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Modification of Physicochemical and Functional Properties of Pumpkin Seeds Protein Isolate (PsPI) by High-Intensity Ultrasound: Effect of Treatment Time

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ABSTRACT: High-intensity ultrasound (HIUS) is reported to modify tecno-functional properties of proteins. This study aimed to investigate the effect of HIUS treatment times on pumpkin seeds protein isolate (PsPI) suspensions as a function of pH, by evaluating particle size, zeta potential, and emulsion and foam properties. Results showed that 5 min of HIUS treatment caused a reduction in the average hydrodynamic diameter of the particles from 5.63 ± 0.08 μm (control) to 1.68 ± 0.15 μm. In addition, an increase of zeta potential in the pH range around the isoelectric point of PsPI was found in all treated samples. However, prolonged HIUS treatment favored the formation of molecular aggregates, and 5 min treatment with HIUS was sufficient to achieve a significant increase in foaming and emulsifying ability compared to the untreated control, especially in the pH range of 2.5−6.5. Thus, the use of HIUS was proved to be an excellent treatment method for enhancing the functional properties of PsPI.

KEYWORDS: *pumpkin seeds protein Isolate, particle size, emulsion and foam properties, zeta potential*

INTRODUCTION

In times of circular economy, scientific interest is increasingly directed toward the utilization of food processing byproducts and wastes, as well as underutilized agricultural products. However, the problems of industrial waste are becoming harder to solve, and much effort will be needed to develop the nutritional and industrial potential of food and agricultural byproducts and wastes.[1]

Pumpkin seeds (*Cucurbita maxima*, L.) are utilized directly for human consumption as snacks after salting moreover, they are widely used in the production of edible oil, which, due to its nutrient profiles, is known to provide several health benefits.[2] The pumpkin seeds represent 3.1% of total pumpkin fruit weight and are a rich source of oil (47.3%) and proteins (33%), showing a high content of sulfur amino acids and low content of phytic acids and trypsin inhibitors.[3] However, the oil extraction process generates as waste a defatted flour with high protein content (up to 65% protein), with a balanced essential amino acid content, which is considered by many authors an interesting and promising source of vegetable proteins that can be both consumed as a dietary supplement or incorporated into other food products.[4−7]

Unfortunately, the procedure to obtain proteins from the defatted pumpkin flour negatively affects their native structure, influencing their solubility and, consequently, their ability to be incorporated into food systems. Moreover, due to the acidic isoelectric point of seed proteins (pI 3.5−5.5), they show poor solubility under acidic conditions (pH 3−6).[8] This precludes their use in acidic foods, such as coffee, acidic beverages, yogurt, and sauces. Therefore, new approaches are needed to improve the physicochemical properties of pumpkin proteins so that they can be used as functional ingredients in foods.

Ultrasound technology is used in food processing for a variety of applications related to food preservation, molecular modification, degassing, foam control, mixing, emulsification, meat tenderization, etc. For many years, ultrasound has been used in the study of proteins to estimate improvements in protein hydration and conformation changes. These parameters can be related to functional properties of proteins in foods, such as solubility, foaming, and flexibility.[9] Recently, several studies have shown that high-intensity ultrasound (HIUS) can improve the functional properties of biopolymers, such as their solubility, interfacial, emulsification, foaming, and gelling properties.[10]

Vargas et al.[11] reported that HIUS processing improves the emulsification properties of whey protein isolate. Zhu et al.[12] studied the effects of HIUS on the chemical and physical properties of walnut proteins. The authors reported that sonication increased the water solubility, decreased the number of large aggregates, and improved the emulsifying properties of walnut proteins. These effects were attributed to the ability of HIUS waves to disrupt the physical bonds between and within spherical protein molecules, resulting in some unfolding and dissociation. Jambrak et al.[13] also reported that ultrasound caused significant changes in particle size and molecular weight of whey proteins and that prolonged treatment of whey protein

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isalate with a 40 kHz ultrasound bath promoted the formation of molecular aggregates.

Despite the large number of publications on the use of ultrasound to modify various proteins of different origins, to date, there are no studies in the literature regarding the application on pumpkin seed proteins. Therefore, the aim of this work was to obtain a protein preparation of pumpkin seed protein isolate (PsPI), using a classic isoelectric precipitation approach, starting from a pumpkin seed matrix, previously defatted to simulate the waste product resulting from oil extraction process from pumpkin seeds. The PsPI thus obtained were subjected to sonication treatments with HIUS for 5, 10, or 20 min, after which the effects on the solubility, surface charge and particle size, as well as on the ability to form foam or emulsions, were studied. In this sense, the work aimed to evaluate whether sonication could affect PsPI to the point of improving their physicochemical and technological properties, making them more suitable for use in food products as functional ingredients, which could lead to increase their application in food processes.

MATERIALS AND METHODS

Materials. Hulled pumpkin seeds were purchased from a local market. Deionized water from a water purification system was used to prepare all solutions; all other reagents and solvents used were of analytical grade.

Preparation of Pumpkin Seeds Protein Isolate (PsPI). Hulled pumpkin seeds were ground by using a coffee mill to obtain a fine powder and defatted with *n*-hexane (1:10 w/v) before the alkaline extraction and isoelectric precipitation of proteins. Briefly, the defatted material was dispersed in deionized water at a final ratio of 1:10 and stirred for 15 min to solubilize the albumin fraction. Then, the pH of the dispersion was raised up to 10.0 by using 1 N NaOH. The dispersion was thoroughly stirred for 1 h at room temperature and then centrifuged at 8000*g* for 20 min. The supernatant fraction was collected, and the pH was adjusted to 5 by adding 1 M HCl drop by drop under stirring. The sample was incubated at 4 °C overnight and finally centrifuged at 8000*g* for 20 min. At each centrifugation step, aliquots of the pellet and supernatant were collected and frozen for further analysis. The pellet, containing the protein isolate (PsPI), was resuspended in water, neutralized at pH 7, and then freeze-dried together with the supernatant aliquots; finally, the obtained powders were stored in a dry place. For the determination of nitrogen content (N %), PsPI powder and pumpkin seed flours, both whole and defatted, were analyzed according to the Kjeldahl method.[14] The amount of protein in each sample was calculated as N % × 6.25.

SDS-PAGE. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli[15] using 15% resolving gel and 5% stacking gel at constant voltage (80 V). The marker used was Precision Plus Protein All blue Prestained Protein Standards Bio-Rad. The samples were prepared in reducing buffer containing β-mercaptoethanol and then denatured at 100 °C for 2 min. After the electrophoretic separation of proteins, the gel were stained with Comassie brilliant blue R-250 0.5% (methanol/acetic acid/water 5:1:4) and destained in methanol/acetic acid/water 1:1:8. A digital camera was used to acquire the image of the gels.

Ultrasonic Treatment. PsPI were suspended in water (1% w/v) at different pH (from 2.5 to 10.5 pH at one-unit intervals) under stirring at room temperature for 60 min. The pH was checked during the stirring and, if necessary, adjusted by the addition of 0.1 M HCl or 0.1 M NaOH. Ultrasonication was carried out using an ultrasound device (LabSonic U, B.Braun) equipped with a 1 cm sonotrode. 20 mL of each solution were placed in a 75 mL glass beaker immersed in an ice bath in order to maintain the temperature below 45 °C and avoid protein denaturation. The ultrasound treatment was applied for 5, 10, and 20 min with a power of 150 W and with 0.5 s on/0.5 s off pulse duration. The sonotrode was immersed approximately 1 cm below the surface of the solution.

