Universidad de Valladolid

Protein extraction from shrimp shells using ultrasound for the development of new foods.

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Abstract

This study explores ultrasound-assisted extraction (UAE) as an eco-friendly method to recover proteins from shrimp by-products like shells and heads, typically discarded. UAE uses water as a solvent, avoiding traditional harmful chemicals. Ultrasound waves induce cavitation, breaking cell structures to release proteins. The study assesses key parameters—time, temperature, and solvent-to-feed ratio—on protein yield and efficiency. UAE yielded 2.6% to 4.26% of raw material, higher than 2.4% from non-sonicated methods, yet only 19.9% of total protein content was extracted, suggesting incomplete protein release. UAE shows promise as a sustainable alternative, enhancing yields with reduced environmental impact. Further research should optimize parameters and explore complementary techniques for maximal protein recovery from shrimp waste.

Keywords: Ultrasound-assisted extraction (UAE); Protein recovery: Shrimp by-products; Sustainable extraction; Cavitation.

Resumen

Este estudio explora la extracción asistida por ultrasonido (UAE) como un método ecológico para recuperar proteínas de subproductos de camarón, como cáscaras y cabezas, que suelen ser desechados. UAE utiliza agua como disolvente, evitando los químicos dañinos de los métodos tradicionales. Las ondas ultrasónicas inducen cavitación, rompiendo estructuras celulares y facilitando la liberación de proteínas. El estudio evalúa parámetros clave—tiempo, temperatura y relación disolvente-a-materia prima—en el rendimiento y eficiencia de extracción. UAE logró rendimientos de 2.6% a 4.26% del material crudo, superior al 2.4% obtenido con métodos no sonificados, aunque solo se extrajo el 19.9% del contenido total de proteínas, indicando una liberación incompleta. UAE muestra potencial como alternativa sostenible, mejorando rendimientos con menor impacto ambiental. Se recomienda investigar más para optimizar parámetros y explorar técnicas complementarias para maximizar la recuperación de proteínas de residuos de camarón.

Palabras clave: Extracción asistida por ultrasonido (EAU); Recuperación de proteínas; Subproductos de camarón; Extracción sostenible; Cavitación.

1. Introduction

The growing demand for sustainable and nutritious food sources has led researchers to explore unconventional protein extraction sources. Shrimp, widely consumed for its nutritional value, is obtained through both aquaculture and wild-capture fisheries. Aquaculture accounts for 63% of the global shrimp supply, playing a critical role in seafood production. In 2020, total shrimp production reached 5.03 million tons, with projections of 7.28 million tons by 2025. Despite its significant production volume, a large portion of shrimp by-products, including shells, heads, and other residues, are typically discarded. While these residues are commonly repurposed for chitin extraction, the valuable protein content is often overlooked and discarded (*Q1 2023 Jan-Mar | GLOBEFISH - Information and Analysis on World Fish Trade | Food and Agriculture Organization of the United Nations | GLOBEFISH | Food and Agriculture Organization of the United Nations*, n.d.; *Shrimp Market Size, Value, Price Trends, Report 2024-2032*, n.d.).

Typically, shrimp are preserved and shipped frozen, either with or without their shells. This processing generates approximately 55% residual material, including shells, viscera, heads, eyes, and legs. While some shrimp waste is used in animal and aquaculture feed, a significant portion is repurposed for the extraction of chitosan and chitin. However, the current method of extraction applies components as hydrochloric acid and sodium hydroxide not allowing the full utilization of the remaining valuable bioactive compounds and increase environmental pollution. Exploring alternative methods for extracting these compounds from shrimp waste could offer promising economic opportunities for the food industry and contribute a substantial environmental benefit by reducing waste highlighting the need for versatile and efficient extraction methods to maximize resource utilization and promote sustainability in food industry (Chandra Roy et al., 2021; Rebouças et al., 2023).

