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REVIEW ARTICLE

Physicochemical properties and functional characteristics of ultrasound-assisted legume-protein isolates: a comparative study

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Abstract Sonicated protein isolates were recovered from *Chenopodium quinua, Phaseoulus vulgaris* and *Lens culinaris* to develop a functional matrix by assessing the physicochemical and functional properties. The plant protein isolates were prepared from powdered materials followed by sonication in alkaline medium using a Box-Behnken design. pH (6–10), a buffer-to-material ratio (5:1 to 15:1) and sonication time (0–20 min) were taken as independent variables, whereas protein yield was taken as the dependent variable. A pH of 9, 20 min treatment, and a buffer-to-material ratio of 5:1 were the optimal extraction conditions for quinoa and black beans, whereas a 1:10 ratio was suitable for lentils. Sonication in alkaline medium caused partial protein unfolding and these isolates; in turn, the molecular weight affected the emulsifying activity and stability. Moreover, sonication had a strong effect on the gelation temperature, emulsifying activity, the water, and oil sorption. Sonication improved protein yield and exposed amino acids such as glutamic acid, aspartic acid, leucine and glycine. In turn, thiol groups were responsible for the increased in gelation temperature. The better gelling property coupled with high emulsifying property of these proteins show potential application as protein emulsifiers in the production of gels, sausages, and pet foods.

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Keywords Protein isolates · Sonication · Physicochemical properties · *Chenopodium quinoa* · *Phaseolus vulgaris* and *Lens culinaris*

Introduction

Proteins are macromolecules displaying amphiphilic properties, as well as excellent biocompatibility, biodegradability, high nutritional value, and a strong ability for interacting with several types of compounds through hydrogen bonding and electrostatic interactions (Zhang et al. 2014). Further, properties such as molecular weight (MW), isoelectric point (IP), solubility, and functionality depend on their source and the sequence of amino acids residues present in their structure, forming hydrophobic and hydrophilic domains followed by segments of variable rigidity or flexibility (Wan et al. 2015).

The unique amino acid sequence of a protein is responsible for the specific 3D organization or folding, resulting in a typical functional characteristic. Therefore, the gelling, foaming, film formation, emulsifying, and coagulation capabilities depend on the chemical composition of the protein and remain fascinating in several science fields (Avramenko et al. 2016; Chang et al. 2016; Li and Tang 2013; Nedovic and Willaert 2013; O'Sullivan et al. 2016; Wan et al. 2015). The functional properties of proteins depend on the hydrophobic and electrostatic interactions, hydrogen bonding, or disulfide bonds, which take place on the protein–protein and protein-water molecules. These interaction forces are mostly acquired when the native protein structure is denatured by thermal treatments, pH, or ionic strength changes. As a result, a partial unfolding occurs, exposing the hidden side chains from the native structure to the external media. Consequently, these

hidden moieties are capable of cross-linking with the medium and determine the loading capability of bioactive compounds (McClements and Jafari 2018; O'Sullivan et al. 2016).

In nature, plant-derived proteins are composed mainly of albumin and globulin-like proteins. Their molecular weight ranges from 5 to 300 kDa, forming pH-dependent rigid structures. Traditionally, they are used as stabilizers of colloidal systems due to their relatively slow diffusion into the oil–water interface creating functional films as compared to the animal counterpart (Nedovic and Willaert 2013). Further, they are considered as "environmentally sound" and have a great functionalization potential, high renewable character, biodegradability and low extraction costs since plant life comprise up to 95% of the world's food supply (Bucko et al. 2016; Lafarga et al. 2018; Primozic et al. 2017). For instance, proteins extracted from plant sources such as quinoa (*Chenopodium quinoa*), black bean (*Phaseolus vulgaris*) and lentil (*Lens culinaris*) have been reported as macromolecules with high-profit potential. They are able to stabilize emulsions, possess gelation capability, have good water and oil adsorption power, worthy foaming capacity and exhibit a suitable hypoallergenic (Bamdad et al. 2006; Bora 2002; Kaspchak et al. 2017; Lafarga et al. 2018; Ramírez-Jiménez et al. 2015; Yang et al. 2018). Quinoa is a pseudo-cereal originated from the Andes in South America, having a protein content ranging from 12 to 23% (p/p) depending on the growth and environmental conditions. These proteins are composed of globulins and albumins, having a molecular weight between 8 and 39 kDa, making them ideal emulsion stabilizers and gelling agents (Kaspchak et al. 2017). Conversely, black beans have a protein content ranging from 20 to 30%w/w and exhibit a barely balanced amino acid composition since they have a low content of sulfur moieties. The main proteins extracted from black beans have a molecular weight ranging from 22 to 186 kDa. These globulins have been used for stabilization of emulsions and as a foaming or gelling agents (Jafari et al. 2016; Makri and Doxastakis 2006; Yang et al. 2018). Likewise, lentils contain globulins-type proteins ranging from 24 to 30% w/w (Majeed et al. 2017). The molecular weight of these proteins varies from 14 to 66 kDa and their functional properties still remain mostly unexplored. Some studies report good water and oil sorption capability, a moderate film formation ability, and potential for stabilization of emulsions (Lafarga et al. 2018; Ramírez-Jiménez et al. 2015; Yang et al. 2018).