Determination of Protein Solubility, Turbidity, and Protein Surface Hydrophobicity. Protein solubility and turbidity were determined in a pH range between 2.5 and 10.5 following the protocols of Shevkani et al.[16] with some modifications. PsPI were suspended at a final concentration of 1% (w/v) in 20 mL of deionized water, and the pH of the suspension was adjusted to the scheduled pH with 0.1 M HCl or 0.1 M NaOH solutions under magnetic stirring for 1 h. After pretreatment with HIUS at 150 W as above-described, the prepared suspensions were measured by a spectrophotometer at 600 nm for the turbidity assessment, or centrifuged at 8000*g* for 10 min for the evaluation of the solubility: the protein content in the supernatant was measured by the Bradford assay using bovine serum albumin (BSA) as protein reference.[17] Each measurement was performed in triplicate.

The protein solubility was calculated as the percentage of protein dissolved in the supernatant with respect to the total content of proteins in the dispersed amount of PsPI preparation (previously evaluated by the Kjeldahl method), according to the following formula:

$$\text{Solubility (\%)} = \frac{\text{amount of proteins in the supernatant}}{\text{amount of proteins in the dispersed PsPI}} \times 100 \quad (1)$$

For the analysis of the protein surface hydrophobicity (PSH), the protocol of Lieske and Konrad[17a,b] was applied with some modification. Briefly, 20 μL of 1:40 diluted PsPI control or HIUS-treated suspensions (samples) or 20 μL of distilled H_2O (Blanks) were placed at the bottom of a 1.5 mL tube; then, for both samples and blanks 20 μL of 0.25% (w/v) Tween 80 or 20 μL of distilled H_2O (for the control tubes) were added. After incubation for 10 min at room temperature with gentle agitation in an orbital shaker, 1 mL of fresh prepared 1× Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Inc., Hercules, California, USA) was added, and the tubes were allowed to stand for further for 12 min to allow the color development. Finally, the absorbance at 595 nm was measured against distilled water. The PSH degree was calculated as follows:

$$\text{PSH(\%)} = \frac{(\text{AS} - \text{AB}) - (\text{AST} - \text{ABT})}{(\text{AS} - \text{AB})} \times 100 \quad (2)$$

where AS and AST are the absorbance of PsPI samples without and with Tween 80, while AB and ABT are the absorbance of Blanks without and with Tween 80, respectively. Each measurement was performed in triplicate.

Determination of Particle Size and ζ-Potential. The particle sizes and ζ-potentials of PsPI samples were measured by dynamic light scattering using a Zetasizer Pro analyzer (Malvern, UK). Prior to analysis, the samples were appropriately diluted at a final concentration of 10 mg of protein/mL with Milli-Q water and the measurements were carried out using standard parameters at 25 ± 1 °C.[18] The values reported are the arithmetic mean ± standard deviation (SD) of three replicates (n = 3).

Foaming Capacity and Stability Analysis. Foaming capacity (FC) and foam stability (FS) were determined according to the method described by Sze-Tao and Sathe with slight modifications.[19] Briefly, 20 mL of each protein solution treated for different times with ultrasound (5, 10, and 20 min) were taken in a 50 mL falcon tube, and the pH was adjusted from 2.5 to 10.5. These protein solutions were homogenized at 12,000 rpm (IKA T18, Germany) for 2 min. For foaming capacity and foam stability evaluation, the total sample volume was taken at 0 and up to 20 min. Foaming capacity and FS were then calculated as follows:

$$\text{Foaming capacity (FC) (\%)} = \frac{V_a - V_b}{V_b} \times 100 \quad (3)$$

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$$\text{Foam stability (FS) (\%)} = \frac{V_{20} - V_b}{V_b} \times 100 \quad (4)$$

where V_a and V_b are the volume (mL) after and before shaking, respectively, and V_{20} is the volume after 20 min.

Preparation of Pumpkin Protein Emulsions. PsPI suspensions (1 % w7v) were prepared at different pH values (from 2.5 to 10.5) as described in "Ultrasonic treatement" paragraph. For the PsPI emulsions, sunflower oil (φ = 0.5) was added to the protein dispersion, and the two-phase system was homogenized at 12,000 rpm (IKA T18, Germany) for 2 min. The heights of the emulsified layer and that of the total contents in the tube were measured. The emulsifying activity index (EAI) was calculated as

$$\text{EAI (\%)} = \frac{\text{height of emulsified layer in the tube}}{\text{height of the total content in the tube}} \times 100 \quad (5)$$

Storage Stability of emultions. The freshly prepared emulsions were poured into glass cylinders (50 mL) at room temperature to test the creaming stability. The creaming index (CI) was determined after 7 days by the following equation[20]:

$$\text{CI (\%)} = \frac{h_s}{h_t} \times 100 \quad (6)$$

where h_s was the height of the serum and h_t was the total height of the emulsion.

Statistical Analysis. All measurements were performed in triplicate, and the resulting values were pressed as means ± SD. The variance between means (ANOVA) was assessed with a 95% confidence interval using statistical software (SPSS16.0). The ANOVA data with $P < 0.05$ were considered statistically significant.

Average matrix (samples × variables) (n = 6) was submitted to principal component analysis (PCA) to study the effect of treatment time and pH on the physicochemical properties. Time and pH were included in the model as supplementary variables. Varimax was applied as the rotation method. XLSTAT was used to analyze the data.

RESULTS AND DISCUSSION

PsPI Electrophoretic Profile. The protein content in ground pumpkin seeds, on a wet matter basis, was 12.46 ± 1.87, because of the high content of fat; in fact, after the defatting treatment, the flour contained 51.53 ± 2.64% of proteins. This value was not satisfying, since fibers, phytates, and ashes were still at high levels in the defatted flour. Thus, alkaline extraction from defatted pumpkin seed flour led to an enrichment of about 40% in protein isolate powder (PsPI). In fact, the protein content in PsPI was found to be 90.32 ± 1.27%. SDS-PAGE analysis of protein fractions taken at each step of alkaline extraction (Figure 1) shows that proteins with high molecular weight (>250 kDa) were not solubilized at pH 10, probably because they were entrapped in the insoluble fiber material (Figure 1, lane 2). After precipitation at pH 5.0, most of the protein species in the alkaline extract were recovered (Figure 1, lane 4) with a yield of 90% of the total proteins. The most abundant proteins in the PsPIs are the cucurbitin (11 S globulin) an hexameric globular protein where each subunit consists of a large acidic subunit of 33 kDa (α-chain) linked to a small basic subunit of 22 kDa (β-chain) through disulfide bonds. The low molecular weight (≤10 kDa) soluble proteins left after precipitation (Figure 1, lane 3) are essentially related to the 2S fraction of pumpkin seeds albumin.[7]

Solubility and Particle Characterization of HIUS-Treated PsPI Suspensions at Different pH. The most important functional properties of proteins in food include their solubility, emulsifying capabilities, and foaming abilities.

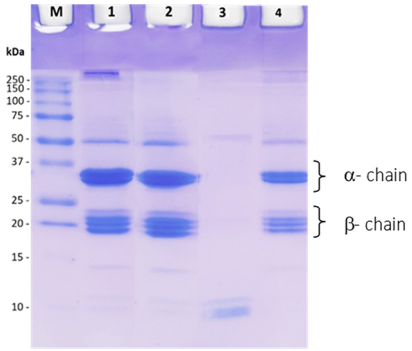

Figure 1. Pumpkin seeds protein profile in SDS-PAGE 15%, under reducing conditions, during alkaline extraction. Lane 1, aqueous suspension of defatted pumpkin seed powder; lane 2, supernatant at pH 10; lane 3, supernatant at pH 5; lane 4, pellet at pH 5. *M*, molecular weight marker (Precision Plus Protein All blue Prestained Protein Standards, Biorad).