Efficient processing of shrimp waste can recover valuable compounds such as protein, flavor compounds, calcium carbonate, chitin, lipids, and astaxanthin. The primary constituents of shrimp waste are approximately 30% protein, 10% minerals and 30% of chitin. (Yek et al., 2022). These bioactive components have applications in various industries, including pharmaceuticals, agriculture, cosmetics, textiles, biology, and food (Olsen et al., 2014; Ruangwicha et al., 2024).

Traditional methods for utilizing shrimp waste often face challenges related to environmental impact, resource inefficiency, and high costs. Commonly used solvent extraction, which uses organic solvents to separate various components, can be effective

but often suffers from issues such as incomplete recovery of bioactive compounds and the environmental hazards associated with solvent use. Additionally, traditional methods such as enzymatic hydrolysis, while useful for breaking down proteins, can be costly and have variable effectiveness depending on the enzyme and process conditions. These practices highlight the need for more sustainable and efficient technologies to better utilize shrimp waste (Giraldo et al., 2024; Mao et al., 2017; Silva et al., 2024).

The protein content in shrimp by-products, comprising approximately 70-80% myogenic fibronectin and 20-30% sarcoplasmic protein, enhances the nutritional quality of seafood protein (Liu et al., 2021). As is shown in Figure 1, shrimp shells consist of three layers: the exocuticle, epidermis, and endocuticle. The epidermis is rich in proteins, lipids, and minerals, while the exocuticle and endocuticle contain dense fibrous plates with protein and chitin. This structural complexity, particularly the dense fibrous network of chitin in the exocuticle and endocuticle, makes the extraction of proteins more challenging, as they are tightly bound within these layers (Deng et al., 2020).

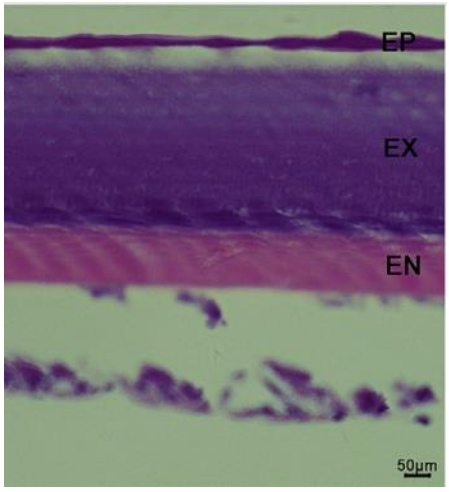

Figure 1. Shrimp shell layers (Deng et al., 2020)

As the global population continues to grow, ensuring nutritional and food security is becoming increasingly challenging. Proteins are essential for promoting healthy aging and balanced diets, particularly as dietary habits evolve due to economic development and urbanization. In wealthier nations, the average daily protein intake from meat is about 30 grams, accounting for roughly a quarter of total protein consumption, with additional contributions from eggs, fish, and dairy products. Conversely, in low and lower middle-income countries, plant-based sources predominantly provide dietary protein, with an average requirement of about 50 grams per person per day. This disparity highlights the need for sustainable protein sources that can be effectively utilized worldwide. Shrimp waste, which is often discarded in large quantities, presents a

valuable opportunity. By extracting proteins and bioactive compounds from shrimp by-products, we can address the protein needs of diverse populations while reducing environmental impact and minimizing waste (Jones, 2023).

Innovative extraction processes are crucial, requiring eco-friendly techniques to maximize protein yield without compromising environmental sustainability or the nutritional and functional properties of the protein. Effective extraction methods should disrupt structural bonds and cell walls to release proteins bound with other biomolecules like polyphenols, fats, fiber, and carbohydrates (Ampofo & Ngadi, 2022). In shrimp waste, proteins are often bound through interactions like hydrogen bonding with polyphenols, hydrophobic interactions with fats, and encapsulation within fibrous networks of chitin and other polysaccharides (Sánchez-Camargo et al., 2011).

