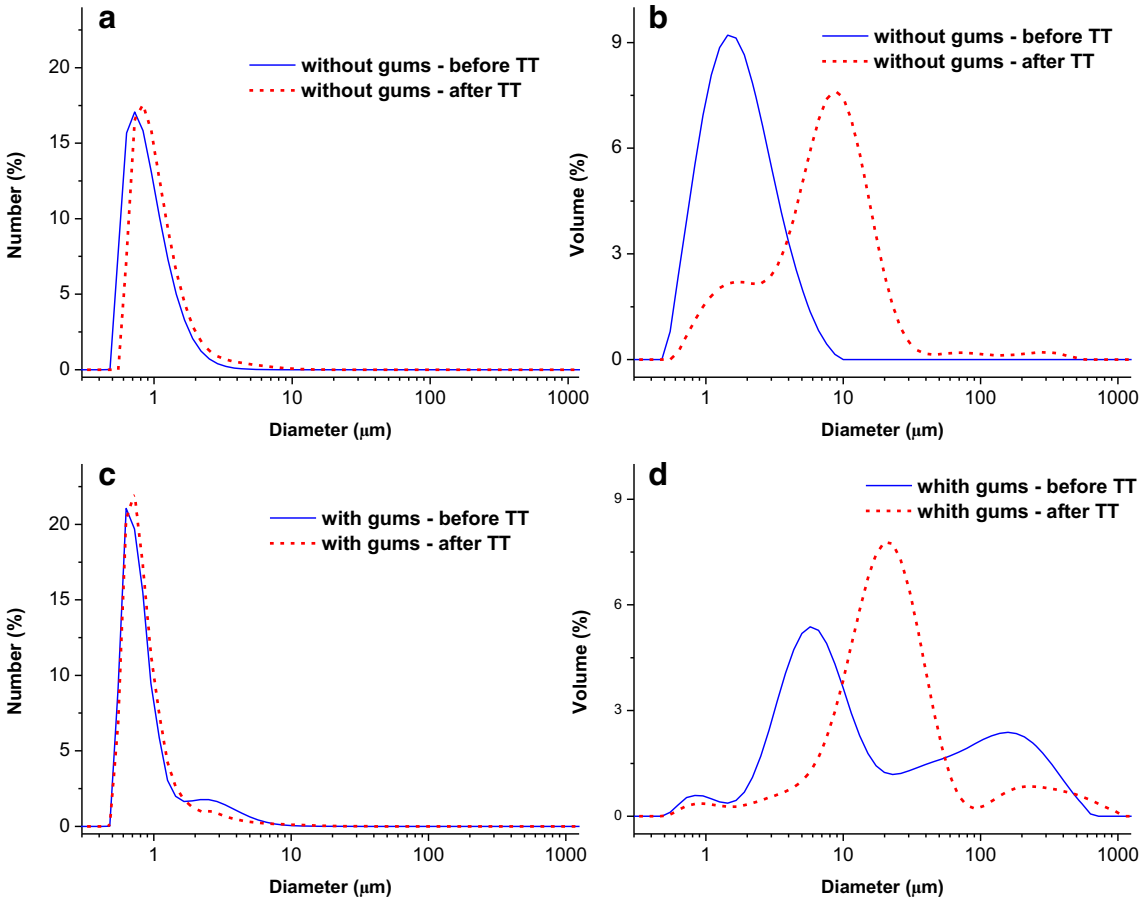

Fig. 2 Particle size distribution of slurries (without gums, panels **a** and **b**) and amaranth-based beverages (with gums, panels **c** and **d**) before (solid line) and after (dotted line) thermal treatment (TT)

interactions when proteins were in native state, while other interactions seemed to be added when proteins were unfolded [30]. Moreover, Ibanoglu [27] stated that following heat-induced denaturation, much stronger interactions can be formed between hydrocolloid gums and proteins, giving rise to stable high molecular weight complexes which inhibit the protein–protein aggregation and therefore increase the solubility of the protein. The formation of soluble or colloidal-stable macrocomplexes may partially explain the increase in viscosity and could improve colloidal stability.

ζ-Potential The absolute values of ζ-potential of amaranth proteins were relatively high for unheated samples, −29.7 ± 0.7 mV. This value is considered high enough to provide stability to dispersed systems, because of electrostatic repulsion [31]. In our system, the negative charges were due to the distance to the isoelectric point and were significantly ($p < 0.05$) increased with the addition of GG and XG, ζ-potential was −30.6 ± 1.2 mV in added samples. This fact indicates the association of GG and XG with amaranth proteins, as it was also concluded from particle size distribution analysis. Thermal treatment increased absolute value of ζ-potential in GG- and XG-added samples (−32.1 ± 1.2 mV), but not in samples that did not contain gums (−28.3 ± 0.4 mV). This result suggests that protein denaturation allowed a higher degree of association between these hydrocolloids and amaranth proteins, which was also proposed in particle size distribution analysis and was stated by Ibanoglu [27].