These properties are related to the molecular size, the structure (amino acid composition and three-dimensional structure), and the charge surface of the protein, thus changes in the protein structure can affect the functional properties of plant proteins.[21,22] Figure 2 shows the effects of different times of exposition to HIUS on the solubility (panel A), turbidity (panel B), ζ-potential (panel C), and the average particle size (panel D) of PsPI powder dispersed in water at different pHs and subjected to HIUS treatment for 5 min (HIUS-5), 10 min (HIUS-10), and 20 min (HIUS-20). Concerning the solubility, regardless of ultrasound treatment, the PsPIs exhibit the typical U-shaped profile, with a higher solubility at extreme pH values, which progressively decreases up to the isoelectric point of proteins (Figure 2A), according to the results reported in the literature for different proteins, especially those of plant origin (Ogunwolu et al.).[23]

In particular, for the untreated PsPI (ctr), the lowest solubility (3%) was found at pH values near the isoelectric point (pH 6.5) where the balance between positive and negative charges of the proteins decreases the electrostatic repulsion, leading to the formation of insoluble protein aggregates,[24] whereas the highest solubility (37%) was observed at pH 2.5 and 10.5 (Figure 2A).

As expected, the turbidity curves showed a completely mirror-like trend compared to those of the solubility (Figure 2B), highlighting how around the isoelectric point, where the solubility was at its lowest point, the turbidity reached its maximum value, precisely because proteins, by forming large aggregates, generated light scattering with consequent reduction in the transparency of the protein suspension.

Following the ultrasound treatment, an increase in solubility with a parallel decrease of turbidity was observed in all the samples even at pH values around the isoelectric point, in fact all the curves, while maintaining the same profile, showed an upward shift with respect to that of the control, and the minimum solubility value increased by about 10 points going from 3% in the control (at pH 6.5) to 13% in the HIUS-5 sample (at pH 5.5). The substantial difference was in the fact that as the sonication time increased, the solubility values at the various pHs were progressively lower, following this trend: HIUS-5 > HIUS-10 > HIUS-20 > PsPI control (Figure 2A). Furthermore, it should be emphasized that in all the sonicated samples, the pH at which the lowest solubility and the highest turbidity were recorded moved to a value of 5.5. However, the

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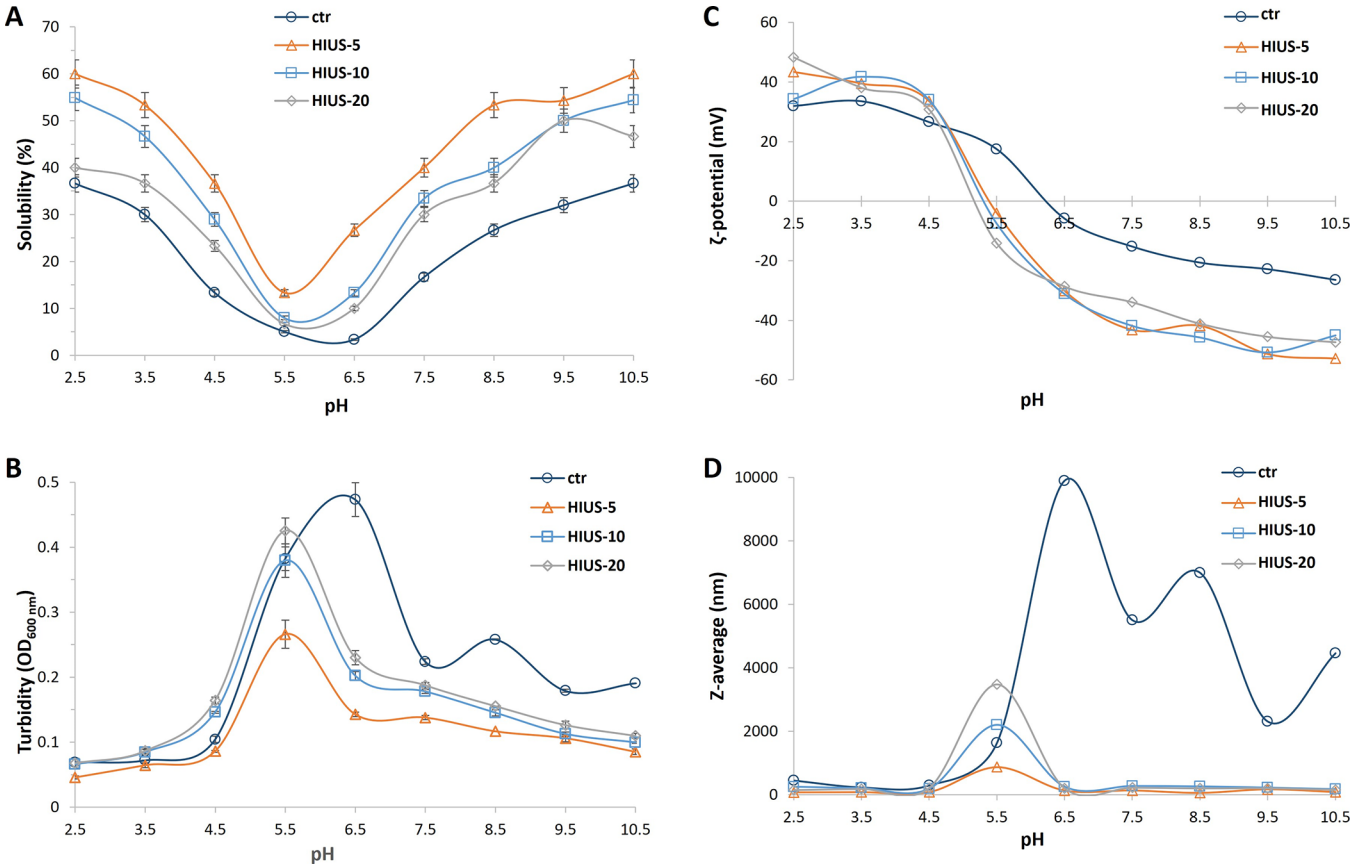

Figure 2. Solubility (A), turbidity (B), ζ-potential (C), and Z-average particle size (D) as a function of pH of PsPI untreated (ctr) and HIUS-treated for 5 min (HIUS-5), 10 min (HIUS-10), and 20 min (HIUS-20). Values are expressed as mean ± standard deviation of three replicates (n = 3).

maximum water solubility was obtained using a sonication time of 5 min (60%) at pH 2.5 and 10.5, which was significantly greater than that of the control sample at the same pH (37%). This result can be explained by hypothesizing that the HIUS waves interrupt the physical interactions that hold the protein molecules together in larger aggregates, thus favoring the release of smaller, and therefore more easily solvable, proteins.[25] Furthermore, increasing the ultrasonic treatment times, the high energy supplied to the solution can lead to the denaturation of the proteins, with consequent exposure of the hydrophobic residues, and this again leads to an aggregation of the molecules with consequent lowering of the solubility.[26] However, other studies have reported an increase in water solubility after sonication for other types of proteins, such as whey proteins,[13] soy proteins,[27] and meat proteins.[28]

Zeta (ζ-)potential is a measure of surface charge of dispersed particles and is a function of the environment of the medium in which the particle is dispersed, such as pH and ionic strength. The magnitude of the ζ-potential provides information about particle stability in the solution and a ζ-potential value >+30 mV or <−30 mV is generally considered to have sufficient repulsive force to attain better physical stability.[29] Similarly, the reduction of particle size increases the surface area of the particles that allows greater interaction with the solvent, which is responsible for an increase in solubility.[13,30]