Ultrasonication, a non-invasive and eco-friendly extraction method, has garnered significant attention for its ability to efficiently break down cell structures and release bioactive compounds from various biological matrices (McDonnell & Tiwari, 2017). Operating at a frequency of 24 kHz, ultrasonic processors induce cavitation within liquids, enhancing extraction efficiency by exposing hidden compounds and reducing processing times (Ultrasonic Homogenizers for Liquid Processing, n.d.).

Ultrasonication employs high-frequency sound waves to create cavitation bubbles in a liquid medium. These bubbles collapse violently, generating intense local energy and shear forces that disrupt cell walls and membranes. This disruption facilitates the release of intracellular compounds, enhancing the extraction of proteins and other valuable biomolecules. Ultrasonic-assisted extraction offers several advantages over conventional methods, including higher extraction yields, shorter processing times, lower energy consumption, and reduced solvent usage (Zhang et al., 2022).

Ultrasound technology is indeed a versatile tool applied across various biological materials for extraction purposes. Initially, it is used broadly to extract bioactive compounds from diverse sources such as plants, algae, and marine organisms. For example, ultrasound is employed to extract essential oils and flavonoids from plants, using conditions such as a frequency of 30 kHz and a power of 250 watts, which have been shown to achieve high extraction yields efficiently (B. Wang et al., 2018; Zorga et al., 2020).

In the case of algae, ultrasound facilitates the extraction of polysaccharides and carotenoids, often utilizing frequencies around 25-40 kHz and power levels between 200-300 watts. These conditions improve yield and extraction speed significantly (Carreira-

Casais et al., 2021; Generalić Mekinić et al., 2019). Additionally, ultrasound is effective in extracting bioactive peptides and omega-3 fatty acids from marine organisms, with studies showing optimal conditions like frequencies of 20-40 kHz and powers of 200-500 watts, which enhance recovery rates and efficiency (Kadam et al., 2013; Quiterio et al., 2022). These specific conditions illustrate the technology's adaptability and effectiveness in optimizing the extraction of valuable compounds across various biological materials.

Research is exploring the optimization of ultrasonic parameters such as frequency, power, temperature, and solvent type to maximize extraction efficiency and protein yield. Frequency refers to the rate at which the ultrasound waves oscillate, typically ranging from 20 to 40 kHz for effective extraction. Power determines the energy applied, with higher power levels (100 to 500 watts) often leading to more efficient cell disruption and protein release. Temperature control is crucial because excessive heat can denature proteins; thus, maintaining an optimal range (usually between 20°C and 40°C) is essential. The choice of solvent also impacts extraction efficiency; aqueous solutions with added salts or acids can enhance protein solubility and extraction yield (Cordero-Clavijo et al., 2024).

Additionally, parameters like cycle and amplitude play a significant role. The duty cycle, which represents the percentage of time the ultrasound is active during the extraction process, can influence the overall efficiency. For instance, a duty cycle of 50% means the ultrasound is on half the time and off the other half, allowing for better control of energy input and preventing overheating. Amplitude, the extent of the oscillation movement, directly affects the intensity of the cavitation process. Higher amplitudes (measured in micrometers) increase the mechanical effects on cell walls, improving the release of intracellular compounds. Studies have shown that optimizing these parameters can lead to significant improvements in extraction yields. For example, using a 50% duty cycle and amplitudes of 30-50 micrometers has been effective in maximizing protein recovery from plant-based substrates while minimizing degradation (Ampofo & Ngadi, 2022).

In conclusion, the application of ultrasound technology for protein extraction from shrimp waste presents a promising solution to address both nutritional and environmental challenges. By optimizing ultrasonic parameters such as frequency, power, temperature, solvent type, cycle, and amplitude, researchers can enhance the efficiency of protein extraction, maximizing yields while maintaining the integrity of the proteins. This approach not only contributes to the sustainable utilization of shrimp by-products but also aligns with the growing demand for eco-friendly and innovative food processing

techniques. The integration of ultrasound-assisted extraction methods holds significant potential for transforming shrimp waste into valuable bioactive compounds, thereby supporting global food security and advancing sustainable practices in the food industry.

