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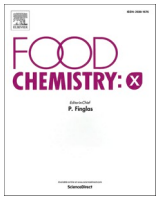

Analytical Methods

Innovative optimized protein extraction from pequi almond cake: A comparison of ultrasound-assisted and alkaline extraction methods.☆

Felipe Pires Ribeiro [a], Rafael Silva Naito [a], Beatriz dos Santos Galvão [a], Walter Fabri Júnior [a], Kayu Kazuo Toyonaga [a], Ivanise Guilherme Branco [a], Sungil Ferreira [b,*], Cassia Roberta Malacrida [a,*]

[a] São Paulo State University (Unesp), School of Sciences and Languages, Dom Antônio Avenue 2100, CEP, 19806-900 Assis, SP, Brazil
[b] University of Arkansas System Division of Agriculture (UADA), Department of Food Science, 2650 Young Ave, Fayetteville, AR, USA

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ABSTRACT

Pequi (*Caryocar brasiliense* Camb.) is a fruit native to the Brazilian Cerrado with significant economic value. Its processing generates by-products often underused, such as its protein-rich almond. This study aimed to optimize and compare protein isolates obtained from defatted pequi almond cake using ultrasound-assisted extraction (UAE) and alkaline extraction (AE). The alkaline pH for protein solubilization was set at 9.5, and the isoelectric precipitation point at pH 4.8. UAE was optimized using a central composite design, with 56 W acoustic power, a solid-to-solvent ratio of 1/15.13 (g/mL), and extraction time of 15 min. UAE resulted in a yield of 47.25 % and protein recovery of 84.28 %, while AE achieved 41.35 % and 70.18 %, respectively. The protein isolate from UAE showed better functional properties, including higher foaming capacity and water absorption. In conclusion, UAE is a promising method for producing protein isolates, offering higher yield, shorter extraction time, and improved functional characteristics.

1. Introduction

The pursuit of sustainable solutions in the industrial sector has led to valorization of by-products generated during food processing. A notable example is pequi (*Caryocar brasiliense* Camb.), a fruit native to the Brazilian Savanna, also known as the Cerrado. Pequi holds economic significance due to its applications in cooking and oil extraction for food, cosmetics, and therapeutic products (Carneiro et al., 2023). However, its processing generates substantial waste, contributing to a higher carbon footprint (Fagundes et al., 2019). The pequi almond is enclosed within a spiny endocarp, and is often discarded due to processing challenges, despite containing approximately 50 % lipids and 25 % proteins (Lima et al., 2007). Oil extraction from the almond produces a defatted, protein-rich cake witch remains largely underutilized in terms of protein extraction and functional characterization.

Valorizing by-products such as pequi almond cake aligns with the principles of a circular economy, aiming to reduce waste and promote a more sustainable production chain. Moreover, the growing demand for plant-based proteins highlights the need to explore new sustainable protein sources (Santos et al., 2024). Despite its high protein content, pequi almond cake remains underutilized due to the lack of optimized extraction strategies.

Alkaline extraction (AE) is the most widely used among various protein extraction methods due to its simplicity and efficiency. However, this method can lead to protein denaturation, reduced functional properties, and undesirable reactions (Bedin et al., 2020). Therefore, alternative techniques such as ultrasound-assisted extraction have been explored to address these limitations and improve efficiency while minimizing adverse effects.

Ultrasound has emerged as an efficient technology for extracting bioactive compounds, including proteins, making it an attractive alternative for optimizing industrial processes (Das et al., 2022). Ultrasound enhances mass transfer and disrupts cell membranes through acoustic cavitation, enabling faster and more efficient protein extraction from plant matrices. It offers additional advantages such as shorter extraction times, lower solvent consumption, and greater process control, contributing to more selective and sustainable extractions (Su & Cavaco-Paulo, 2021).

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* Corresponding authors.
E-mail addresses: sunfer@uark.edu (S. Ferreira), cassia.malacrida@unesp.br (C.R. Malacrida).

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Previous studies highlight the potential of ultrasound-assisted extraction (UAE) to enhance both protein recovery and the functional properties of various plant-based sources. For example, Ly et al. (2018) applied UAE to defatted rice bran and observed protein concentrations 1.67 times higher than those obtained by alkaline extraction, along with extraction rates up to 15.9 times faster, depending on the process conditions. Similar results were reported by Golly et al. (2020), who optimized UAE for walnut meal and achieved increases of 30.15 % in yield and 16.27 % in protein purity, as well as a 25 % reduction in extraction time and improvements in emulsifying and foaming properties. Other studies, such as those by Wang et al. (2020) with pea protein and Sert et al. (2022) with pumpkin seed cake, also demonstrated significant improvements in protein recovery and functional properties compared to alkaline extraction.

Despite the proven effectiveness of ultrasound in extracting proteins from various plant sources, its application to pequi almonds remains unexplored. Furthermore, no studies have focused on optimizing this process to improve yield, enhance protein recovery, or assess potential changes in protein functionality. Given the high production volume of this raw material and the need for more efficient extraction methods, this study aims to bridge this gap by optimizing the conditions for ultrasound-assisted extraction. The findings not only highlight the potential of ultrasound for protein extraction, but also contribute to valorizing pequi almonds, promoting their full utilization and expanding their applications in the food industry.

2. Materials and methods

2.1. Sample preparation

Pequi almonds used in this study were obtained from Montes Claros, Minas Gerais, Brazil, during the 2023 harvest. After chopping, the almonds were dehydrated in a hot-air oven at 40 °C for 24 h. Oil was first extracted by cold pressing using a hydraulic press at 0.5 tons/cm^2 for one hour. The extracted oil was then properly stored. The almond residue underwent Soxhlet extraction to remove the remaining oil using petroleum ether as the solvent at 50 °C for 6 h. The resulting material was then ground in a knife mill to produce defatted pequi almond flour (DPAF), sifted through a 30-mesh sieve (595 μm), and stored in a desiccator.

2.2. Protein extraction methods

2.2.1. Conventional alkaline extraction

The pH values used for both alkaline extraction (AE) and ultrasound-assisted extraction (UAE) were determined based on the protein solubility curve of DPAF, tested over a range from pH 2 to 10. Protein isolate was obtained using AE, following the method described by Bedin et al. (2020), with modifications. DPAF was dispersed in distilled water at a flour-to-water ratio of 1/20 (g/mL), and the pH was adjusted to the selected alkaline value using 1.0 M NaOH for the initial pH correction, followed by 0.1 M NaOH for fine adjustments to reach the target pH more precisely. The mixture was homogenized at room temperature with a magnetic stirrer at 300 rpm for 30 min. The pH was continuously monitored and adjusted as needed to maintain stability throughout the process. After homogenization, the mixture was centrifuged at 3000 ×g for 15 min. The supernatant was collected, and the pellet was discarded. The pH of the supernatant was then adjusted to the isoelectric point for protein precipitation, following the same stepwise approach, using 1.0 M HCl for coarse adjustment and 0.1 M HCl for fine tuning. The solution was stirred again at room temperature for 30 min, followed by a second centrifugation at 3000 ×g for 15 min. The resulting protein-rich precipitate was collected and dried in an oven at 40 °C for 24 h.