Activity of Trypsin Inhibitors Valdés-Rodríguez et al. [32] reported the occurrence of a protein proteinase inhibitor in amaranth extracts. Thus, it is of interest to verify this activity in the final product. Our results indicate that 0% activity of trypsin inhibitors in both untreated and thermal-treated ABB. Since the processing conditions prior to heat treatment are compatible with the stability of the amaranth trypsin inhibitor (soluble and stable at alkaline pH at room temperature), these results suggest that the trypsin inhibitor content was undetectable in raw material and that ABB was safe in regard to this anti-nutritional factor.

Physical and Microbiological Stability of Amaranth-Based Beverage

Colloidal Stability Non-added amaranth slurries and samples added with two levels of GG and XG were stored at 20 °C for

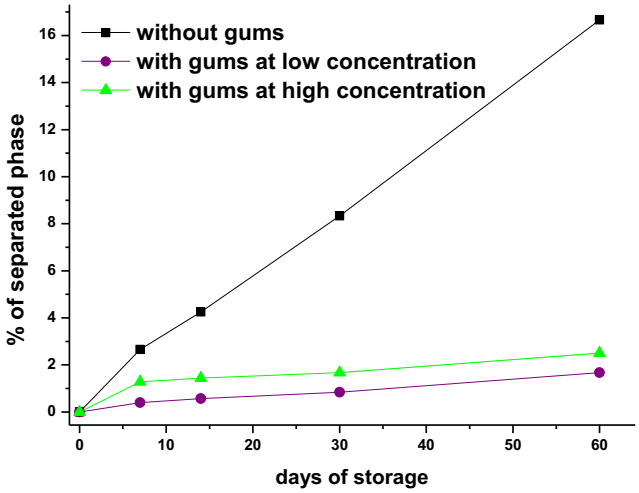

Fig. 3 Colloidal stability of slurries (without gums, squares) and amaranth-based beverages (with gums at low concentration, circles) and sample with high gums concentration (triangles)

60 days. Separation of phases was analyzed. Colloidal stability was evaluated by recording the magnitude of the clarification front (top of the tubes) as a function of time (Fig. 3). Sedimentation was verified as clarification in the top of the tubes and was much faster in non-added samples. After 60 days, the clarified height corresponded to 16.6% of the sample length for samples without gums. GG- and XG-added samples were the more stable, since clarification front was 1.6 and 2.5% after 60 days for the lowest and the highest gums concentrations, respectively. The gum-induced stabilization was probably due to the presence of macroaggregates formed by association between gums and amaranth proteins, which were colloidal stabilized because of electrostatic repulsion, which in turn was due to high absolute values of ζ-potential, as seen previously. The combination of GG and XG is chosen to stabilize particles of plant origin, especially GG is very effective to stabilize plant proteins at neutral pH [33]. Noteworthy, the more stable sample was that with the lowest levels of GG and XG, for which the clarification front was smaller and slower. Considering that viscosity was enhanced directly proportional to gums concentration (Table 2), this result indicates that the stabilizing effect of gums was not directly related to the increase in viscosity, but rather to interactions with amaranth components. Considering the effects on colloidal stability and viscosity, with the aim of obtaining a stable beverage with no excessive viscosity, the lowest level of hydrocolloids added was confirmed as the choice to formulate ABB.

Conclusions

Our results indicate that it is possible to formulate a nutritionally good ABB with appropriate stability characteristics. From a nutritional point of view, ABB had macrocomponent contents very similar to those of skimmed milk in terms of proteins of high biological value, lipids and carbohydrates. It also contained soluble fiber that contributes to the functionality of the product. This composition would give ABB advantages compared to other plant-based milks previously developed [8, 33]. The stability properties of the ABB would be based mainly on those of amaranth proteins and those that arise from their association with GG and XG, such as formation of macroaggregates with high absolute value of ζ- potential. The proteins present in ABB, particularly 11S and P globulins, are capable of interact forming aggregates which increase in size as a consequence of heat treatment and form macrocomplexes with the GG and XG. In these aggregates, the proteins are slightly denatured, due to the protective effect exerted by gums, and interact through hydrogen bonds, hydrophobic and electrostatic interactions, and disulfide bonds. The aggregates, in addition to increasing the viscosity of the product, would be more repulsive among them due to the greater ζ-potential, which contributes to the physical stability of ABB. A better understanding of the system will emerge from future consumer acceptance analysis, from mineral bioavailability assays and from the study of the bioactive properties of the proteins after the ABB gastrointestinal digestion process, aspects that are currently being developed in our laboratory.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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