2. Objectives

The aim objective is to harness ultrasound-assisted extraction (UAE) as an innovative, eco-friendly technique for protein recovery from shrimp by-products using water as a solvent and analyzing the key operational parameters to enhance extraction efficiency, increase protein yield, and promote the sustainable utilization of seafood waste specifically shrimp shell powder.

To achieve the objectives of the study, the following specific objectives were proposed:

1. To determine the acoustic power delivered by the ultrasound equipment used for extraction and the influence of temperature.
2. To analyze the effect of key extraction parameters, including time, temperature, and solvent-to-feed (SF) ratio, in the protein extraction and global extraction yield from shrimp waste under fixed ultrasound parameters.
3. To compare the efficiency of ultrasound-assisted extraction (UAE) with conventional extraction methods in terms of protein yield and extraction yield.

3. Materials and methods

3.1 Shrimp waste preparation and characterization

The raw materials used in this study consisted of a combination of various shrimp byproducts, including beards, eyes, legs, shells, and tails, provided by Gambastar S.L. (Valdorros, Burgos). These materials were kept frozen at -20°C until they were ready for processing, ensuring the preservation of their quality. Prior to use, the shrimp waste was thoroughly rinsed with tap water to remove any residual impurities. The cleaned waste was then dried in an oven (Memmert, Innsbruck, Austria) at 105°C for 24 hours to eliminate all moisture content. Following the drying process, the shrimp waste was finely ground using a knife mill (SM 100, Retsch, Haan, Germany) to produce a uniform shell powder.

This shell powder underwent comprehensive characterization to determine its composition, including water extractives, ash, protein, amino acids, fatty acids, and chitin content, as summarized in Table 1. Water extractives were determined by extraction in Soxhlet with type III water for 8 h and a solvent-to-feed ratio of 63.3 mL/g. To quantify

the mineral content, the ash content was measured using the AOAC 938.08 method with some modifications (*AOAC Official Method 938.08 Ash of Seafood PDF | PDF*, n.d.). The fatty acid content was determined following an adapted AOAC 948.16 method, while the chitin content was assessed using the AOAC 978.10 method, also with necessary adjustments (*AOAC INTERNATIONAL*, n.d.). The protein content was determined by the bicinchoninic acid assay (BCA) in the liquid extracts generated during the chitin determination, as well as in the water extractives. This method will be explained later in detail.

Table 1. Approximate composition from shrimp waste in dry shell basis (ds. b)

Component	**% (ds. b)**
Water extractives	9.3 ± 0.6
protein	2.0 ± 0.2
Fatty acid	2.12 ± 0.02
Ash	39.6 ± 0.2
Chitin	16.9 ± 0.8
Protein	19.4 ± 0.9
Total	87 ± 3

The particle size distribution of the shell powder was analyzed using laser diffraction (Mastersizer 2000, Malvern Panalytical, Malvern, UK) in dry mode which revealed that the volume-weighted mean particle size (D [4,3]) was 286 µm, with a surface-weighted mean (D [3,2]) of 68 µm. The particle size distribution percentiles were recorded as d (0.1) = 40 µm, d (0.5) = 249 µm, and d (0.9) = 579 µm.

The preparation and characterization of the raw material was performed by Mauricio M. de Sousa Ribeiro, doctoral student in the PressTech group from the Department of Chemical Engineering and Environmental Technology, where this Master Thesis was developed.

3.2 Ultrasonic Processing

3.2.1 Acoustic Power Determination

To determine the effective ultrasound energy delivered to the system, the Acoustic power, was calculated based on the initial and final temperatures recorded during each test, according to the calorimetric test used by Huerta et al, 2020, following the next formula:

$$P = \frac{m * Cp * \Delta T}{\Delta t}$$

Where:

- m is the mass of the sample,
- Cp is the specific heat capacity of the sample (4,184 J / g ºC),
- ΔT is the change in temperature over the time interval Δt\Delta tΔt.