2.2.2. Ultrasound-assisted extraction

For the UAE procedure, defatted flour was first dispersed in distilled water, and the pH of the solution was adjusted to the selected alkaline value using a 0.1 and 1 M NaOH solution. The mixture was then subjected to ultrasound treatment using a Sonic Ruptor 400 ultrasonic processor (Omni International, USA), equipped with a 13 mm titanium probe operating at 20 kHz. The extraction was performed at 25 °C in a temperature-controlled water bath.

After ultrasound application, the pH was readjusted to the alkaline value, and the mixture was centrifuged at 3000 ×g for 15 min. The supernatant was filtered to remove solid residues, concentrating the solubilized proteins. Protein precipitation followed the same procedure described for alkaline extraction (Section 2.3.1). All UAE extractions were performed with a solvent volume of 50 mL for standardization and reproducibility.

2.3. Optimization of ultrasound-assisted extraction

2.3.1. Determination of acoustic energy transmitted by ultrasound

Evaluating the acoustic energy absorbed in liquids presents a challenge in processes involving ultrasound application. While the electrical energy consumed by the ultrasound generator is relatively easy to determine, this measure does not directly reflect the amount of energy effectively absorbed by the medium. Therefore, it is essential to determine the real absorbed energy to evaluate the process efficiency (Margulis & Margulis, 2005). In turn, the acoustic power (AP), energy efficiency (EE), ultrasonic intensity (UI), and acoustic energy density (AED) transmitted by ultrasound were determined before optimizing the ultrasound-assisted extraction parameters using the calorimetric method described by Tiwari (2015). This method calculates the acoustic power transmitted to the solution from the temperature increase caused by ultrasound, as expressed in Eq. 1. Distilled water was used as the medium for power determination, which was measured in increments of 20 % of nominal power, up to a maximum of 100 %.

In Eq. 1, dT/dt was calculated by measuring the temperature of the medium at 5-s intervals over a total period of 90 s. These measurements were taken using a digital thermometer (SH 113, J. Prolab®, Brazil). Cp represented the specific heat of water (4.184 kJ kg^{-1} K^{-1}), m was the mass of water used (0.05 kg), and AP was the ultrasonic acoustic power found (W). Cp and m were assumed constant over the temperature range of the experiment. EE was calculated by dividing AP by the nominal power of the ultrasonic equipment (NP), as shown in Eq. 2. UI was calculated by dividing AP by the area of the transducer's emitting face (A = 1.13 cm^2), as shown in Eq. 3. AED was determined by dividing AP by the sample volume (V = 50 cm^3), as shown in Eq. 4.

$$AP = mCp[dT/dt] \quad (1)$$

$$EE = AP/NP \quad (2)$$

$$UI = AP/A \quad (3)$$

$$AED = AP/V \quad (4)$$

2.3.2. Experimental design

For the optimization of ultrasound treatment, the solid-to-liquid ratio (g/mL) and ultrasound application power (W) were optimized to increase the yield (%) and the amount of recovered protein (%) in the final product. This optimization process was conducted using a central composite design (CCD) strategy, employing the MINITAB® statistical program. The ultrasound power of each experiment followed the acoustic power results, while the solid-to-solvent ratio had a minimum value of 1/12 and a maximum value of 1/6. The ultrasound application time for this step was fixed at 10 min.

The protein isolate yield was calculated according to Eq. 5, where Mdf represents the mass of DPAF, Mip the mass of the protein isolate, and Y the percentage yield of the process. The recovered protein percentage refers to the protein fraction present in DPAF retained in the

final isolate after extraction, as described in Eq. 6, where Mpi corresponds to the mass of proteins in the isolate, Mpf to the mass of proteins in DPAF, and RP to the recovered protein percentage. Protein recovery is a key indicator of process efficiency, reflecting the method's ability to preserve proteins. Protein concentration was determined using the Kjeldahl method, following the methodology described by AOAC (Association of Official Analytical Chemists) (2000).

$$Y = \frac{Mip}{Mdf} * 100 \quad (5)$$

$$RP = \frac{Mpi}{Mpf} * 100 \quad (6)$$

Next, time intervals of 5, 10, and 15 min were tested to investigate the influence of ultrasound application time on protein extraction after the CCD experiments. These tests were conducted using the optimized parameters of solid-to-solvent ratio and acoustic power. The best extraction time was selected by comparing the yield (%) and recovered proteins (%) of the protein isolate obtained at each time interval. Additionally, a comparison of the yields and protein recovery with the alkaline extraction (AE) method was performed.

2.4. Effect of extraction methods on functional properties of pequi protein isolates

2.4.1. Proximate composition analysis

The protein isolates obtained from both alkaline extraction (AE) and ultrasound-assisted extraction (UAE), using optimized parameters, were subjected to proximate composition characterization following the standard procedures described by AOAC (Association of Official Analytical Chemists) (2000). Moisture was determined by oven-drying at 105 °C until constant weight. Ash content was determined by incinerating the dried samples at 525 °C in a muffle for 6 h. The crude protein content was measured by the Kjeldahl method, using the value 5.18 as the nitrogen conversion factor, which is the standard established for almonds (AOAC (Association of Official Analytical Chemists), 2000). Crude fat content was determined using Soxhlet extraction with petroleum ether as the solvent, at 60 °C for 6 h. Carbohydrate + fiber content was calculated by difference. The proximate composition of DPAF was also analyzed and compared with the protein isolates, enabling evaluation of the efficiency between both extraction processes.

2.4.2. Protein solubility of the DPAF and Pequi protein isolates

The solubility of proteins from DPAF was evaluated to determine the optimal pH values for the AE and UAE processes; more specifically, the alkaline pH at which maximum solubilization occurs and the isoelectric point at which solubility is minimized. Identifying these pH values is crucial for optimizing both the extraction and protein recovery steps, thereby enhancing the process efficiency.

The methodology was based on the procedure described by Bera and Mukherjee (1989), with adaptations. DPAF dispersions (100 g/L) were prepared, and the pH was adjusted between 2 and 10 using 0.1 and 1 M HCl or 0.1 and 1 M NaOH. Samples were shaken at 25 °C for 2 h in an orbital shaker to ensure proper protein solubilization. Subsequently, they were centrifuged at 3000 ×*g* for 15 min; this duration was selected to allow for complete sedimentation of insoluble material and clear separation of the supernatant. The protein concentration in the supernatant was determined using the Bradford (1976), with bovine serum albumin (BSA) as the standard. Protein solubility at each pH was calculated using Eq. 7. Absorbance was measured at 595 nm with a UV–Vis spectrophotometer (UV-M51, Bel Photonics®, Brazil).

$$Protein\ Solubility\ (\%) = \frac{Total\ Solubilized\ Protein}{Total\ Protein} * 100 \quad (7)$$

The same procedure was applied to evaluate the solubility of the pequi protein isolates, using dispersions at a concentration of 10 g/L. The total protein content used for the solubility calculation was determined by the Kjeldahl method, as reported in section "3.3.1 Proximate Composition" for both DPAF and the protein isolates.