Conventional methods (leaching) used for protein extraction have mostly focused on evaluating the effect of the use of different buffers (phosphate, citrates, or Tris–HCL buffers) and adjuvants (acetone, phenol or enzymes), that favor the increase in the extraction yields. It was seeking also, to obtain purer protein isolates from the extraction of the different sources (Álvarez et al. 2018; Chaurasiya and Umesh Hebbar 2013). Chaurasiya and Umesh Hebbar (2013), studied optimizing some factors that affect the process of extracting bromelain from the pineapple nucleus (Ananas comosus L.) (Chaurasiya and Umesh Hebbar 2013). Among the factors studied were the buffer type, pH (4–7), temperature (5–35 °C), and buffer concentration (0.005–1 M). The authors reported that neither buffer concentration, pH, nor temperature in the studied range, had a significant effect on bromelain extraction yield. However, the buffer type used was the parameter that modified the extraction, the buffer that mixt the dibasic sodium phosphate, and monobasic potassium phosphate reported the highest yield (Chaurasiya and Umesh Hebbar 2013). The use of the adjuvants seeks to increase the percentage of extraction performance, including the use of enzymes. Ramakrishnan et al. (2013) reported the enzymatic extraction of proteins from mackerel fish waste using the enzyme Alkalase at three concentrations (0.5, 1 or 2%) and four hydrolysis times (1, 2, 3 and 4 h). The authors reported a good result; almost 80% of the total protein was recovering in the form of peptides and free amino acids in a time of 4 h of hydrolysis with an enzyme concentration of 2% (Ramakrishnan et al. 2013). However, the time need for reaching this extraction yield rate is high compared to other techniques. In addition, due to enzymatic hydrolysis, the functional properties of proteins such as gelling, emulsifying or foaming capacity are modified, or in some cases, the properties are lost (Álvarez et al. 2018).

Recently, novel techniques have been used to enhance protein extraction efficiency. These methods involve high-pressure, microwave-assisted, and ultrasound-assisted extractions (Álvarez et al. 2018; Zhu et al. 2018). The last treatment applies an acoustic wave with a frequency of high intensity, equal to or greater than 20 kHz (Álvarez et al. 2018; Zhu et al. 2018). The effects generated for the ultrasound on liquid–liquid or liquid–solid systems extraction are mainly attributed to the formation of small gas bubbles in the system (cavitation) that collapse violently. This effect leading to the formation of micro-jet and regions with high temperatures (up to 5000 °C) and pressures (up to 50 MPa) due to dissipated energy. Micro-jets collide violently with the material dispersed in the extraction system, which together with the pressure and temperature differentials favor the mass transfer of the material dispersed to the solvent, managing to extract with more efficient the proteins from sources (Álvarez et al. 2018; Zhu et al. 2018).

However, intense cavitation and prolonged ultrasound exposure times result in physical alterations in protein structure and the turn, affecting their functional properties

(Zhu et al. 2018). Chen et al. (2018), used high-intensity ultrasound to accelerate the conjugation of whey protein with acacia gum through Maillard's reaction. The application of ultrasound generated a denaturation of the protein, modifying its secondary structure and exposing the amino groups distributed in its structure, favoring the reaction of Maillard, which led to a noticeable increase in the solubility and emulsifying properties of the protein (Chen et al. 2018). The goal of this study was to evaluate the physicochemical and functional properties of proteins extracted from quinoa, black beans, and lentils by sonication and compare those properties with a convectional extraction method (leaching).

Materials and methods

Materials

The seeds of quinoa, black beans, and lentils were obtained from a farmers market of Medellín (Colombia). The fresh seeds were oven-dried (model IMP180, Thermo Fisher Scientific) at 37 °C for 48 h followed by milling, passed through a number 60 mesh (250 μm size), and stored in desiccators until use. Boric, hydrochloric, and sulphuric acids, methylene blue, copper sulfate hepta-hydrate, methyl red, methylene blue, sulphate, potassium hydroxide, sodium hydroxide, petroleum ether, potassium hydroxide, tris (hydroxymethyl) aminomethane, sodium biphosphate, phenol, acetonitrile (HPLC grade), methanol (HPLC grade), fluoraldehyde o-phthaldialdehyde (OPA), (9-fluorenylmethyl) chloroformate (FMOC), ethylenediamine tetraacetic acid, sodium dodecyl sulphate, glycerin, acrylamide, bis-acrylamide, coomassie blue, 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) were of analytical grade.

Chemical composition of seeds and macronutrients

The obtained powder was analyzed for moisture, protein, ash and lipids content according to the AOAC (Association of Official Analytical Chemists) (Lynch 1998). The protein content was determined by the Kjeldahl method (AOAC 2011.04), whereas the moisture content was evaluated by a gravimetry heating the sample at 120 °C until reaching constant weight (AOAC 934.06). The ash content was determined by calcination in an oven at 550 °C until reaching constant weight (AOAC 942.05). The lipid content was determined by Soxhlet extraction with ether (AOAC 922.06). The polysaccharide content was calculated from the subtraction of all compounds from 100%. All the analyses were performed in triplicate.

Ultrasound-assisted protein extraction

The operational conditions for the ultrasound-assisted extractions (UAPE) were optimized for each vegetable source using a Box–Behnken experimental design (BBD), employing the Design Expert Software® (Vs. 8.0.6, Stat-Ease, USA). pH (6–10), a buffer (Tris–HCL)-to-material ratio (5:1 to 15:1or 50 mL: 10 g to 150 mL: 10 g), and sonication time (0–20 min) were taken as independent variables in an experimental matrix composed of 17 runs (Table 1). The ultrasound was applied with an ultrasound bath Elmasonic E30h to the frequency of 37 kHz, power of 320 W, and a temperature of 25 °C constant operational conditions. The protein yield (%w/v) was taken as the main dependent variable and calculated by the Biuret method using a UV/Vis spectrophotometer (UV-1700, Shimadzu Europe®) (Drochioiu et al. 2016; Tontul et al. 2018).

In order to optimize the extraction conditions and investigate the effect of the independent variables the least squares method was used. Further, the second-order polynomial equation was adjusted to the experimental data by comparison of the determination coefficient (r^2) and the adjusted determination coefficient (r^2-adj). The analysis of variance (ANOVA) was used to investigate the statistical significance of the independent variables in the models obtained (with a 95% confidence level). The extraction conditions were optimized in order to obtain the highest protein yield from each vegetable source.