To establish this 180 ml of water type III was used in the same ultrasonic processor used later for the extraction. Also, the same jacketed vessel was used, although without controlling the temperature with a water bath.

This was a UP400S processor shown in Figure 1 (Hielscher Ultrasonics GmbH, Teltow, Germany). The apparatus operates at a frequency of 24 kHz, providing high-intensity ultrasonic waves suitable for efficient extraction processes equipped with a sonotrode of a diameter of 22 mm that provides a maximum amplitude, and extent of the oscillation movement, of 100 mm.

The tests for acoustic power measurement were carried out under the same extraction conditions. The cycle was fixed at 1, meaning that the ultrasounds were continuously applied, the amplitude at 100% and sonotrode depth was 4.5 cm. Temperature was registered every 15s for a total time of 6 minutes.

Figure 1. UP400S processor (Hielscher Ultrasonics GmbH, Teltow, Germany).

3.2.2 Ultrasonic extraction

The ground shrimp shell powder was mixed with 180 ml of type III water to form a slurry; it is important to mention that this water was previously heated up to operating temperature with a heating plate (IKA RCT Classic magnetic stirrer/hotplate) for experiments performed above ambient temperature . The mass of raw material processed was varied from 18 g to 3 g according to the solvent-to-feed ratio that was ranged as follows: 10:1, 20:1, 40:1, and 60:1 (v/w).

The slurry was placed in a jacketed glass beaker of 200 mL and subjected to ultrasonic treatment under the following conditions: 100% amplitude and, duty cycle (percentage of time ultrasound is active) of 1 meaning continuous application of ultrasounds. The

temperature at which extractions occur was controlled using an external thermostatic bath (Selecta Tectron 200) at 20°C, 40°C, and 50°C, and was continuously measured with a thermocouple connected to a digital display (Omron E5GN). The duration of the sonication process was set to 5, 15 and 25 minutes. The ultrasonic probe was immersed into the slurry to a depth of approximately 4.5 cm as shown in Figure 2.

Figure 2. UP400S processor (Hielscher Ultrasonics GmbH, Teltow, Germany) with the sample and the water bath (Selecta Tectron 200).

All experiments were performed at least in duplicate, and the various analyzed variables are expressed as a means with standard deviation.

After sonication, the samples were subjected to a mechanical separation process for separation of the solid residue and liquid extract for further characterization. Centrifugation was the selected process: the extract was centrifuged at 3122 g (Magnus 22 R from Orto Alresa, Daganzo, Spain) in a 750 mL vessel for 20 minutes. An aliquot of the liquid about 10 mL was taken and kept at 4ºC until analysis. The solids were then washed with Type III water and centrifuged again, frozen at -20ºC, and freeze-dried for 24 to 48 hours until a dried powder was obtained.

3.2.3 Conventional extraction

As a reference, a conventional extraction was carried out in the same vessel, in the absence of ultrasounds, at 40ºC, controlled by the external water bath, with a solvent-to-feed ratio of 20:1 and with a stirring plate set to 750 rpm for agitation.

The experiment was conducted in duplicate with a single treatment duration of 25 minutes; Liquid samples were taken (approximately 6 ml) at 5, 15, and 25 minutes to determine the protein content. The overall yield was only measured at the 25-minute mark, after centrifugation of the slurry and freeze drying of the solid, as for the extraction experiments with ultrasounds.

3.3 Extraction performance

3.3.1 Global Extraction Yield Determination

The Global Extraction Yield was determined gravimetrically by weighing the solids obtained after the lyophilization (freeze-drying) process.