2.4.3. Least gelation concentration

Gelation capacity was assessed by determining the least gelation concentration (LGC), following the method described by Ghribi et al. (2015), with modifications. Protein isolate dispersions at concentrations ranging from 2 to 20 % (*w/v*) were prepared using either distilled water or Tris-HCl buffer (pH 8.5), and homogenized with a vortex mixer for 1 min. The samples were heated in a water bath at 100 °C for 60 min, then immediately cooled in an ice bath and stored at 4 °C for 2 h. After refrigeration, the tubes were inverted to evaluate gel formation. The LGC was defined as the lowest concentration at which the sample did not flow upon inversion.

2.4.4. Emulsifying activity and emulsion stability

Emulsifying activity (EA) and emulsion stability (ES) were determined based on a modified method described by Yasumatsu et al. (1972), by evaluating the proportion of the separated phases after centrifugation. To determine EA, 0.5 g of protein isolate was suspended in 7.5 mL of distilled water, followed by the addition of 7.5 mL of soybean oil. The mixture was homogenized using an Ultra-Turrax (IKA® Ultra Turrax® Tube Drive) at 20000 rpm for 2 min to form an emulsion. The emulsions were transferred to tubes (11.8 cm in height, 1.5 cm inner diameter) and centrifuged at 750 ×*g* for 5 min. EA was calculated as the percentage of the emulsified layer (top phase) relative to the total sample height, according to eq. 8, where He is the height of the emulsified layer (cm), and Ht is the total height of the sample (cm).

$$EA\ or\ ES\ (\%) = \frac{He}{Ht} * 100 \quad (8)$$

For ES, emulsions were prepared following the same procedure. After preparation, the emulsions were subjected to heat treatment in a thermostatic bath at 80 °C for 30 min and subsequently cooled to room temperature. Emulsions were transferred again to tubes of the same dimensions and centrifuged under the same conditions. ES was then calculated using the same eq. 8, representing the emulsion's thermal stability based on its resistance to phase separation after heating.

2.4.5. Water and oil absorption capacity

Water absorption capacity (WAC) was evaluated following the method of Brishti et al. (2020), with adaptations. One gram of protein isolate was dispersed in 10 mL of distilled water and the mixture was homogenized in a vortex mixer for 5 min. The sample was then centrifuged at 3000 ×*g* for 20 min in pre-weighed centrifuge tubes. The supernatant was discarded after centrifugation, and the tubes containing the sediment were weighed again to determine WAC.

Oil absorption capacity (OAC) was determined following the method of Brishti et al. (2020), also with modifications. One gram of the sample was mixed with 10 mL of soybean oil and homogenized in a vortex mixer for 5 min. The sample was then centrifuged at 3000 ×*g* for 20 min in pre-weighed centrifuge tubes. The supernatant was then discarded, and the tubes were weighed again. WAC and OAC were calculated according to Eq. 8, where W2 represents the weight of the tube with the sediment (g), W1 the weight of the tube with the dry sample (g), and W0 the weight of the dry sample (g). The results are expressed in grams of water/oil absorbed per gram of protein isolate.

$$Water \Big/ Oil\ absorption\ capacity\ \left(\frac{g}{g}\right) = \frac{W2 - W1}{W0} \quad (9)$$

2.4.6. Foaming capacity and foam stability

The foaming capacity (FC) and foam stability (FS) were evaluated following the method described by Saricaoglu (2020), with modifications. First, protein isolate dispersions (15 g/L) were prepared in

distilled water with the pH adjusted to 7.0. Samples of 15 mL of these dispersions were agitated at 11000 rpm for 2 min using an Ultra-Turrax homogenizer (NT 138, Novatecnica®, Brazil). The solutions were transferred to 50 mL graduated cylinders, and the total volume was recorded as V0. The mixtures were then left to stand at room temperature for 60 min, and the volume was recorded again (V60) to determine foam stability. FC was expressed as the percentage increase in foam volume after agitation, while FS was calculated based on the remaining foam volume after resting intervals. FC and FS calculations were performed according to Eqs. 9 and 10, with V0 as the total suspension volume after agitation, V1 as the initial suspension volume, and V60 as the foam volume after 60 min of rest.

$$FC\ (\%) = \frac{V0 - V1}{V1} * 100 \quad (10)$$

$$FS\ (\%) = \frac{V60 - V1}{V0 - V1} * 100 \quad (11)$$

2.5. Statistical analyses

The data obtained from the experimental design were analyzed and interpreted using the MINITAB 18 statistical program (Mini Tab Inc., USA). All other analytical determinations were performed in triplicate and subjected to analysis of variance (ANOVA) with a confidence level of 5 %. Tukey's multiple comparison test was employed to compare means when treatment effects were significant in the ANOVA.

3. Results and discussion

3.1. Protein solubility of DPAF

According to the solubility curve of DPAF (Fig. 1), it was observed that the proteins exhibited the lowest solubility indices at pHs 4, 4.8, and 5, with values close to 18 %, with the minimum solubility recorded at pH 4.8 (17.3 %). There was a progressive increase in solubility above this range, reaching maximum values at pHs 9.5 (80.4 %) and 10 (83.2 %). This behavior in response to pH is consistent with that observed for other plant proteins, such as pea, soy and rice proteins, characterized by reduced solubility at the isoelectric point, typically located around pH 4.8 (Zhao et al., 2020).

pH 9.5 was selected for the protein extraction steps given the potential adverse effects of elevated pH on the integrity of essential amino acids (Zhang et al., 2020) and the absence of significant differences at pH 10. This pH was chosen to maximize protein solubility while minimizing the risk of nutritional degradation. Moreover, pH 4.8 was selected as the point of minimal protein solubility for isoelectric precipitation, maximizing protein isolate recovery.

3.2. Ultrasound-assisted extraction

3.2.1. Calculation of ultrasound acoustic power

Table 1 presents the relationship between nominal power and acoustic power (AP), alongside data on energy conversion efficiency (EE), ultrasonic intensity (UI), and acoustic energy density (AED) at different ultrasound amplitudes. The conversion efficiency ranged from 8.91 to 19.33 %, with the lowest and highest values recorded at 160 W

Table 1
Acoustic power (W) as a function of nominal power (in percentage) of the equipment.