Protein composition, amino acid content (AA)

The amino acids profile for each protein isolates was determined by high-performance liquid chromatography (HPLC). Proteins were submitted to previous acid hydrolysis with 6 M HCl for 24 h. An HPLC (Agilent® 1200 serial) composed of a binary pump, 1100 degasser, UV–visible detector, and a Zorbax Eclipse Plus® AAA-C_{18} column (Zorbax Eclipse Plus® C_{18}, 4.6 × 150 mm and particle size of 5 μm) and Zorbax Eclipse Plus® AAA-C_{18} pre-column (4.6 ID × 12.5 mm) were employed. The column temperature was 40 °C and the mobile phases used were composed of (A) 40 mM NaH_2PO_4 Buffer (pH 7.8) and, (B) acetonitrile/methanol/water (10:45:45) at a flow rate of 0.2 mL min^{-1} (injection volume of 72 mL). The linear gradient varied from 100 to 50% B for 20 min, followed by 100% A from 20 to 25 min, and subsequently kept constant with 100% A from 30 to 36 min. 25 μL of each hydrolyzed sample was injected at a flow rate of 2.0 mL/min. The quantification of amino acids was performed by online derivatization using o-phathalaldehyde (OPA) for primary amino acids, and 9-fluorenyl-methyl chloroformate (FMOC) for side chain amino acids. The former was detected at 338 nm and the latter at 262 nm.

Table 1 The experimental matrix for the UAPE from quinoa, lentils and black beans according to the Box–Behnken experimental design

Treatment number	pH	Ultrasound time (min)	Buffer-to-material ratio (X:1)	Quinoa Protein (%)	Lentils Protein (%)	Black beans Protein (%)
1	6.0	10.0	5.0	2.23 ± 0.01	6.87 ± 0.03	10.18 ± 0.05
2	8.0	0.0	15.0	1.71 ± 0.22	8.57 ± 0.02	9.27 ± 0.02
3	6.0	0.0	10.0	2.81 ± 0.05	10.15 ± 0.04	6.77 ± 0.08
4	8.0	20.0	15.0	3.09 ± 0.11	10.33 ± 0.04	8.94 ± 0.03
5	6.0	10.0	15.0	2.54 ± 0.14	9.07 ± 0.03	8.12 ± 0.03
6	10.0	10.0	5.0	3.70 ± 0.07	6.83 ± 0.02	9.56 ± 0.03
7	8.0	10.0	10.0	3.12 ± 0.05	7.26 ± 0.03	8.82 ± 0.05
8	10.0	0.0	10.0	2.78 ± 0.09	7.75 ± 0.04	8.87 ± 0.03
9	8.0	10.0	10.0	3.48 ± 0.14	7.59 ± 0.02	7.83 ± 0.03
10	6.0	20.0	10.0	3.77 ± 0.05	8.16 ± 0.02	9.68 ± 0.04
11	10.0	10.0	15.0	2.95 ± 0.12	9.29 ± 0.02	8.52 ± 0.02
12	10.0	20.0	10.0	3.70 ± 0.07	8.60 ± 0.01	8.73 ± 0.07
13	8.0	10.0	10.0	2.93 ± 0.07	7.47 ± 0.03	8.37 ± 0.04
14	8.0	20.0	5.0	4.54 ± 0.05	6.78 ± 0.02	10.32 ± 0.04
15	8.0	10.0	10.0	3.12 ± 0.05	7.48 ± 0.02	7.55 ± 0.06
16	8.0	10.0	10.0	3.12 ± 0.07	7.38 ± 0.01	7.60 ± 0.01
17	8.0	0.0	5.0	3.48 ± 0.03	6.71 ± 0.04	10.26 ± 0.05

Values are expressed as mean ± standard deviation (n = 3)

Proteins characterization

The proteins extracted with the optimized conditions from each sources were precipitated by adding sufficient ammonium sulfate to cause saturation (100%) without causing denaturation. Subsequently, the solution was centrifuged at 12,000 g for 10 min (Hermle Z206A) followed by dialysis using a 3 kDa cellulose membrane (Fisher®)for two days under cooling replacing the media (Type II water) every 12 h. The same procedure was carried out skipping the ultrasound treatment in order to evaluate the effect of sonication. These non-sonicated proteins were labeled as PE. The sonicated proteins (UAPE) and PE were characterized using following methods.

Protein yield (EY)

It was determined by comparing the protein concentration in the supernatant utilizing the Biuret method (Kim et al. 2018). Protein yield was calculated using the following equation:

$$\mathrm{EY}(\%) = (\text{soluble protein in the extract/total protein from the plant source}) \times 100\%. \quad (1)$$

Isoelectric point (IP) and particle size (PS)

The isoelectric point was determined from the z-potential vs. pH plot and the IP corresponds to the point where the potential becomes 0 mV. The z-potential and particle size were measured on 0.1% protein solutions at a pH range from 2 to 10 using a Zetasizer (Nano ZS90 Malvern®) (Tang and Sun 2010). pH was adjusted with 0.1 M NaOH and HCl solutions.

Molecular weight (MW)

It was determined by gel electrophoresis on polyacrylamide-sodium dodecyl sulfate plate (12%, SDS-PAGE) using a Mini-Protean® system (Bio-Rad, UK) and run at 150 V. Proteins were then stained with Coomassie blue R-250. A protein weight marker (11–245 kDa, Biolabs®, England) was used as a reference standard (Laemmli 1970).

Free sulfhydryl (SH) content

Sulfhydryl groups of protein isolates were determined according to the method of Ellman (1959) and modified by Tang et al. (2009). Briefly, 15 mg of the isolated protein was suspended in 5.0 mL of Tris buffer (0.086 M Tris, 0.09 M glycine, 4 mM EDTA, pH 8.0) and 50 µL of Ellman reagent was added (4 mg DTNB (5,5-dithio-bis-(2-

nitrobenzoic acid) to 1 mL of Tris–glycine buffer). The absorbance of the complex thus formed was read at 412 nm (UV-1700, Shimadzu®) after 1 h of storage under darkness at room temperature (25 ± 1 °C). The SH content was calculated using the extinction coefficient of 2-nitro-5-thiobenzoate (NTB) at 412 nm (13,600 M^{-1} cm^{-1}) and expressed as µmol/g protein.