PsPI powder showed good dispersibility and stability in solution at pH equal to or below 4.5 without HIUS treatment. In fact, the Z-average value of particle size in the solution was below 300 nm (Figure 2D) and the ζ-potential >+30 mv (Figure 2C). At pH 5.5 the size increased to 1 μm and at pH 6.5 particles higher than 9 μm were observed, corresponding to the maximum protein aggregation, also confirmed by the ζ-potential value that was close to zero (Figure 2C). At pH higher than 6.5, the ζ-potential became more negative until it reached the maximum value of −26.4 ± 0.3 mV at pH 10.5. However, the particle size was still higher than 2 μm. HIUS treatments significantly improved both the dispersibility and stability of PsPI powder. In fact, the PsPI powder dispersed at different pHs and treated with HIUS showed nanometric dimensions at all the considered pHs, except at the isoelectric point, regardless of the HIUS exposure time (Figure 2D). According to these results, an increase of turbidity associated with a reduction of solubility was also found in the range of pH 5.5−8.5 for the control and 4.5−6.5 for HIUS-treated PsPI (Figure 2B). These results can be attributed to the ability of HIUS waves to break some of the physical forces that hold protein molecules together in larger aggregates, releasing smaller soluble proteins.[25,31]

Similar results were obtained for chickpea proteins treated for different times and different power levels.[32] The authors reported that 30 min of HIUS treatment at 150 W had a good ability to break up larger aggregates of plant proteins. However, a further increase of the power had no significant effect on particle size distribution. This is because the effect of HIUS on protein aggregates is limited when the particle size is reduced to 0.1−10 μm, a phenomenon also observed in lupin proteins by Lo et al.[33] Moreover, it is interesting to note the effectiveness of HIUS to change surface charges of PsPI. In

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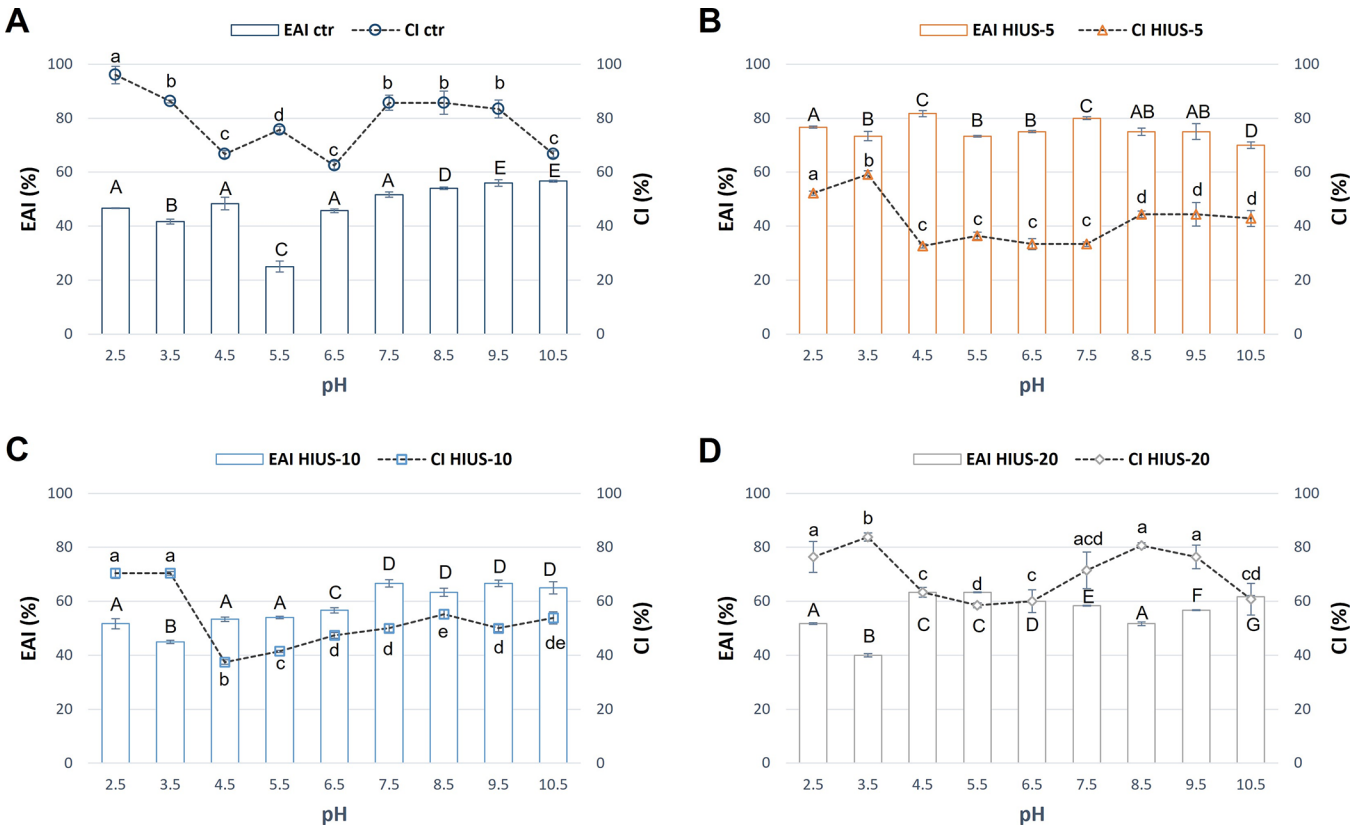

Figure 3. Emulsifying activity index (EAI, bars) and creaming index (CI, lines) of PsPI untreated (ctr, A) and HIUS-treated for 5 min (HIUS-5, B), 10 min (HIUS-10, C), and 20 min (HIUS-20, D) at a sonication power of 150 W (pulse duration: 0.5 s on/0.5 s off). Values are expressed as mean ± SD (n = 3). Samples marked with different letters are significantly different ($p < 0.05$). Capital letters refer to the EAI (bars) while lower case letters refer to CI (lines).

fact, an increase, in absolute value, of the ζ-potential in both acidic and basic conditions was found (Figure 2C), confirming the improvement of the PsPI solution stability. In fact, it is well-known that a net positive or net negative charge on protein surface results in an electrostatic repulsive force that counteracts the protein aggregation, improving the stability of solutions.

Other studies described similar results for HIUS treatment of black bean proteins,[34] BSA,[35] whey proteins,[13] and meat proteins.[28] The authors suggest that HIUS treatment induces irreversible changes in the protein structure and conformation with a clear increase of charged residues located on the surface of the protein molecule, improving the interaction with the solvent that stabilizes the solution. Experimental evidence regarding the hydrophobicity of the protein surface seems to confirm an active role of the HIUS treatment in the conformational modification of PsPIs. In fact, at pH 8.5, the calculated PSH degree for untreated proteins was 22.7 ± 0.5%. After sonication, PSH decreased to 17.5 ± 0.3, 15.3 ± 0.4, and 17.0 ± 0.6%, respectively, for HIUS-5, HIUS-10, and HIUS-20. This PSH reduction could be related to the disruption of molecular assemblies caused by ultrasonic waves, which is linked to a greater exposure of the hydrophilic residues of PsPI. At this pH value (8.5), the surface charge of the proteins is still sufficient to maintain the PsPI in solution in a disaggregated form (Figure 2D).

However, at pH 5.5 (close to the isoelectric point) where the neutralization of the superficial charges occurs, an increase in the average particle size was found due to protein aggregation (Figure 2D). It is worth to note that the size of aggregates increased along with the increase of HIUS treatment.