Nominal power (W)	dT/dt (°C/s)	AP (W)	EE (%)	UI (W/cm^2)	AED (W/cm^3)
80 (20 %)	0.026 ± 0.0006	8.64	10.79	7.64	0.17
160 (40 %)	0.047 ± 0.0008	14.26	8.91	12.62	0.29
240 (60 %)	0.100 ± 0.0006	33.24	13.85	29.41	0.67
320 (80 %)	0.171 ± 0.0021	57.35	17.92	50.75	1.15
400 (100 %)	0.231 ± 0.0101	77.32	19.33	68.42	1.55

Notes: Values are presented as means (n = 3) ± standard deviation.

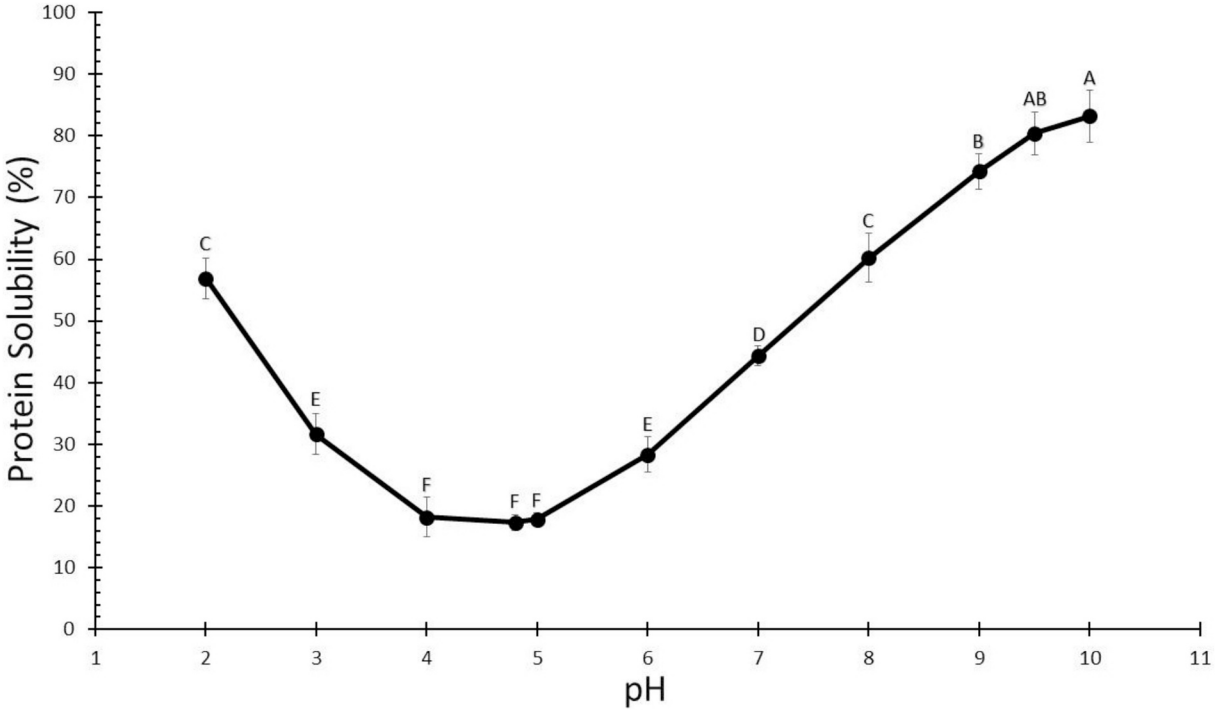

Fig. 1. Solubility curve of the defatted pequi almond flour (DPAF) proteins. Data are presented as means ± standard deviation ($n = 3$), and means with different letters are significantly different (Tukey's test, $p < 0.05$).

and 400 W, respectively. These results align with previous studies, which show that conversion efficiency is moderate in ultrasonic systems, being affected by wave attenuation in the liquid medium (Son et al., 2011). The efficiency of 19.33 % observed in this study is comparable to values found in the literature, such as Contamine et al. (1995), who reported 26 % under similar conditions. Although increasing nominal power favors conversion, phenomena such as acoustic attenuation and streaming limit efficiency at lower amplitudes.

The UI ranged from 7.64 to 68.42 W/cm^2, and the AED oscillated between 0.17 and 1.55 W/cm^3, both reaching maximum values at 400 W (Table 1). These parameters are fundamental for estimating the energy introduced into the system, which is critical for experimental design. Moreover, they can serve as valuable guidelines to optimize future extraction processes and industrial scaling of the extraction system (Tiwari, 2015).

3.2.2. Experimental design

A CCD was employed with two factors to assess the influence of the solid-to-solvent ratio (g/ml) and ultrasonic acoustic power (W) on yield (%) and protein recovery (%) of the final product in order to optimize the process. Acoustic power values corresponding to 40 % and 80 % amplitude of the ultrasound were used as minimum and maximum for the CCD. The adequacy of the model equations was evaluated by the coefficient of determination (R^2), and the significance of each term in the equation was assessed based on *p*-values, with values below 0.05 indicating the significance of the term. The yield and protein recovery results for the 13 extractions are presented in Table 2.

The highest yield (46.15 %) and protein recovery (81.88 %) were obtained in experiment 9, which used a power of 57.35 W and a solid-to-solvent ratio of 1/12 (g/ml). In contrast, the lowest yield (29.01 %) and protein recovery (49.06 %) were observed in experiment 10, conducted with a power of 14.26 W and a solid-to-solvent ratio of 1/6 (g/ml). The regression equations for yield and protein recovery are shown in Eqs. 11 and 12, where Y1 is yield, Y2 is protein recovery, X1 is the solid-to-solvent ratio, and X2 is the acoustic power.

$$Y1 = 19,90 + 71X1 + 0,728X2 - 240X1^2 - 0,00411X2^2 - 1,423X1.X2 \quad (12)$$

$$Y2 = 38,9 + 86X1 + 1,243X2 - 385X1^2 - 0,00748X2^2 - 2,06X1.X2 \quad (13)$$

The analysis of variance and determination coefficients (R^2) for the second-order polynomial model applied to extraction yield and protein recovery showed good data fit, with R^2 values of 0.93 and 0.92, respectively (Table 3). Both results indicate that the fitted model explains the observed experimental variation well. Notably, both the linear and quadratic terms of acoustic power significantly influenced both extraction yield and protein recovery ($P < 0.05$), highlighting the critical role of this parameter in the extraction process.

Table 2
Central Composite Design (CCD) to optimize ultrasound-assisted protein extraction, including experimental variables and responses.