Fat absorption capability (FAC) and water holding capacity (WHC)

These tests were executed according to the method described by Boye et al. (2010). FAC was determined weighing ~ 0.5 g of sample and mixing with 3 mL of sunflower oil in a Falcon tube followed by homogenization for 1 min. Subsequently, samples were centrifuged at 4000 g for 30 min, and the supernatant was discarded. The resultant residue in the tubes was weighed and the FAC was calculated as follows:

$$\mathrm{FAC}(\%) = (\text{Weight of fat absorbed by the sample /weight of sample}) \times 100\% \quad (2)$$

The WHC was assessed by weighing 1 g of sample and adding sufficient water to saturate the sample without forming a liquid dispersion. Samples were then homogenized and centrifuged at 2000 g for 10 min. The supernatant was removed and the WHC was expressed as the percentage of water absorbed by the proteins.

Emulsifying capacity

It was determined according to the Boye et al. (2010) methodology (Boye et al. 2010). Briefly, 1.5 mL of oil was added to 4.5 mL of a protein solution (0.5% w/v) prepared in 0.01 M phosphate buffer (pH 7). The mixture was homogenized at 20,000 rpm at room temperature for 2 min using a 5 mm dispersing shaft (ds-500/5, Dlab). Subsequently, 250 µL of the emulsion was mixed with 50 mL of 0.1% sodium dodecyl sulfate solution, and the resulting absorbance was read at 500 nm employing a UV/VIS spectrophotometer (UV-1700, Shimadzu®). The emulsifying activity index (EAI) and emulsifying stability index (ESI) were calculated as described by Pearce and Kinsella (1978):

$$EAI\left(m^2/g\right) = \frac{2 * 2.303 * A_0 * N}{c * \varphi * 10000} \quad (3)$$

$$ESI\left(m^2/g\right) = \frac{A_0 * t}{\Delta A} \quad (4)$$

where A_0 corresponds the absorbance of the diluted emulsion right after homogenization, N is the dilution factor (× 150), c is the protein height per volume (g/mL), φ is the oil volume fraction of the emulsion, ΔA is the change in absorbance between 0 and 10 min (A_0–A_{10}) and t is the time interval of 10 min (Boye et al. 2010).

Gelation temperature (Tgel)

The viscoelastic properties were measured in order to examine the dynamic formation of a gel network as described by Zhao et al. (2014). The storage module (G′) and the loss module (G″) were selected as the dynamic rheology parameters determined on a rheometer (MCR92, Anton Paar Corporation, Austria). A 5% protein concentration was measured using a C-CC27/T200XL concentric cylinder geometry. In order to induce the gel formation, samples were subjected three stages of a heating ramp: (1) first, from 25 °C to 80 °C, followed by (2) 80 °C for 3 min, and a cooling step (3) from 80 °C to 25 °C. The heating and cooling rates were 1 °C/min. Measurements were registered at a shear strain of 0.1% and an angular frequency of 1.0 s^{-1} where proteins remained within the linear viscoelastic region.

Statistical analysis

Data variability was conducted by analysis of variance (ANOVA), the test for comparison of means using the LSD (least significant difference) and the Fisher's test. Moreover, the Principal Component Analysis (PCA) and correlation matrix analyses were performed (Euclidean square) using the Minitab software® (vs. 17.2.1). The multivariate analysis allows for condensing the data set contained in the original variables into a smaller set of new vectors with a minimum loss of data. PCA is the most successful method for the factor analysis. There are various methods to determine the optimum number of factors such as the Scree test, proportion of variance, analysis of residuals and a priori hypotheses. In this paper, the Kaiser–Guttman rule was employed due to its simplicity and availability in various computer packages (Esmaeili and Shokoohi 2011).

Results and discussion

Chemical composition of vegetable material

The chemical composition of the raw materials obtained from the three vegetable included (1) carbohydrates 70.06 ± 0.16, 60.22 ± 0.37, 67.96 ± 0.48%; (2) Ashes 4.81 ± 0.39, 6.10 ± 0.38, and 3.52 ± 0.28; (3) crude protein 16.17 ± 0.25, 22.97 ± 0.12, and 22.10 ± 0.35; (4) fat 0.05 ± 0.05, 0.14 ± 0.08, and 0.12 ± 0.10; (5) and moisture 8.91 ± 0.39, 5.77 ± 0.11, and 6.30 ± 0.49 for quinoa, lentil and black beans, respectively. These

vegetable sources were abundant mainly in carbohydrates and protein, and the resulting protein content in dry basis was 16.25%, 23.02% and 22.16% for quinoa, lentil, and black beans, respectively. This protein content is in agreement with those studies reported previously of 14.03 ± 0.25% and 23.04 ± 0.25% for quinoa and black beans, respectively (Elsohaimy et al. 2015; Santiago-Ramos et al. 2018). Interestingly, the protein content of lentils is more substantial than the value reported by other researchers (15.3 ± 0.1%) (Li et al. 2019). This phenomenon is explained by seasonal factors such as soil composition, harvest time, sowing and environmental conditions. It is worthwhile mentioning the high nutritional potential of these powders as a good source of protein as compared to other plants such as rice (7.5%), corn (13.4%) and barley (11%) (Elsohaimy et al. 2015).