This effect can be explained considering that probably, increasing the time of HIUS treatment, the proteins were subjected to greater conformational changes, leading to the exposure of a greater number of hydrophobic residues. This finding is confirmed by the higher PSH value, for longer exposure times, in the HIUS-20 treatment, indicating a partial protein denaturation leading to a more open conformation, which brings the hydrophobic residues of the inner core outward. Therefore, when the net charge decreases near the isoelectric point, the hydrophobic forces prevail over the electrostatic forces and the proteins form larger aggregates.[25,31] These results suggest that 5 min of HIUS treatment represents the best condition for a high stability of the PsPI solution at all the tested pHs.

Emulsifying and Foaming Properties of HIUS-Treated PsPI. Charge and/or hydrophobicity changes of proteins surface can affect the behavior of proteins at oil/water and air/water interfaces and are of fundamental importance in food systems as emulsions and foams. Thus, EAI, CI, FC, and FS of the PsPIs subjected to the different HIUS treatments were investigated at different pHs, and results are reported in Figures 3 and 4.

The untreated PsPI emulsions showed an EAI in the range of 42−57% at all the selected pH values, except at pH 5.5 where the lowest value (25%) was observed; while for the same sample, the CI, that is inversely related with the emulsion

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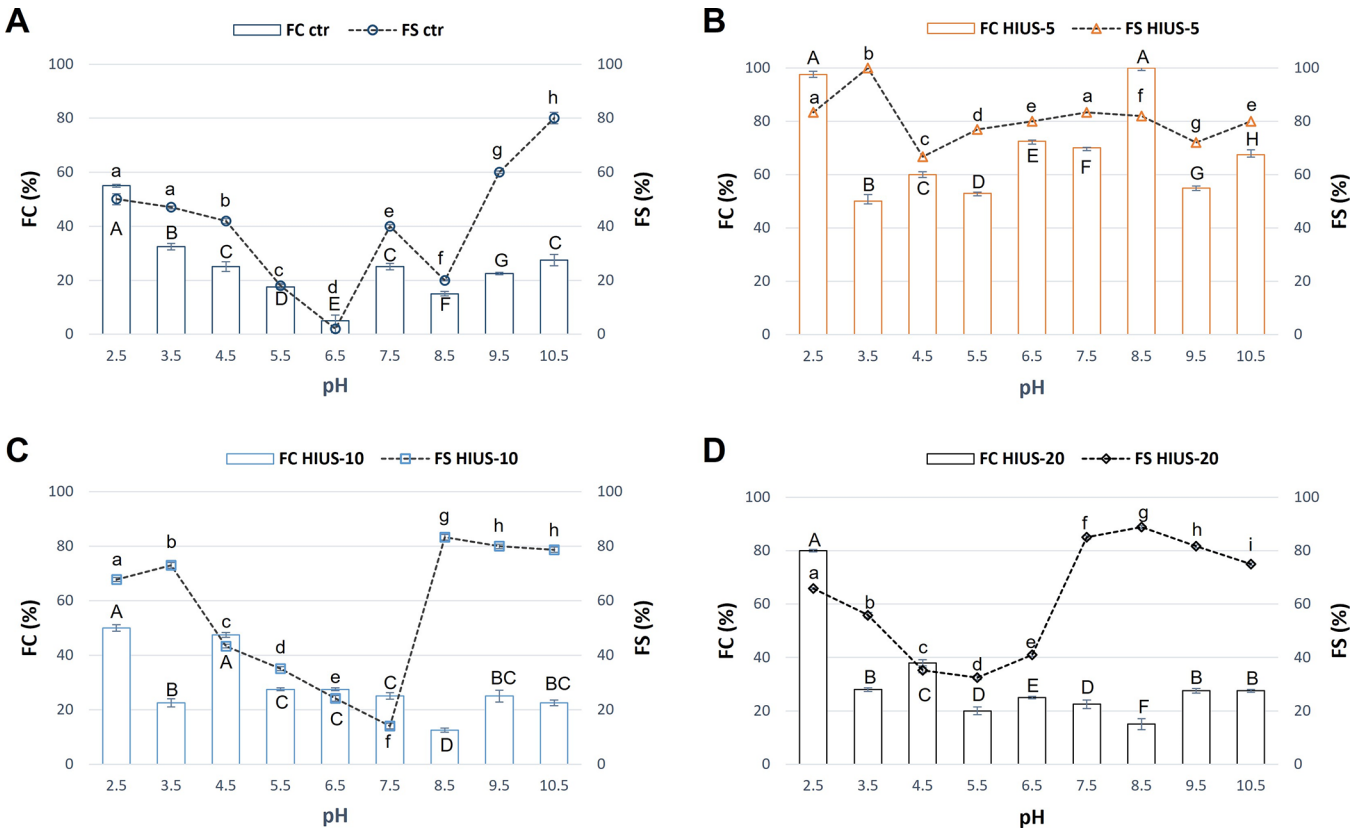

Figure 4. Foaming capacity (FC, bars) and foam stability (FS, lines) of PsPI untreated (ctr, A) and HIUS-treated for 5 min (HIUS-5, B), 10 min (HIUS-10, C), and 20 min (HIUS-20, D) at a sonication power of 150 W (pulse duration: 0.5 s ON/0.5 s off). Values are expressed as mean ± SD (n = 3). Samples marked with different letters are significantly different (p < 0.05). Capital letters refer to the FC (bars) while lower case letters refer to FS (lines).

stability, resulted around to the 70−80% with a maximum (96%) at pH 2.5 (Figure 3A). These results highlight the poor propensity of native PsPI to generate and stabilize emulsions. Conversely, when the PsPI were subjected to HIUS treatments, an important improvement of the EAI was observed. Specifically, in the samples treated for 5 min (HIUS-5) EAI increased to 73−82% (Figure 3B) at all the pH values, while the threshold of 60% was exceeded at pH values greater than 7.5 for HIUS-10 samples (Figure 3C) and between pH 4.5 and 6.5 as well as at pH 10.5 for HIUS-20 (Figure 3D). What it is important to underline is that at the isoelectric point of PsPI (pH 5.5), the emulsifying capacity of the proteins was sensibly improved since the EAI passed from 25% in the control (ctr) to 73, 54, and 63% in HIUS-5, HIUS-10, and HIUS-20 samples, respectively.

The HIUS treatments also improved the ability of PsPI to stabilize the emulsion; in fact, the CI, in the control samples fluctuated between 63 and 97%, with the lowest values between pH 4.5 and 6.5 (Figure 3A), strongly decreased in HIUS-5 and HIUS-10 samples, where the CI assumed values even lower than 40% in the intermediate pH points (4.5−7.5 for HIUS-5, and 4.5−5.5 for HIUS-10) (Figure 3B,C). The trend of CI for HIUS-20 samples along the pH was similar to that of the control but with lower values (Figure 3D). However, it is interesting to underline that in the emulsions obtained with untreated PsPI, the CI reached its stable value after 1 day, whereas the creaming rate of the emulsions obtained by PsPI subjected to HIUS treatment was slower, reaching the final value after 1 week.