Experiment	X1	X2	Y1	Y2
1	1 / 8	35.80	39.05	70.08
2	1 / 8	35.80	37.70	66.26
3	1 / 8	5.34	29.97	53.52
4	1 / 8	35.80	38.64	68.25
5	1 / 8	35.80	41.39	71.41
6	1 / 6	57.35	39.29	69.22
7	1 / 18	35.80	41.09	73.23
8	1 / 8	66.27	42.95	74.61
9	1 / 12	57.35	46.15	81.88
10	1 / 6	14.26	29.01	49.06
11	1 / 5.43	35.80	37.76	66.13
12	1 / 8	35.80	40.44	70.93
13	1 / 12	14.26	30.76	54.32

Variables: X1 = *Solid-to-solvent ratio (g/mL), X2 = Acoustic power (W).*
Responses: Y1 = *Yield (%), Y2 = Protein recovery (%).*

The sum of squares for extraction yield was 297.15, with acoustic power being the most influential factor, showing significance in both its linear ($P = 0.000$) and quadratic effects ($P = 0.026$). The solid-to-solvent ratio also had a significant impact on yield ($P = 0.034$), while the interaction between the solid-to-solvent ratio and acoustic power was not significant ($P = 0.197$). The absence of significant lack of fit ($P > 0.05$) indicated that the model adequately described the experimental data. Similarly, protein recovery was primarily influenced by acoustic power, with significant effects in both the linear (P = 0.000) and quadratic ($P = 0.035$) terms. The solid-to-solvent ratio was again significant (P = 0.026), while the interaction between the solid-to-solvent ratio and acoustic power was not significant ($P = 0.329$). The absence of significant lack of fit for this variable (P > 0.05) further confirmed the model's precision.

According to the contour plots (Fig. 2), the optimal conditions for maximizing extraction yield and protein recovery occurred between 50 and 66 W of acoustic power and solid-to-solvent ratio of 1/15.3 to 1/9.6 (Fig. 2a) and 1/15.3 to 1/10.5 (Fig. 2b), respectively. Increasing power and reducing the solid-to-solvent ratio favored both parameters.

Using the MINITAB® response optimization function, the software indicated that the ideal conditions for maximizing extraction were 56 W of acoustic power and a solid-to-solvent ratio of 1/15.13 (w/v), with predicted yields of 46.37 % and protein recovery of 81.86 %. The UAE performed under these optimized conditions resulted in an average yield of 45.22 % and protein recovery of 79.77 %, constituting values close to those predicted by the software, and thereby confirming the adequacy of the statistical model used.

Additional experiments were conducted after optimizing the ultrasonic power and solid-to-solvent ratio to evaluate the impact of extraction time (5, 10, and 15 min) on yield and protein recovery. The results are presented in Table 4, and show that both parameters increased progressively with longer sonication times, reaching maximum values at 15 min. Unlike other plant matrices, which may exhibit decreased efficiency with prolonged ultrasound exposure, no reduction in protein recovery was observed within the tested range. Sonication time can influence protein extraction outcomes in different ways: while moderate durations promote cell disruption and compound diffusion, excessive treatment may induce protein aggregation, denaturation, or reduced cavitational intensity (Das et al., 2022). Although this study did not evaluate the structural integrity of the extracted proteins, the absence of negative effects on recovery suggests that 15 min of ultrasound was an effective condition for this process. The impact of longer exposure times on protein structure and functionality should be addressed in future studies.

Based on these findings, a comparison between alkaline extraction and ultrasound-assisted extraction clearly demonstrates the superior performance of ultrasound at all evaluated times (Table 4). Alkaline extraction yielded 41.35 % with a protein recovery of 70.18 %. In contrast, ultrasound resulted in higher yields even at shorter extraction times: 45.02 % in 5 min, 45.22 % in 10 min, and 47.25 % in 15 min, with protein recovery ranging from 79.77 % to 84.28 %. These results indicate that ultrasound not only increased yield by up to 14.3 % and protein recovery by up to 18.0 %, but also significantly reduced processing time, highlighting its high efficiency for protein extraction.

This enhanced performance is largely attributed to the physical and chemical effects induced by high-intensity ultrasound (HIUS), typically operating at frequencies between 20 and 100 kHz. Acoustic cavitation during sonication generates extreme local conditions, with pressures of up to 50 MPa and temperatures approaching 5000 K, resulting in turbulence, microjets, and shock waves (Das et al., 2022). These phenomena lead to cell wall disruption, pore formation, surface erosion, and increased contact area between the solvent and biomass, thereby enhancing solvent penetration, diffusion, and intracellular protein release. Ultrasound may also induce structural changes, such as protein

Table 3
Regression coefficient estimates for yield (%) and protein recovery (%) responses (X1: solid-to-solvent ratio and X2: acoustic power).

Source	Yield (%)					Protein Recovery (%)				
	Sum of squares	DF	Mean square	F	P	Sum of squares	DF	Mean square	F	P
Model	297.15	5	59.43	18.54	0.001*	947.22	5	189.44	15.25	0.001*
Linear										
X_1	22.18	1	22.18	6.92	0.034*	97.63	1	97.63	7.86	0.026*
X_2	242.92	1	242.92	75.79	0.000*	751.67	1	751.67	60.5	0.000*
Quadratic										
$X_1.X_1$	1.20	1	1.20	0.38	0.559	3.10	1	3.10	0.25	0.632
$X_2.X_2$	25.34	1	25.34	7.91	0.026*	83.91	1	83.91	6.75	0.035*
Interaction										
$X_1.X_2$	6.53	1	6.53	2.04	0.197	13.69	1	13.69	1.1	0.329
Residual	22.44	7	3.21	–	–	86.96	7	12.42	–	–
Lack of fit	13.80	3	4.60	2.13	0.239	68.94	3	22.98	5.1	0.075
Pure error	8.64	4	2.16	–	–	18.02	4	4.51	–	–
Cor total	319.59	12	–	–	–	1034.18	12	–	–	–
	$R^2 = 0.9298$					$R^2 = 0.9159$				

Notes: *Indicates significance at $p \leq 0.05$.

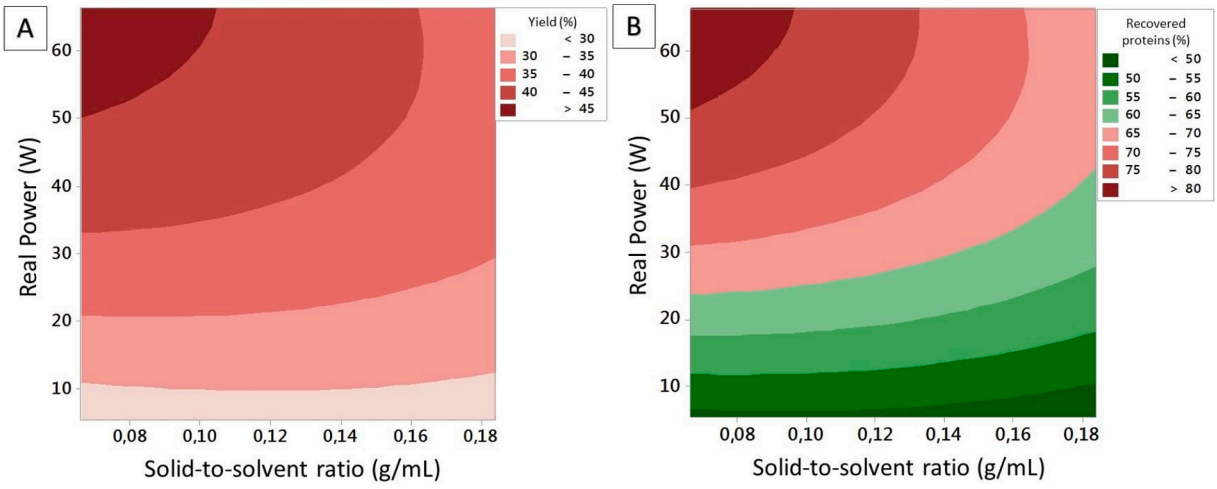

Fig. 2. Contour plots of (a) yield and (b) protein recovery as a function of acoustic power and solid-to-solvent ratio.