Ultrasound-assisted protein extraction (UAPE) and protein solubility

Table 1 lists the experimental matrix and protein yield obtained from each source. Each matrix contained 17 experimental runs performed in triplicate. The extraction experimental conditions affected the amount of protein obtained from the three sources. The highest extraction yield was obtained at a pH of 8 and a sonication treatment of 20 min. A slightly alkaline pH was the most critical factor for protein extraction since it guaranteed a high extraction yield. Further, this pH increased solubility, but in turn contributed to partial denaturation. Conversely, at acidic pH the isoelectric point is reached decreasing protein solubility and hence diminishing the mass transfer process. The vegetable source, solvent, and buffer pH also affected the protein yield. On the other hand, prolonged sonication treatments led to increased transfer in the protein extraction processes, in all sources studied. This increase attributed to the effect generated by cavitation in the extraction buffer, generating micro-jets that impact the plant material and temperature and pressure differentials that favor the migration of proteins from plant material to the buffer (Zhu et al. 2018). Other studies have shown also reported that sonication increased the extraction efficiency (from 37 to 57%) of protein from other sources such as wheat germ (Zhu et al. 2009). Thus, the intense cavitation and mechanical effect caused by ultrasounds is responsible for the enhancement of protein extraction. As a result, particles are broken down into smaller particles creating a larger surface area available for solvent uptake improving the mass transfer process (Zhu et al. 2009).

The ANOVA Table for protein yield (Fig. 1) shows that most quadratic models were statistically significant ($p < 0.05$) and showed an excellent fit to the experimental data as indicated by the high correlation coefficient (Quanhong and Caili 2005). Further, the response surface plots show that the increase of sonication time leads to an increase in protein yield. This effect was more striking for quinoa proteins. The three independent variables studied in each design were statistically significant in their linear interaction and quadratic terms.

The optimization process was conducted based on the quadratic models obtained (Eqs. 3 and 4) in order to maximize protein yield from each vegetable source. The optimal values predicted by the models were a (1) 20 min treatment time, pH of 9 and a 5:1 buffer-to-material ratio for quinoa and black beans; whereas lentils required a 10:1 ratio. The predicted protein yield at these conditions was 4.42%, 9.55%, and 10.32% for quinoa, lentil, and black beans, respectively. Three validation runs were conducted at these optimal conditions resulting in protein yields of 4.10 ± 0.18, 7.64 ± 0.03, and 9.70 ± 0.10 for quinoa, lentil and, black beans, respectively. The relative and absolute errors obtained (7.22%, 20.02%, and 6.01% for quinoa, lentil and black bean, respectively) indicates that the models describe and predict protein yield with a high statistical confidence. This optimal extraction conditions were employed for the extraction of proteins that were subsequently assessed and characterized in their functionality.

Protein composition (AA)

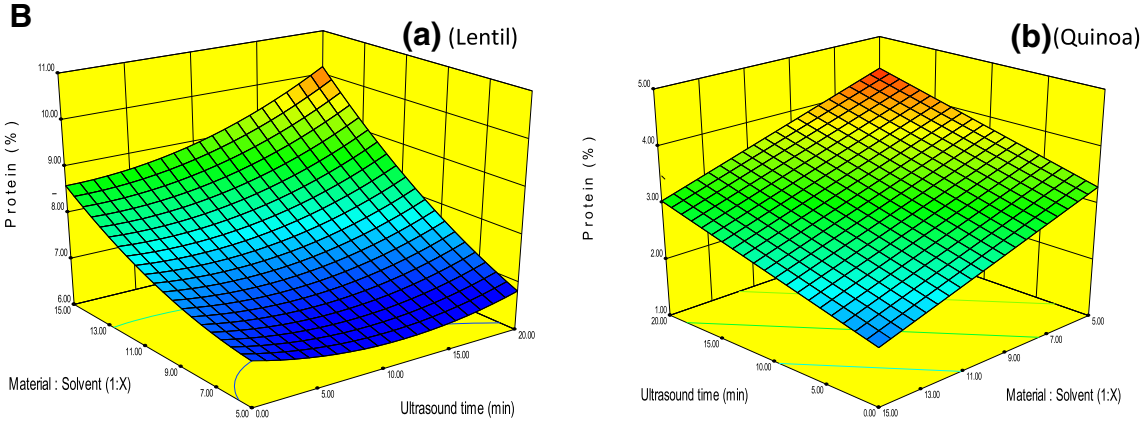
The three proteins extracts were mainly composed of a group of five essential amino acids including glutamic acid, aspartic acid, leucine, glycine and arginine, ranging from 50 to 55% in total (Fig. 2). Interestingly, ionized acidic amino acids predominate at neutral pH. This finding agrees with other reports for proteins extracted from vegetable sources such as soy, sunflower and potato (Nesterenko et al. 2014; Pęksa et al. 2018) having arginine, glutamic and aspartic acids as the primary amino acids found in these protein sources (Nesterenko et al. 2014; Pęksa et al. 2018).

It is well known that moieties found in the protein structure and the ionized regions along with the hydrophobic and hydrophilic regions are responsible for the functional properties such as emulsion stabilization, gelling and foaming capacity. Moreover, ionized amino acids such as Glu, Asp, Arg, Lys and His, contain ionic groups able to form electrostatic interactions, and hence, contribute to particle stability of emulsified systems. Conversely, Cys and Met are amino acids that contain sulfur and SH moieties which are capable of forming gels as a consequence of protein denaturation. Therefore, it is expected a remarkable gelling and emulsifying activities for proteins obtained from lentils, black beans and quinoa. This phenomenon is explained by the formation of steric

A

Variable	Quinoa	Lentil	Black bean
	p-value	*p-value*	*p-value*
Model	< 0.001	< 0.001	0.002
A-pH	> 0.050	0.028	0.560
B-ultrasound time (min)	< 0.001	< 0.001	0.044
C- Buffer-to-material ratio (X:1)	< 0.001	< 0.001	0.002
AB	> 0.050	> 0.050	0.019
BC	> 0.050	0.001	> 0.050
A^2	> 0.050	0.012	> 0.050
B^2	> 0.050	0.001	> 0.050
C^2	> 0.050	0.001	0.006
Lack of Fit	0.549	0.139	0.527
r^2	0.911	0.992	0.82
r^2-adj	0.897	0.985	0.731