The behavior observed in the HIUS-treated PsPI can be associated with the exposure of internal hydrophobic groups on the protein surface that improve the amphiphilic properties of HIUS-treated proteins. These phenomena, also confirmed by the changes in superficial charge (Figure 2C), lead to an increase in the ability of the proteins to migrate at the oil/water interface to generate a protective barrier around fat droplets. Moreover, the PsPI size reduction observed in HIUS-treated samples (Figure 2D) is able to generate a more homogeneous and thinner layer of protein at the oil/water interface in which the hydrophobic group interacts with the oil and the charged groups interact with water, generating a high net charge on the droplet surface which prevents their coalescence.[36] Similar results were observed in HIUS-treated nut proteins,[12] egg proteins,[37] soy proteins,[27,38] and peanut proteins.[10]

Foam formation is governed by three factors, including transportation, penetration, and reorganization of molecules at the air/water interface. To exhibit good FC, a protein must be capable of migrating rapidly to the air/water interface, unfolding and rearranging at the interface.[39] Dickinson[40] reported that the FC of a protein was improved as a result of increasing its flexibility and exposure to more hydrophobic residues, which enhance the capacity to decrease surface tension. FC is related to the ability of the proteins to form liquid/air dispersions while FS represents the percentage of foam volume remaining compared to the initial foam volume after a given time and is an important parameter for evaluating the stability of whipped foods.[41]

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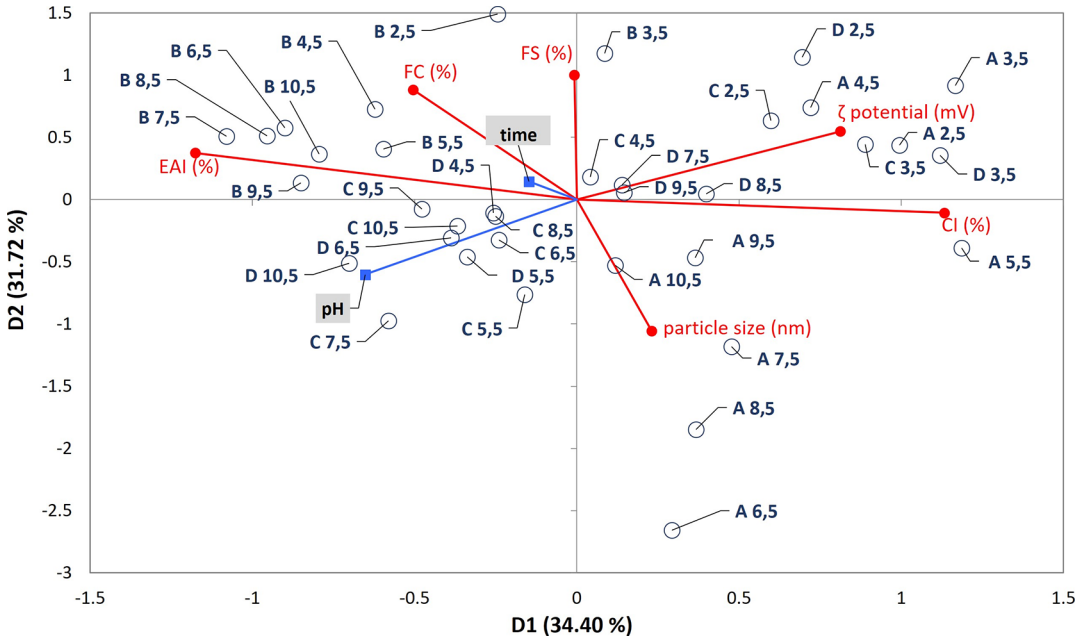

Figure 5. Principal component analysis biplot. Empty circles (○), samples (A, ctr; B, HIUS-5, C, HIUS-10, D, HIUS-20; pH is indicated alongside); full circles (●), active variables (ζ-potential, particle size, EAI, CI, FC, FS); full square (■), supplementary variables (pH and time).

Results about foaming properties of PsPI, reported in Figure 4, showed that untreated PsPI exhibited FC values lower than or near to 30% at all the considered pHs except for pH 2.5 in which FC was 55% (Figure 4A). Moreover, for this sample, the lowest FC value was observed at pH 6.5, which also corresponds to the highest protein aggregation level (Figure 2D). Evidently, the large particle size reduces the capability of proteins to migrate to the air/water interface and the exposition of hydrophobic residues involved in the particle aggregation.[42]

The HIUS treatment of PsPI significantly improved the FC only in HIUS-5 treated samples which even achieved values of 97.5 and 100% at pH 2.5 and 8.5, respectively (Figure 4B), while no significant improvements were observed for the HIUS-10 and HIUS-20 samples (Figure 4C,D). Similar results were described for HIUS-treated whey proteins[13] and soy protein isolates.[43] In addition, with regard to the FS parameter, the best results were recorded for the HIUS-5 sample, which showed 100% FS at pH 3.5, while FS settled around 80% at pH between 5.5 and 8.5 and at pH 2.5 (Figure 4B). For longer HIUS treatments, the trend of the FS values followed that of the control (Figure 4A) with the difference that in HIUS-10 and HIUS-20 at basic pH, FS was equal to or higher than the 80% (Figure 4C,D).

It is reported by Xiong et al.[44] that HIUS treatment may cause partial unfolding of pea protein, reduction in particle size and increase in hydrophobicity: these changes improved FS from 58.0 to 73.3% for up to 10 min of treatment with respect to the untreated samples.

This result could be attributed to partial denaturation during HIUS treatment. Indeed, prolonged ultrasound exposure may lead to partial unfolding of the structure of proteins, resulting in increased exposure of hydrophobic groups to the protein surface, which increases the possibility of aggregation between proteins. It is known that the application of HIUS causes changes in the structures of proteins that can alter their basic functionality.

For example, Gülseren et al.[35] reported that after 45 min of ultrasound, the percentage of α-helix increased from 61.1 to 74.5, while the percentage of β-sheet and β-turn decreased by 2.8 and 1.6%, respectively. Similar results have also been reported by researchers who have applied sonication to other types of proteins, such as nut protein, black bean protein, and chicken myofibrillar proteins.[12,28,34] It has also been reported that sonication reduces the fluorescence emission intensity of proteins, which is indicative of a change in protein tertiary structure and/or aggregation state.[45] Changes in tertiary structure have been reported for egg proteins[37] and soybean proteins.[46] These effects are attributed to the ability of high-intensity ultrasonic waves to disrupt the physical bonds between and within globular protein molecules, thus leading to some unfolding and dissociation. Sonication can therefore be used to improve the functional properties of proteins.

Principal Component Analysis. The correlation between particle size, ζ-potential, emulsion and foaming properties at different pHs of suspensions/emulsions prepared with PsPIs treated by HIUS for different times was investigated through PCA.

Figure 5 shows the obtained PCA biplot. The first principal component (PC1) of the PCA described 34.4% of the variation in terms of the physicochemical properties of the samples; thus, most of the interpretable variation could be described along PC1. The addition of a second component (PC2) increased the explained variance to 66.12%. As can be observed in the second quadrant, the control samples (indicated by the letter "A") were characterized by a dominant granulometry in the range of pH 5.5−10.5. The pH variation from acidic to alkaline, especially near the isoelectric point, of the untreated samples of PsPI (A), is reflected in an increase in particle size associated with the formation of aggregates. However, at pH below the isoelectric point, they are more characterized by a high ζ-potential (first quadrant) (Figure 2), according to the previously reported results about particle size and ζ-potential analysis.