Table 4
Comparison of yield and protein recovery between alkaline extraction (AE) and ultrasound-assisted extraction (UAE) at different ultrasound application times.

Extraction Method	Ultrasonic Extraction Time (min)	Yield (%)	Protein Recovery (%)
AE	–	41.35 ± 0.68^a	70.18 ± 1.66^a
UAE	5	45.02 ± 0.82^b	80.01 ± 1.49^b
	10	45.22 ± 0.64^b	79.77 ± 1.11^b
	15	47.25 ± 0.87^c	84.28 ± 1.56^c

Notes: Values are means (n = 3) ± standard deviation. Different letters in the same column indicate significant differences (Tukey's test, $P < 0.05$).

unfolding, further improving extraction efficiency (Tiwari, 2015).

Moreover, at the molecular level, ultrasonic cavitation promotes the disruption of non-covalent interactions, such as hydrogen bonds, hydrophobic interactions, and van der Waals forces that stabilize protein aggregates. This disruption facilitates the disaggregation of peptide complexes and may alter the functional properties of proteins (Wang et al., 2020). The shear forces and localized turbulence generated by the collapse of cavitation bubbles contribute to the fragmentation of large molecular assemblies into smaller and more soluble peptide fractions, often with molecular masses below 1 kDa. In some cases, these effects may also induce conformational changes in secondary and tertiary structures, exposing reactive or hydrophilic groups. Although peptide bond cleavage and alterations in the primary structure remain debatable, the physical effects of ultrasound clearly promote structural reorganization, enhancing protein recovery and functional applicability (Tian et al., 2020).

Such effects make ultrasound-assisted extraction (UAE) a particularly effective strategy for structurally complex plant matrices. Wang et al. (2020) optimized UAE for pea proteins, achieving 82.6 % recovery, while traditional alkaline extraction only yielded 71.6 %. Similarly, Sert et al. (2022) applied UAE to pumpkin seed cake and improved protein recovery from 43.6 % to 57.8 %. These improvements are mainly associated with disruption of protein–polysaccharide complexes and increased protein solubility due to the exposure of hydrophilic residues (Su & Cavaco-Paulo, 2021). Given that pequi almond cake has a high fiber content and dense cell walls (Holland et al., 2020), UAE stands out as a promising approach to maximize protein yield and recovery from this agro-industrial by-product.

3.3. Effect of extraction methods on functional properties of protein isolates

3.3.1. Proximate composition

Table 5 presents the proximate compositions of DPAF and the protein isolates produced via AE and UAE. The protein percentage in the isolates is significantly higher than that of the defatted flour. DPAF exhibited a protein content of 46.45 %, while the AE-derived isolate had 78.82 %, representing an increase of approximately 69.7 % compared to the flour. The UAE-derived isolate exhibited a protein content of 82.84 %, corresponding to an increase of about 78.3 % compared to DPAF, indicating the efficiency of the extraction processes in concentrating proteins. Both isolates show significantly lower carbohydrate content than the flour, suggesting a relative concentration of proteins compared to carbohydrates during the extraction processes.

When comparing the two extraction processes, UAE produced isolates with significantly lower moisture and lipid contents than AE. This result can be attributed to the effect of acoustic cavitation generated during ultrasound, improving overall extraction capabilities. Ultrasonic waves during cavitation cause the formation and subsequent collapse of bubbles in the liquid medium, resulting in high-energy microjets. This phenomenon facilitates the rupture of cellular membranes and the release of intracellular compounds, promoting more effective removal of lipids during extraction (Deng et al., 2022). The reduction of residual lipids is particularly relevant, as these compounds can negatively affect functional properties such as foaming and emulsion stability by competing with proteins at the interface and hindering the formation of stable structures (Lazdiņa et al., 2025). Regarding moisture, cavitation may increase water removal efficiency during centrifugation and drying due to the breakdown of cellular structures, leading to a concentrate with lower moisture content.

3.3.2. Protein solubility of the isolates

Solubility is one of the most critical functionalities of proteins, as other properties such as emulsification, foaming, and gelling are generally closely linked to it (Kang et al., 2020). Therefore, the solubility of protein isolates obtained through alkaline extraction (AE) and ultrasound-assisted extraction (UAE) was evaluated across a pH range of 2–10. As shown in Fig. 3, both isolates exhibited similar solubility profiles, with no significant differences throughout the pH spectrum.

The lowest solubility was observed at pH 5, with values approaching zero. This result is expected, as it is close to the isoelectric point (pH 4.8), where the net protein charge is minimal, promoting intermolecular aggregation and consequently reducing solubility. Similar to DPAF, solubility increased under both acidic and alkaline conditions. The highest solubility values were recorded at pH 10, reaching 95.23 % for AE and 97.40 % for UAE. At pH levels far from the isoelectric point, proteins carry either a positive or negative net charge, leading to electrostatic repulsion, reduced aggregation, and improved solubility (Bessada et al., 2019).

Despite the similarity in solubility profiles between the isolates, this result may reflect a balance between the opposing effects induced by ultrasonic cavitation. On one hand, ultrasound can disrupt aggregates, reduce particle size, and expose hydrophilic groups, thereby enhancing solubility. On the other hand, when applied at high intensity or for prolonged periods, it may lead to excessive denaturation and the formation of insoluble aggregates (Rahman & Lamsal, 2021). Furthermore, although solubility is a prerequisite for various functional properties, it is not the sole determinant of protein performance in applications such as foaming, emulsification, or oil and water absorption. In this study, despite comparable solubility levels, the UAE and AE isolates exhibited distinct functional behaviors, particularly in foaming capacity and oil absorption. These differences may be attributed to variations in molecular characteristics, including surface hydrophobicity, molecular flexibility, conformational structure, and particle size, which directly influence interfacial activity and ultimately affect the functional properties of the isolates (Kang et al., 2020).