B

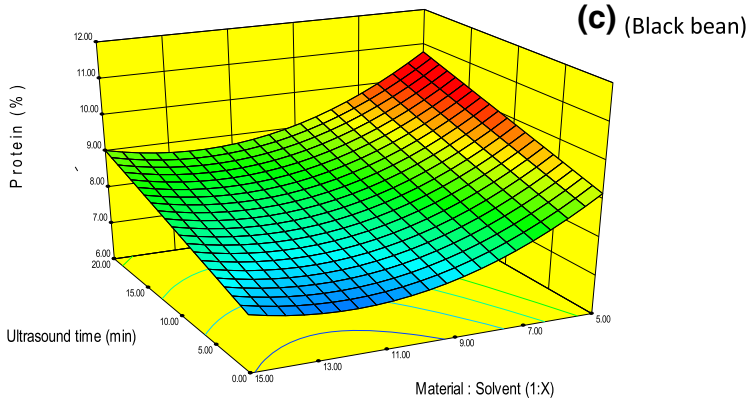


Lentils (%) = 9.92 - 0.78*A -0.13*B - 0.08*C

+8.32x10^{-3}*BC + 0.05*A^2 + 3.93 x10^{-3}*B^2

+0.01*C^2

Quinoa (%) = 4.01 +0.06*B - 0.13*C



Black bean (%) = 9.89 + 0.44*A + 0.35*B - 0.95*C - 0.04*AB + 0.04*C^2

Fig. 1 (Panel **A**) ANOVA Table for protein yield from each source. (Panel **B**) Response surface plots for the effects of Buffer-to-material ratio vs. ultrasound time on protein yield from: **a** lentil, **b** quinoa, and **c** black beans

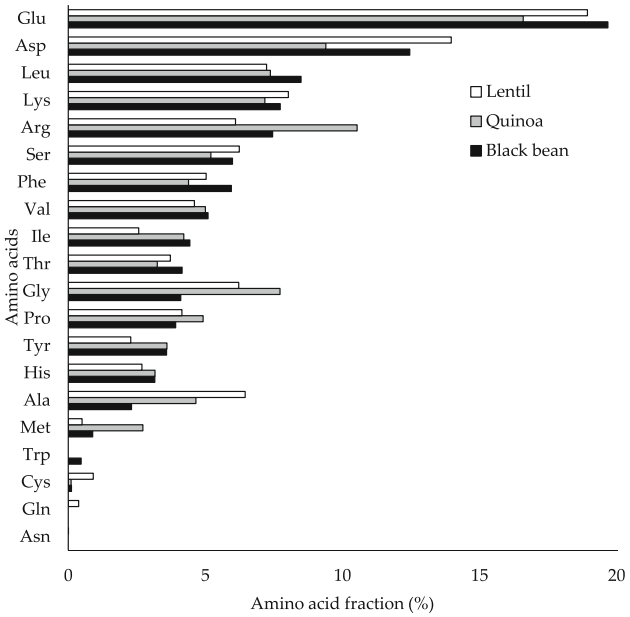


Fig. 2 Amino acid composition of quinoa, black bean and lentil proteins (g/100 g of protein isolate)

and electrostatic interactions (Jafari et al. 2016; Makri and Doxastakis 2006; Yang et al. 2018).

Physicochemical properties of proteins obtained by optimized UAPE and EP

The functional properties and characteristics of proteins obtained by UAPE and PE are shown in Table 2. The extraction type and protein source were the independent variables that affected the parameters studied. Further, properties such as MW, IP and SH were highly related to the plant source. On the contrary, properties such as EY, PS, WHC, FAC, EAI, ESI and Tgel were dependent on both the plant source and the extraction treatment. In general, a two-fold increase in protein yield was reached when sonication was employed. The cavitation effect explains this in the ultrasound-assisted extraction that generated the micro-jets and temperature and pressure differentials, which favored the protein transfer from the plant-powder to the buffer phase. Further, the results obtained for the PE extractions from the three sources were comparable to those obtained by other authors (Karaca et al. 2011; Ladjal-Ettoumi et al. 2016; Mäkinen et al. 2016; Pellegrini et al. 2018; Sharif et al. 2017; Tang and Ma 2009).

Interestingly, the type of extraction did not show statistically significant differences ($p < 0.05$) on properties such as MW, IP, SH, PS (except quinoa) and ESI (except lentils) for the three protein sources. The MW, IP, SH, PS are properties related to the primary structure from the protein, and its values are not affected by the application of ultrasound in the extraction processes (Fig. 3, Table 2). On the other hand, sonication showed statistically significant effects ($p < 0.05$) for the properties functional assessed; WHC, FAC, EAI, ESI, and Tgel that they are related to the secondary structure from the protein (Table 2, Fig. 4).

The micro-jets generated in the ultrasound-assisted extraction collide with the proteins suspended in the medium (Zhu 2016; Zhu and Li 2018). This physical effect in the extraction processes generates a partial denaturation of the proteins, generating an unfolding of the secondary structure and exposing the internal radical groups of the

Table 2 Characterization of quinoa, black bean and lentil proteins obtained by optimized UAPE and PE