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Moreover, the biplot analysis showed that PsPI samples treated with HIUS for 5 min (HIUS-5, indicated by the letter "B") were characterized by EAI, FC, and FS at all the investigated pH values; in fact, almost all the "B" samples are localized in the fourth quadrant. This indicates that HIUS-5 pretreatment could have a significant effect on the functional properties of PsPI. With increasing pH and HIUS treatment time at 10 min (HIUS-10, indicated by "C") and 20 min (HIUS-20, indicated by "D"), the ζ-potential value decreased, which is shown by the negative correlation between time and pH and ζ-potential, since "C" and "D" samples are distributed in the first and third quadrant. With the decrease of ζ-potential, and thus of the electrostatic repulsion between proteins, an increase in particle size was observed, and this effect was mainly observed at alkaline pH and for treatment times longer than 5 min (Figure 5, samples "C" and "D" in the fourth quadrant). However, by keeping the times at low rates (5 min), EAI, FC, and FS were roughly constant at each pH. PCA showed that HIUS processing affected the different considered indices, which is due to the remarkable effect on the functional properties of the pumpkin seed protein isolates.

In conclusion, in this study, it was demonstrated that solubility, particle size, ζ-potential, as well as emulsifying and foaming properties of PsPI samples were significantly affected by HIUS treatment. The solubility increased by more than 20 points at extreme pH values. The particle size was reduced to nanometric dimensions in all the pH ranges except at the isoelectric point (pH 5.5). In parallel, the ζ-potential moved to more negative values associated with more stability of particles in solution. As a consequence, emulsifying properties were improved, and the best values were obtained with the samples treated for 5 min at a 150 W power level. The FC and FS values of ultrasound-treated protein samples gradually decreased with increasing time treatment, which resulted from the exposure of hydrophobic regions for ultrasound caused partial unfolding of PsPI. Particularly, after the sonication for 5 min, PsPI suspension proteins achieved a maximum emulsifying activity with the lowest creaming index, even at acidic pH (including di isoelectric point pH 5.5). Moreover, FC and FS were also at the highest values with respect to all other samples. These results suggested that 5 min ultrasonic treatment might be a useful approach to modify functional properties of PsPI at desired values, above all in the pH range more interesting for the food industry (3.5−5.5), which concerns a large part of foods like beverages, sauces, yogurt, and so on. Anyway, further studies for the detailed stability mechanisms are still needed to enable the use in food processes.

■ ASSOCIATED CONTENT

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

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Author Contributions

A.R.: investigation; methodology; formal analysis; writing-original draft. S.M.: investigation; methodology. A.S.: supervision; conceptualization; writing review and editing; visualization. P.M.: supervision; conceptualization; writing-review and editing. P.D.P.: investigation, supervision; conceptualization; writing review and editing. All authors have read and agreed to the published version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

HIUS, high-intensity ultrasound; PsPI, pumpkin seeds protein isolate

■ REFERENCES

(1) Rifna, E. J.; Misra, N. N.; Dwivedi, M. Recent advances in extraction technologies for recovery of bioactive compounds derived from fruit and vegetable waste peels: A review. *Crit. Rev. Food Sci. Nutr.* **2023**, *63* (6), 719−752.

(2) Lestari, B.; Meiyanto, E. A review: the emerging nutraceutical potential of pumpkin seeds. *Indonesian J. Cancer Chemoprevent.* **2018**, *9* (2), 92−101. (2018)

(3) Ahmad, G.; Khan, A. A. Pumpkin: Horticultural Importance and Its Roles in Various Forms; a Review. *Int. J. Hort. Agric.* **2019**, *4* (1), 1−6.

(4) Murkovic, M. *Pumpkin seed oil, in Gourmet and Health-Promoting Specialty Oils*; Moreau, R. A.; Kamal-Eldin, A., Eds.; AOCS Press: Urbana, IL, 2009; pp 345−358.

(5) Peričin, D.; Radulović-Popović, L.; Vaštag, Ž.; Mađarev-Popović, S.; Trivić, S. Enzymatic hydrolysis of protein isolate from hull-less pumpkin oil cake: Application of response surface methodology. *Food Chem.* **2009**, *115*, 753−757.

(6) Vaštag, Ž.; Popović, L.; Popović, S.; Krimer, V.; Peričin, D. Production of enzymatic hydrolysates with antioxidant and angiotensin-I converting enzyme inhibitory activity from pumpkin oil cake protein isolate. *Food Chem.* **2011**, *124*, 1316−1321.

(7) Bučko, S.; Katona, J.; Popović, L.; Vaštag, Ž.; Petrović, L.; Vučinić-Vasić, M. Investigation on solubility, interfacial and emulsifying properties of pumpkin (*Cucurbita pepo*) seed protein isolate. *LWT-Food Sci. Technol.* **2015**, *64* (2), 609−615.

(8) Rezig, L.; Chibani, F.; Chouaibi, M.; Dalgalarrondo, M.; Hessini, K.; Guéguen, J.; Hamdi, S. Pumpkin (*Cucurbita maxima*) seed proteins: sequential extraction processing and fraction characterization. *J. Agric. Food Chem.* **2013**, *61* (32), 7715−7721.

(9) Ozuna, C.; León-Galván, M. F. *Cucurbitaceae* seed protein hydrolysates as a potential source of bioactive peptides with functional properties. *Biomed. Res. Int.* **2017**, *2017*, No. 2121878.

(10) Zhang, Q. T.; Tu, Z. C.; Xiao, H.; Wang, H.; Huang, X. Q.; Liu, G. X.; Liu, C. M.; Shi, Y.; Fan, L. L.; Lin, D. R. Influence of ultrasonic