Table 5
Proximate composition of defatted flour and protein isolates obtained by alkaline extraction (AE) and ultrasound-assisted extraction (UAE).

Determination (%)	Defatted Flour	Protein Isolate (AE)	Protein Isolate (UAE)
Moisture	8.50 ± 0.15^{a}	6.64 ± 0.10^{b}	4.12 ± 0.57^{c}
Ash	3.52 ± 0.29^{a}	1.76 ± 1.24^{b}	1.93 ± 0.09^{b}
Lipid	2.19 ± 0.45^{a}	2.03 ± 0.26^{a}	0.40 ± 0.13^{b}
Protein	46.45 ± 0.33^{a}	78.82 ± 0.87^{b}	82.84 ± 3.26^{b}
Carbohydrate	38.89 ± 0.75^{a}	9.71 ± 0.90^{b}	10.90 ± 3.11^{b}

Notes: Values are presented as means ($n = 3$) ± standard deviation. Different letters in the rows indicate significant differences (Tukey's test, $p < 0.05$).

3.3.3. Least gelation concentration

Neither of the protein isolates obtained by AE or UAE exhibited gel-forming ability at the tested concentrations (2 to 20 %, *w/v*), whether dissolved in distilled water or in Tris-HCl buffer (pH 8.5). These results indicate that the applied conditions were insufficient to induce gelation. Protein gel formation typically occurs through protein unfolding and aggregation, constituting a process in which heating exposes reactive groups that form intermolecular bonds with neighboring proteins, leading to a three-dimensional network responsible for gel structure (Hettiarachchy et al., 2013).

However, according to Silvestrini et al. (2017), pequi almonds are predominantly composed of 2S albumins, which are highly compact proteins rich in disulfide bonds, conferring high resistance to thermal denaturation. This characteristic limits the unfolding necessary for protein network formation, which may have contributed to the lack of gelation observed in the isolates. Furthermore, the low solubility of the isolates at higher concentrations may have limited the availability of proteins in the dispersed phase, compromising gel network development. Therefore, strategies which enhance protein solubility may be required to fully explore the gelling potential of these materials.

3.3.4. Water and oil absorption capacities

Water absorption capacity (WAC) and oil absorption capacity (OAC) represent the amount of water or oil that 1 g of sample can absorb. The ability of protein molecules to associate with water or oil molecules without dissolving is of great importance for the texture and quality of food products (Qadir & Wani, 2023). Water or oil can be absorbed by proteins through two different mechanisms. The first involves the binding of protein molecules to water or oil molecules, which not only depends on the hydrophilic or hydrophobic nature of the proteins but also on the exposure of these groups to the surrounding environment.

The second mechanism is the physical entrapment of liquid molecules within the three-dimensional structure of the proteins (Wouters et al., 2016). As shown in Table 6, no significant difference was observed in oil absorption capacity between the isolates obtained by AE (1.23 g/g) and UAE (1.27 g/g). However, WAC showed a significant difference between the proteins obtained by UAE (1.41 g/g) and AE (0.88 g/g). This suggests that protein denaturation may have occurred, exposing hydrophilic sites, which allowed water molecules to bind to the proteins through hydrogen bonds (Qadir & Wani, 2023).

3.3.5. Emulsifying activity and emulsion stability

The emulsifying properties of proteins can be attributed to their amphiphilic nature, meaning they exhibit both hydrophilic and hydrophobic characteristics, allowing them to form a thin film around oil droplets dispersed in water, thereby preventing structural alterations (Argel et al., 2020). The surface charge of protein-coated oil droplets can vary depending on whether the pH of the emulsion is above or below the protein's isoelectric point. Electrostatic repulsions between droplets contributed to emulsion stability, while pH values near the isoelectric point promote aggregation and coalescence, leading to instability (Lam

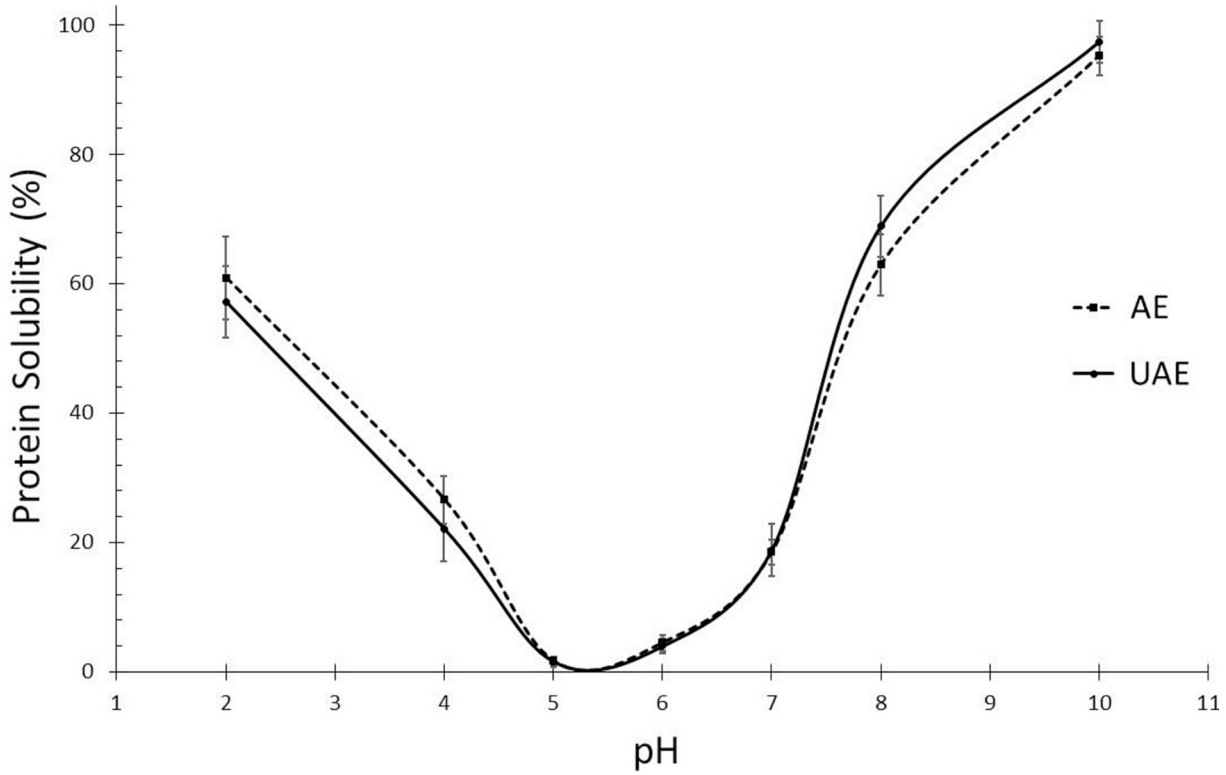

Fig. 3. Solubility curve of protein isolates obtained by alkaline extraction (AE) and ultrasound-assisted extraction (UAE).