Source	Quinoa		Black bean		Lentil	
Treatment	PE	UAPE	PE	UAPE	PE	UAPE
EY (%)	$16.24^{a} \pm 0.02$	$39.34^{b} \pm 0.01$	$31.61^{a} \pm 0.01$	$67.71^{b} \pm 0.01$	$23.19^{a} \pm 0.01$	$57.57^{b} \pm 0.06$
MW (kDa)	58 and 46–32	58 and 46–32	80–32	80–32	46–32	46–32
IP	$4.00^{a} \pm 1.00$	$5.00^{a} \pm 1.00$	$3.50^{a} \pm 1.00$	$3.50^{a} \pm 1.00$	$4.00^{a} \pm 1.00$	$5.00^{a} \pm 1.00$
PS (d.nm, pH 9)	$239.97^{a} \pm 22.86$	$353.73^{b} \pm 17.90$	$344.86^{a} \pm 10.98$	$304.93^{a} \pm 34.64$	$317.80^{a} \pm 26.04$	$265.93^{a} \pm 43.82$
SH (μM SH/g)	$11.48^{a} \pm 0.24$	$11.93^{a} \pm 0.45$	$18.13^{a} \pm 1.09$	$19.16^{a} \pm 0.477$	$21.03^{a} \pm 0.38$	$21.12^{a} \pm 0.27$
WHC (%)	$86.84^{a} \pm 3.69$	$116.55^{b} \pm 8.70$	$64.14^{a} \pm 15.71$	$254.02^{b} \pm 4.44$	$154.42^{a} \pm 21.73$	$182.09^{b} \pm 3.56$
FAC (%)	$222.96^{a} \pm 11.91$	$241.10^{b} \pm 12.56$	$242.25^{a} \pm 20.78$	$241.77^{a} \pm 18.47$	$176.65^{a} \pm 4.28$	$229.79^{b} \pm 7.12$
EAI (m^2/g)	$16.08^{a} \pm 0.26$	$18.53^{b} \pm 0.48$	$10.41^{a} \pm 0.22$	$11.81^{b} \pm 0.13$	$46.26^{a} \pm 2.76$	$51.05^{b} \pm 18.38$
ESI (min)	$17.29^{a} \pm 0.27$	$16.66^{a} \pm 0.79$	$15.34^{a} \pm 2.60$	$14.08^{a} \pm 0.12$	$11.10^{a} \pm 0.14$	$29.22^{b} \pm 1.11$
Tgel (°C)	$58.75^{a} \pm 1.00$	$60.61^{b} \pm 1.00$	$78.97^{a} \pm 1.00$	$75.42^{b} \pm 1.00$	$66.13^{a} \pm 1.00$	$62.39^{b} \pm 1.00$

Values are expressed as mean ± standard deviation (n = 3)

EY extraction yield, *MW* molecular weight, *IP* isoelectric point, *PS* particle size, *SH* thiol groups, *WHC* water holding capacity, *FAC* fat absorption capability, *EAI* emulsifying activity index, *ESI* emulsifying stability index, *Tgel* gelation temperature, *PE* non-ultrasound extraction, *UAPE* ultrasound-assisted extraction

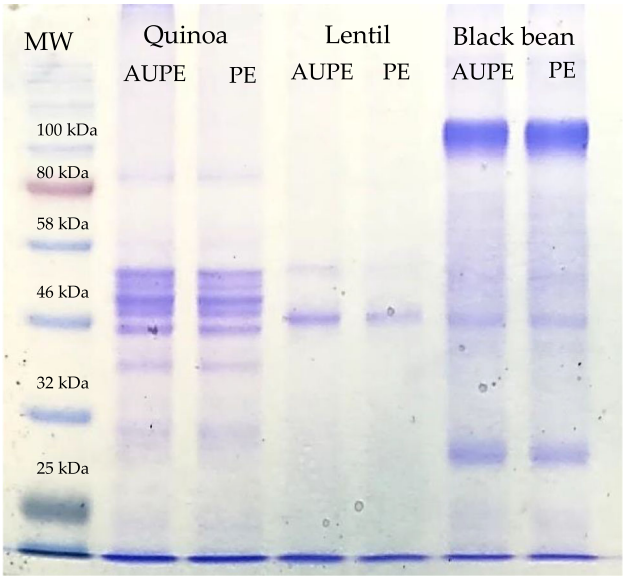


Fig. 3 Molecular weight by electrophoresis of quinoa, black bean and lentil proteins obtained by optimized UAPE and PE

protein to increase hydrophobic, H-bridge, electrostatic and disulfide interactions. This explains the changes between AUPE and PE in functional properties. However, this effect of the micro-jets on the protein structure does not achieve the breaking of covalent and peptide bonds; therefore, the use of ultrasound in the extraction processes does not affect the properties related to the primary structure of the proteins (Li et al. 2016).

These results are comparable with other reports (Zhu et al. 2018). Zhu et al. (2018) evaluated the impact of ultrasound on the molecular, physicochemical, and functional properties of walnut protein isolate (Zhu et al. 2018). A decrease in α-helix and an increase in β-sheet, β-turn, and random coil content in the walnut protein secondary structure were detected using circular dichroism spectroscopy. These changes in protein secondary structure justified the increase of water-solubility (+ 22%), emulsifying activity index (+ 26%), and emulsifying stability index (+ 41%) of the walnut proteins after sonication (Zhu et al. 2018). Further, the ultrasound used did not affect the properties related to the primary structure of the protein, such as molecular weight. These results suggest that ultrasound is a valuable tool for improving the functional attributes of proteins that are related to the protein secondary structure.

Figure 4 shows the progression of viscoelastic properties (G′, storage module) at a 5% protein level during the gel formation for the three different vegetable sources obtained by PE and AUPE. This plot depicts the ability of proteins to form an adequate network structure upon heat treatment. The behavior of the storage module in the three protein isolates was constant up to a heating temperature of approximately 50 °C. Once this temperature was exceeded, the mobility of the protein structure due to thermal agitation is increased, promoting the formation of a gel network. This phase transition occurred due to the formation of disulfide bonds and electrostatic interactions between the moieties exposed (Felix et al. 2016). The gelling profiles obtained for these proteins are similar to those found by the protein products. However, ultrasounds generated an increase in the storage module of the proteins and rendered marked differences in gelation. This effect is explained by the previous denaturation caused by sonication in the proteins, deploying the structure of the proteins to induce new electrostatic interactions, which are reflected in the formation of a new gel network, as seen by the increase of the storage module (Felix et al. 2016). Therefore, sonication generates a structural modification of proteins, which changes the gelling formation process and hence the emulsifying capacity and the water sorption capacity as listed in Table 2.