The extraction yield was calculated using the following formula:

$$\%Extraction\ yield = \left(\frac{Mass\ of\ liophilized\ extracted\ solid}{Sample\ mass}\right) * 100$$

Here, the "Mass of lyophilized extracted solids" is the weight of the dried solids obtained after lyophilization, and the "Sample mass" is the original mass of the sample before extraction.

3.4 Extract characterization

3.4.1 Protein content determination

The bicinchoninic acid assay (BCA) kit from Thermo Scientific (Reference 23226) was used to quantify protein content. The procedure is as follows: a working reagent (WR) was prepared by mixing reagents A and B in a 50:1 ratio. Subsequently, 0.1 mL of the sample or distilled water (used as a blank) was combined with 2 mL of the WR solution in a microcentrifuge tube. The mixture was then vortexed using a Stuart SA3 vortex mixer (Bibby Scientific, Stone, Staffordshire, UK) to ensure thorough mixing. The tubes were incubated at 37°C for 30 minutes before cooling to room temperature in a Fisherbrand Isotem. Absorbance measurements were taken at 562 nm using a UV-Vis spectrophotometer (UV-2550, Shimadzu, Kioto, Japan). To construct a standard curve, albumin standards ranging from 1000 to 25 µg/mL were prepared from an initial BSA solution of 2000 µg/mL. Each sample was analyzed in duplicate using an adequate dilution, and the protein content was expressed as grams of protein per 100 grams of dry shell (g/100 g of DS).

To quantify the protein concentration in the extracts, the following calculations were performed:

Standard Curve Generation: The absorbance values of the BSA standards were plotted against their known concentrations to generate a standard curve every time this essay was performed, as follows in Table 2

Table 2. BCA standard curve.

Sample	Water (µL)	BSA (µL)	Final Concentration (µg/mL)	Abs (562nm)
A	1000	1000	1000	1.009
B	150	450 (A)	750	0.754
C	300	300 (A)	500	0.542
D	450	150 (A)	250	0.262
E	450	150 (C)	125	0.128
F	400	100 (E)	25	0.018

Sample Concentration Calculation: Using the linear equation derived from the standard curve (y = mx + b, where y is the absorbance, m is the slope, and b is the y-intercept), the protein concentration in the unknown samples was calculated by substituting their absorbance values into the equation.

Final Protein Concentration: The calculated concentrations were then adjusted according to the dilution factor (2, 4, 8, and 10) used in the assay to obtain the final protein concentration in the original extract.

3.4.2 Free Amino Acid Content Determination

For the quantification of free amino acids (AA) using the ninhydrin method, 0.4 mL of the sample or distilled water (as a blank) was mixed with 0.2 mL of ninhydrin reagent (N7285-Sigma-Aldrich) in a 2 mL microcentrifuge tube. The mixture was gently stirred before being incubated at boiling water temperature for 10 minutes. After cooling to room temperature, 1.4 mL of 95% ethanol was added to each tube. The absorbance was then measured at 570 nm using a UV-Vis spectrophotometer, with glycine (0.03–0.007 mg/mL) serving as the standard curve. Each sample was measured in duplicate, and the AA content was expressed as grams of AA per 100 grams of dry shell (g/100 g of DS).

The formula used to calculate the amount of free amino acids (AA) using the ninhydrin method is generally based on the Beer-Lambert law, which relates the measured absorbance to the concentration of amino acids in the sample. The basic formula is:

$$Concentration\ of\ AA = \ ((A - Ablank) * Vtotal * F)/(\epsilon * l * Vsample)$$

Where:

A: Absorbance of the sample measured at 570 nm.

Ablank.: Absorbance of the blank (solution without amino acids).

Vtotal: Total volume of the ninhydrin reagent added (in mL).

Vsample: Volume of the sample used in the assay (in mL).

F: Dilution factor, if the sample has been diluted before the assay.

ϵ: Molar extinction coefficient specific to the ninhydrin-AA complex at 570 nm (in M^-1 cm^-1).

l: Path length of the cuvette (in cm), generally 1 cm.