Table 6
Functional properties of pequi almond protein isolates obtained by AE and UAE.

	AE	UAE
EA (%)	41.89 ± 1.51[a]	41.89 ± 0.19[a]
ES (%)	40.61 ± 0.20[a]	40.47 ± 0.31[a]
WAC (g/g)	0.88 ± 0.04[a]	1.41 ± 0.09[b]
OAC (g/g)	1.23 ± 0.02[a]	1.27 ± 0.14[a]
FC (%)	7.23 ± 4.39[a]	23.33 ± 5.77[b]
FS (%)	43.33 ± 11.55[a]	75.00 ± 12.50[b]

Notes: Values are presented as means ($n = 3$) ± standard deviation. Different letters in the rows indicate significant differences (Tukey's test, $p < 0.05$).

& Nickerson, 2013).

As shown in Table 6, emulsifying activity and emulsion stability had values of 41.89 % and 40.61 % for AE, and 41.89 % and 40.47 % for UAE, respectively. These results indicate no significant differences between extraction methods regarding emulsifying activity and emulsion stability. Although this outcome is somewhat unexpected considering the pronounced differences observed in other functional parameters, two factors may help explain this behavior.

First, according to Kanterewicz et al. (1987), optimal emulsifying capacity is generally achieved when water absorption is approximately twice that of oil absorption. In the present study, the oil absorption capacity did not differ significantly between the extraction methods, while the water absorption was notably higher in UAE isolates, resulting in a higher WAC/OAC ratio (1.11 vs. 0.72). This suggests enhanced hydrophilic interaction potential. However, this increased water affinity may not have reached a functional threshold sufficient to improve emulsification, or it may have been counterbalanced by other structural changes.

Secondly, as reported by Shokri et al. (2022), ultrasound treatment can partially unfold proteins, reducing particle size and increasing surface hydrophobicity, thereby enhancing interfacial activity. Yet, under certain conditions, this structural modification may also promote reaggregation of unfolded proteins via hydrophobic interactions. Such reaggregation could reduce protein mobility and impair the formation of stable interfacial films, ultimately offsetting potential emulsifying improvements. Therefore, the lack of difference in EA and ES among the two isolates may result from a balance between enhanced water absorption capacity and structural reorganization effects that limit emulsifying performance.

3.3.6. *Foaming capacity and stability*

Foams are gas dispersions in a continuous phase, and in food systems they are typically air bubbles dispersed in water. The interface between water and air is thermodynamically unstable, as interactions between water molecules are more favorable than those between water and air, leading to rapid foam collapse (Wouters et al., 2016). Two main mechanisms contribute to foam destabilization: disproportionation, which refers to the disappearance of smaller air bubbles due to pressure gradients, causing diffusion into larger bubbles, and coalescence, where bubbles merge due to drainage of the liquid film that separates them (Qadir & Wani, 2023; Wouters et al., 2016).

Foam formation occurs when proteins adsorb at the air-water interface and undergo structural rearrangements. These changes reduce surface tension, stabilizing the air bubbles and preventing their collapse (Qadir & Wani, 2023; Tan et al., 2015).

Protein isolates obtained by UAE exhibited higher foaming capacity and stability compared to those extracted by AE (Table 6). This difference suggests that ultrasound-assisted extraction may have altered the proteins' three-dimensional structure, increasing the exposure of hydrophilic and hydrophobic regions. These exposed residues may enhance electrostatic repulsion between the proteins at the interface, preventing bubble coalescence. The resulting reduction in surface tension facilitates air entrapment, promoting both foam formation and stability (Tan et al., 2015; Wouters et al., 2016). Additionally, polar lipids can interfere with the formation and stabilization of foams by disrupting protein adsorption at the air-water interface (Lazdiņa et al., 2025). Consequently, the lower lipid content in the UAE isolates may

contribute to their enhanced foaming properties.

Furthermore, ultrasonic treatment has been shown to induce partial protein unfolding, increasing the exposure of hydrophobic and hydrophilic residues and enhancing protein flexibility. These modifications improve the protein adsorption rate and efficiency at the air-water interface and result in more cohesive interfacial films, improving both foam formation and resistance to coalescence and disproportionation (Shokri et al., 2022).

4. Conclusion

This study demonstrated the effectiveness of UAE as an efficient alternative to the conventional AE method for obtaining protein isolates from DPAF. UAE resulted in higher yields (47.25 %) and protein recovery (84.28 %) while significantly reducing processing time compared to AE, underscoring its viability as a sustainable and efficient technology.

The chosen extraction parameters at the end of the optimization process were pH 9.5 for the protein solubilization step and pH 4.8 for the isoelectric precipitation step. The optimal conditions for ultrasound parameters included an acoustic power of 56 W, a solid-to-solvent ratio of 1/15.13, and a 15-min ultrasound application time. The experimental validation confirmed the robustness of the statistical model, with results close to those predicted by the optimization software.

Based on the improved technological properties of the UAE-derived isolates, their application in food formulations that require functional ingredients with water retention and stable foaming capabilities is particularly promising. These isolates could be effectively used in the development of meat analogues, where texture, juiciness, and moisture retention are essential attributes. Furthermore, their use in aerated systems such as mousses, chocolates, and beers may enhance foam stability and overall sensory quality.

Therefore, UAE emerges as a promising technology for the valorization of agro-industrial by-products such as DPAF, aligning with the United Nations Sustainable Development Goals by promoting more efficient and sustainable processes. The results of this study provide relevant insights for the development of new plant-based protein products and processes with potential applications in the food and biotechnology industries.

Future studies should further investigate the structural impacts of ultrasound on proteins through techniques such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), Fourier-transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM), in order to better understand conformational changes during extraction. Additionally, assessments of nutritional relevance, including amino acid profiling, glycoprotein detection, and bioactivity assays, could clarify possible degradation effects or functional modifications with relevance to human nutrition. Economic feasibility studies for scaling up UAE to industrial applications are also recommended.

CRediT authorship contribution statement

Felipe Pires Ribeiro: Writing – original draft, Validation, Investigation, Conceptualization, Writing – review & editing, Visualization, Methodology, Formal analysis. **Rafael Silva Naito:** Writing – review & editing, Validation, Formal analysis, Writing – original draft, Investigation. **Beatriz dos Santos Galvão:** Writing – original draft, Writing – review & editing, Visualization. **Walter Fabri Júnior:** Writing – review & editing. **Kayu Kazuo Toyonaga:** Writing – review & editing. **Ivanise Guilherme Branco:** Resources. **Sungil Ferreira:** Supervision, Methodology, Writing – review & editing, Project administration, Conceptualization. **Cassia Roberta Malacrida:** Project administration, Conceptualization, Supervision, Writing – review & editing, Resources, Methodology.

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Declaration of competing interest

The authors declare hat they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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