treatment on the structure and emulsifying properties of peanut protein isolate. *Food Bioprod. Proc.* **2014**, *92* (1), 30−37.
(11) Vargas, S. A.; Delgado-Macuil, R. J.; Ruiz-Espinosa, H.; Rojas-López, M.; Amador-Espejo, G. G. High-intensity ultrasound pretreatment influence on whey protein isolate and its use on complex coacervation with kappa carrageenan: Evaluation of selected functional properties. *Ultrason. Sonochem.* **2021**, *70*, No. 105340.
(12) Zhu, Z.; Zhu, W.; Yi, J.; Liu, N.; Cao, T.; Lu, J.; Decker, E. A.; Mc Clements, D. J. Effects of sonication on the physicochemical and functional properties of walnut protein isolate. *Food Res. Int.* **2018**, *106*, 853−861.
(13) Jambrak, A. R.; Mason, T. J.; Lelas, V.; Herceg, Z.; Herceg, I. L. Effect of ultrasound treatment on solubility and foaming properties of whey protein suspensions. *J. Food Eng.* **2008**, *86* (2), 281−287.
(14) Lynch, J. M.; Barbano, D. M. Kjeldahl nitrogen analysis as a reference method for protein determination in dairy products. *J. AOAC Int.* **1999**, *82* (6), 1389−1398.
(15) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227* (5259), 680−685.
(16) Shevkani, K.; Singh, N.; Kaur, A.; Rana, J. C. Structural and functional characterization of kidney bean and field pea protein isolates: A comparative study. *Food Hydrocoll.* **2015**, *43*, 679−689.
(17) (a) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72* (1−2), 248−254. (b) Lieske, B.; Konrad, G. A new approach to estimate surface hydrophobicity of proteins. *Milchwissenschaft* **1994**, *49* (12), 663−666.
(18) Vera, A.; Valenzuela, M. A.; Yazdani-Pedram, M.; Tapia, C.; Abugoch, L. Conformational and physicochemical properties of quinoa proteins affected by different conditions of high-intensity ultrasound treatments. *Ultrason. Sonochem.* **2019**, *51*, 186−196.
(19) Sze-Tao, K. W. C.; Sathe, S. K. Walnuts (*Juglans regia* L). Proximate composition, protein solubility, protein amino acid composition and protein in vitro digestibility. *J. Sci. Food Agric.* **2000**, *80* (9), 1393−1401.
(20) Hosseini, R. S.; Rajaei, A. Potential Pickering emulsion stabilized with chitosan-stearic acid nanogels incorporating clove essential oil to produce fish-oil-enriched mayonnaise. *Carbohydr. Polym.* **2020**, *241*, No. 116340.
(21) Su, J.; Cavaco-Paulo, A. Effect of ultrasound on protein functionality. *Ultrason. Sonochem.* **2021**, *76*, No. 105653.
(22) Stefanović, A. B.; Jovanović, J. R.; Dojčinović, M. B.; Lević, S. M.; Nedović, V. A.; Bugarski, B. M.; Knežević-Jugović, Z. D. Effect of the controlled high-intensity ultrasound on improving functionality and structural changes of egg white proteins. *Food Bioprocess. Technol.* **2017**, *10* (7), 1224−1239.
(23) Ogunwolu, S. O.; Henshaw, F. O.; Mock, H. P.; Santros, A.; Awonorin, S. O. Functional properties of protein concentrates and isolates produced from cashew (*Anacardium occidentale* L.) nut. *Food Chem.* **2009**, *115* (3), 852−858.
(24) Deng, Q. C.; Wang, L.; Wei, F.; Xie, B. J.; Huang, F. H.; Huang, W.; Shi, J.; Huang, Q.; Tian, B.; Xue, S. Functional properties of protein isolates, globulin and albumin extracted from Ginkgo biloba seeds. *Food Chem.* **2011**, *124*, 1458−1465.
(25) Arzeni, C.; Martínez, K.; Zema, P.; Arias, A.; Pérez, O. E.; Pilosof, A. M. R. Comparative study of high intensity ultrasound effects on food proteins functionality. *J. Food Eng.* **2012**, *108* (3), 463−472.
(26) Morel, M. H.; Dehlon, P.; Autran, J. C.; Leygue, J. P.; Bar-L'Helgouac'h, C. Effects of temperature, sonication time, and power settings on size distribution and extractability of total wheat flour proteins as determined by size-exclusion high-performance liquid chromatography. *Cereal Chem.* **2000**, *77* (5), 685−691.
(27) Hu, H.; Wu, J.; Li-Chan, E. C.; Zhu, L.; Zhang, F.; Xu, X.; Fan, G.; Wang, L.; Huang, X.; Pan, S. Effects of ultrasound on structural and physical properties of soy protein isolate (SPI) dispersions. *Food Hydrocoll.* **2013**, *30* (2), 647−655.
(28) Wang, J. Y.; Yang, Y. L.; Tang, X. Z.; Ni, W. X.; Zhou, L. Effects of pulsed ultrasound on rheological and structural properties of chicken myofibrillar protein. *Ultrason Sonochem.* **2017**, *38*, 225−233.
(29) Bhattacharjee, S. DLS and zeta potential−what they are and what they are not? *J. Controlled Release* **2016**, *235*, 337−351.
(30) Ojha, K. S.; Tiwari, B. K.; O'Donnell, C. P. Effect of ultrasound technology on food and nutritional quality. In *Advances in food and nutrition research*; Toldrá, F., Ed.; Academic Press: New York, NY, 2018; vol *84*, pp 207−240.
(31) Tang, C. H.; Ma, C. Y. Heat-induced modifications in the functional and structural properties of vicilin-rich protein isolate from kidney (*Phaseolus vulgaris* L.) bean. *Food Chem.* **2009**, *115* (3), 859−866.
(32) Bi, C. H.; Chi, S. Y.; Zhou, T.; Zhang, J. Y.; Wang, X. Y.; Li, J.; Shi, W. T.; Tian, B.; Huang, Z. G.; Liu, Y. Effect of low-frequency high-intensity ultrasound (HIU) on the physicochemical properties of chickpea protein. *Food Res. Int.* **2022**, *159*, No. 111474.
(33) Lo, B.; Kasapis, S.; Farahnaky, A. Effect of low frequency ultrasound on the functional characteristics of isolated lupin protein. *Food Hydrocoll.* **2022**, *124*, No. 107345.
(34) Jiang, L.; Wang, J.; Li, Y.; Wang, Z.; Liang, J.; Wang, R.; Chen, Y.; Ma, W.; Qi, B.; Zhang, M. Effects of ultrasound on the structure and physical properties of black bean protein isolates. *Food Res. Int.* **2014**, *62*, 595−601.
(35) Gülseren, İ.; Güzey, D.; Bruce, B. D.; Weiss, J. Structural and functional changes in ultrasonicated bovine serum albumin solutions. *Ultrason Sonochem.* **2007**, *14* (2), 173−183.
(36) Zayas, J. F. *Functionality of proteins in food*, 1st edition; Springer Science & Business Media. Springer: Berlin, Heidelberg, 1997.
(37) Xiong, W.; Wang, Y.; Zhang, C.; Wan, J.; Shah, B. R.; Pei, Y.; Zhou, B.; Li, J.; Li, B. High intensity ultrasound modified ovalbumin: Structure, interface and gelation properties. *Ultrason sonochem.* **2016**, *31*, 302−309.
(38) Zhou, M.; Liu, J.; Zhou, Y.; Huang, X.; Liu, F.; Pan, S.; Hu, H. Effect of high intensity ultrasound on physicochemical and functional properties of soybean glycinin at different ionic strengths. *Innov. Food Sci. Emerg. Technol.* **2016**, *34*, 205−213.
(39) Halling, P. J. Protein-stabilized foams and emulsions *Crit. Rev. Food Sci. Nutr.* **1981**, *13*, 155, .
(40) Dickinson, E. Protein adsorption at liquid interfaces and the relationship to foam stability. In *Foams: Physics, chemistry and structure*; Springer Series in Applied Biology; Wilson, A. J., Ed.; Springer: London, 1999; pp 39−53.
(41) Pezeshk, S.; Rezaei, M.; Hosseini, H.; Abdollahi, M. Impact of pH-shift processing combined with ultrasonication on structural and functional properties of proteins isolated from rainbow trout by-products. *Food Hydrocoll.* **2021**, *118*, No. 106768.
(42) Zou, H.; Zhao, N.; Sun, S.; Dong, X.; Yu, C. High-intensity ultrasonication treatment improved physicochemical and functional properties of mussel sarcoplasmic proteins and enhanced the stability of oil-in-water emulsion. *Colloids Surf. A: Physicochem. Eng. Asp.* **2020**, *589*, No. 124463.
(43) Morales, R.; Martínez, K. D.; Ruiz-Henestrosa, V. M. P.; Pilosof, A. M. Modification of foaming properties of soy protein isolate by high ultrasound intensity: Particle size effect. *Ultrason. Sonochem.* **2015**, *26*, 48−55.
(44) Xiong, T.; Xiong, W.; Ge, M.; Xia, J.; Li, B.; Chen, Y. Effect of high intensity ultrasound on structure and foaming properties of pea protein isolate. *Food Res. Int.* **2018**, *109*, 260−267.
(45) Bermúdez-Aguirre, D.; Corradini, M. G.; Mawson, R.; Barbosa-Cánovas, G. V. Modeling the inactivation of *Listeria innocua* in raw whole milk treated under thermo-sonication. *Innov. Food Sci. Emerg. Technol.* **2009**, *10* (2), 172−17.
(46) Huang, L.; Ding, X.; Dai, C.; Ma, H. Changes in the structure and dissociation of soybean protein isolate induced by ultrasound-assisted acid pretreatment. *Food Chem.* **2017**, *232*, 727−732.

https://doi.org/10.1021/acsfoodscitech.3c00253
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