Because of the delicacy this essay that is carried out under N_2 atmosphere to avoid degradation of Ninhydrin reagent, it was made by Mauricio M. de Sousa Ribeiro, doctoral student in the PressTech group. Further, it was not possible to perform it for all the samples, due to a discontinuation of supply.

3.5 Solid Characterization

3.5.1 Particle size determination

The particle size distribution of shrimp shell powder after extraction of selected samples was determined using a Malvern Mastersizer 3000 with a Hydro 2000MU (A) accessory. Samples were analyzed in water as the dispersant, with the refractive index of the particles set to 1.572 (CaCO3, calcite) and the absorption index set to 0.1. The refractive index of the dispersant was 1.331. Particle size distributions were reported in volume and the following percentiles were used for characterization: d (0.1), meaning that the 10% in volume of the particles have a mean diameter below this value, d (0.5), and d (0.9). Also, the volume-weighted mean particle size (D [4,3]) and surface-weighted mean (D [3,2]) values were reported.

4. Results and discussion

4.1 Acoustic Power Measurement

The results in Figure 3 (Table I, Annex I shows raw data) demonstrate a clear correlation between temperature and acoustic power (P). Initially, the power stabilizes around 115 W between 20°C and 30°C. However, between 30°C and 40°C, the power begins to decline by roughly 10%. After 40°C, the power decreases almost linearly, reaching about 55 W by 65°C. This suggests that higher temperatures negatively affect the efficiency of

the ultrasonic system, reducing its power output as the temperature increases (Kazys & Vaskeliene, 2021).

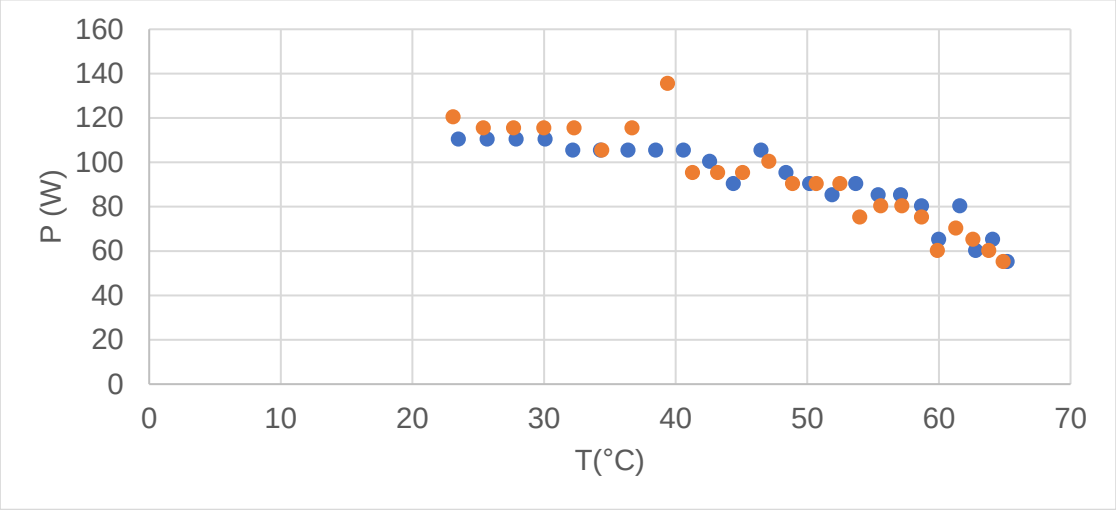

Figure 3. Effect of Temperature on Acoustic Power at 100% Amplitude and Cycle 1 from two independent measurements.

This result is consistent with the known effects of temperature on ultrasonic efficiency. At higher temperatures, the viscosity and density of the medium (water in this case) decrease, which can lead to a reduction in cavitation activity, the primary mechanism responsible for delivering energy in ultrasonic systems. As cavitation decreases, less energy is transferred to the system, resulting in lower acoustic power output (Ahmed K. Abu-Nab et al., 2023).