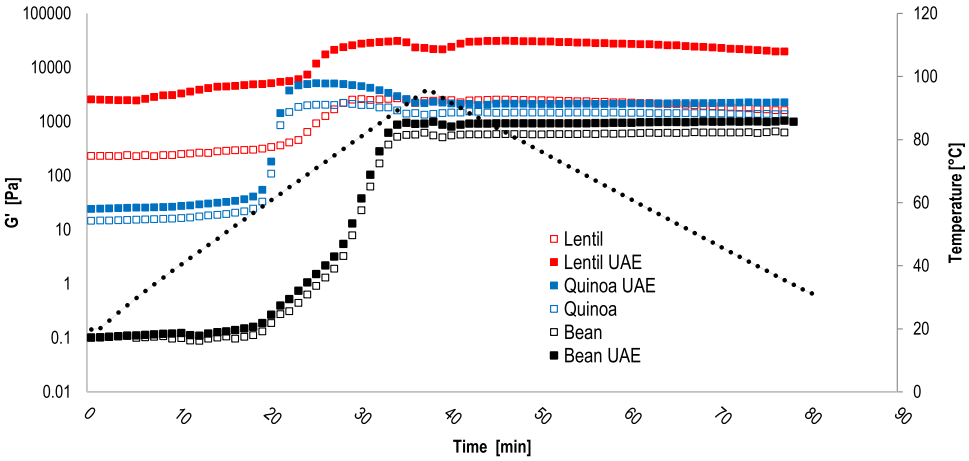


Fig. 4 Variation of storage module (G′) with time in a temperature ramp test of quinoa, black bean and lentil proteins obtained by UAPE and PE

Parameter	Correlation coefficient
Source & SH	0.899
Source & MW	-0.866
Source & EAI	-0.964
Treatment & EY	0.856
MW & EAI	0.806

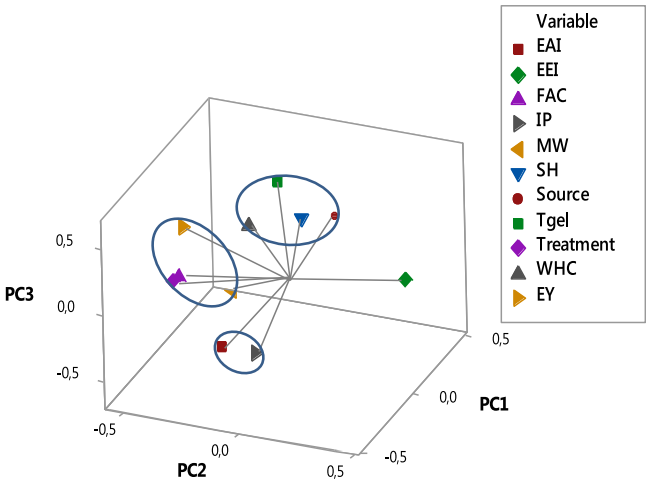


Fig. 5 Multivariate analysis for the process of extraction and characterization of quinoa, black bean and lentil proteins: PCA and properties with significant correlation. EY: extraction Yield, MW: molecular weight, IP: isoelectric point, PS: particle size, SH: thiol group, WHC: water holding capacity, FAC: oil absorption capability, EAI: emulsifying activity index, ESI: emulsifying stability index, Tgel: Gelation temperature

Multivariate analysis of protein properties

The global functional properties of proteins extracted from quinoa, lentils, and black beans are depicted in the principal component plot of Fig. 5. The PCA determined that the first three components describe 85.3% of the variability among the data. This plot shows three main clusters for all properties studied. One cluster is composed of the protein source, which was mainly associated with the SH groups, gelling temperature, and water sorption ability. This indicates that proteins isolated from lentils had a major gelling and water sorption activity as compared to quinoa proteins. The second cluster is characterized by the extraction treatment (PE or UAPE), protein yield, oil sorption, and in a very lower degree molecular weight. Thus, sonication in alkaline media denaturized the protein exposing hydrophobic moieties, increasing oil sorption. Conversely, the third cluster shows the protein isoelectric point strongly related to the emulsifying capacity. In fact, proteins from lentils showed the largest isoelectric point and hence the largest emulsifying activity. This activity is related to the ability to absorb and disperse oils. Further, the molecular weight influences the speed at which the molecule is adsorbed in the oil/water interface, and thus, the smaller the molecule, the higher the absorption rate resulting in a stable emulsion as occurred for proteins extracted from lentils. Interestingly, the correlation analysis confirmed the results obtained by the PCA. In fact, proteins isolated from lentils showed the largest content of sulfur moieties (i.e., cysteine and methionine), the lowest molecular weight, and the largest emulsifying activity. Further, sonication in alkaline medium implied the largest protein solubility and hence the largest protein extraction. Moreover, a high protein molecular weight was reflected in a lower emulsifying capacity.

Conclusion

The application of ultrasounds for the extraction of proteins from quinoa, black beans, and lentils proved to be very efficient in terms of protein yield. A 37 kHz, 320 W, 20 min treatment, pH of 9, and a powder-to-solvent ratio of 1:5 for quinoa and black beans, and 1:10 for lentils were found as optimal. Sonication also caused partial protein denaturation attributed to the cavitation effect and formation of a micro-jet system. The molecular weight, particle size, isoelectric point, and SH groups were not significantly affected by the application of ultrasounds. Conversely, the gelation temperature, emulsifying activity, and the sorption of water and oil in proteins were statistically affected by the application of the ultrasounds. Therefore, this technology is suggested for increasing the extraction yield and the functional properties of proteins obtained from these plant sources.

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