At temperatures around 40–60°C, cavitation activity typically begins to diminish in water as found in Raso's 1999 previous studies, leading to reduced ultrasonic energy transfer. This explains the sharp drop in power observed in the graph between 40°C and 60°C. More recent studies such as those by Badjona et al. on acoustic cavitation in liquids have demonstrated that the cavitation threshold decreases with increasing temperature, leading to lower efficiency of energy delivery (Badjona et al., 2024; Raso et al., 1999).

Similar studies that utilize calorimetric methods for acoustic power determination, such as those by Martínez-Ramos, Corona-Jiménez, and Ruiz-López (2021) have demonstrated the same power decline trend as temperature increases. This trend is important to consider for optimizing ultrasonic processes, as it implies that beyond a certain temperature, the efficiency of ultrasonic extraction decreases unless temperature control is employed (Martínez-Ramos et al., 2021).

4.2 Ultrasonic Extraction:

4.2.1 Establishment of the mechanical separation procedure

Initially, the separation of the solid and the extract after ultrasound (US) treatment was attempted using different filtration methods. The first approach used a quantitative laboratory filter (particle retention size around 8µm), followed by a quantitative filter paper 434 from Anoia (particle retention: 2-3 µm), and finally, a nylon syringe filter (particle retention sizes 0.2-0.45 µm). These filtration techniques were chosen based on the methodology outlined in the thesis of Mauricio M. de Sousa Ribeiro, which involved protein extraction using microwave processing with water as the solvent (*De-Souza-Ribeiro*, n.d.). However, none of these methods proved successful as the filtration process was exceedingly slow and ultimately impractical for completing the separation.

In the next experiment, a different type of filter (hardened quantitative filter paper, slow flow rate, particle size retention of 8µm was used. However, this method also failed to separate the particles effectively.

To verify the possible effect of US on the particle size that hampers the filtration process particle size distribution was determined by laser diffraction. Figure 4 illustrates the effects of ultrasound treatment under varying time and temperature conditions on the raw material. The raw material exhibits a relatively narrow particle size distribution, with a distinct peak around 100 µm. After ultrasound treatment in Trials 1 and 2 (5 min, SF 20 mL/g, 20°C), the particle size distribution broadens, with a significant shift toward smaller particle sizes around 10 µm, as evidenced by the emergence of bimodal peaks. This trend suggests effective particle size reduction, consistent with findings in similar studies where short-duration, low-temperature ultrasound treatments promote the disintegration of particles into finer sizes by inducing cavitation and shear forces such as those discussed in (Kanani et al., 2023; Xie et al., 2020).

Trial 3, which involved a longer ultrasound exposure (25 min) at a higher temperature (40°C), shows a different particle size distribution, with fewer smaller particles and a broader distribution around larger sizes. This observation suggests that extended ultrasound treatment, particularly at elevated temperatures, may not enhance particle size reduction to the same degree as shorter treatments. Prolonged exposure can lead to the agglomeration of smaller particles or thermal effects that counteract further disintegration, as observed in research by (J. Wang et al., 2024), where extended ultrasound treatment led to particle size re-agglomeration.

Given the findings, filtration was discarded as a viable separation method due to the broad size distribution, the variability in particle size (as indicated by higher spans), and

the likelihood of smaller particles could complicate downstream processes like filtration or separation by clogging the filtration medium. Similar challenges with the filtration of fine particles produced by ultrasound treatment have been documented, as in the work of (A. Yang et al., 2022). Therefore, centrifugation was chosen instead to obtain the solid yield. Centrifugation is more efficient at separating fine particles generated by ultrasound and prevents clogging and processing losses, as supported by studies like those of Pradhan et al, and Zhu et al., 2016 where centrifugation is often preferred for recovering solids from fine dispersions produced by ultrasound processing (Pradhan et al., 2022; Zhu et al., 2016).

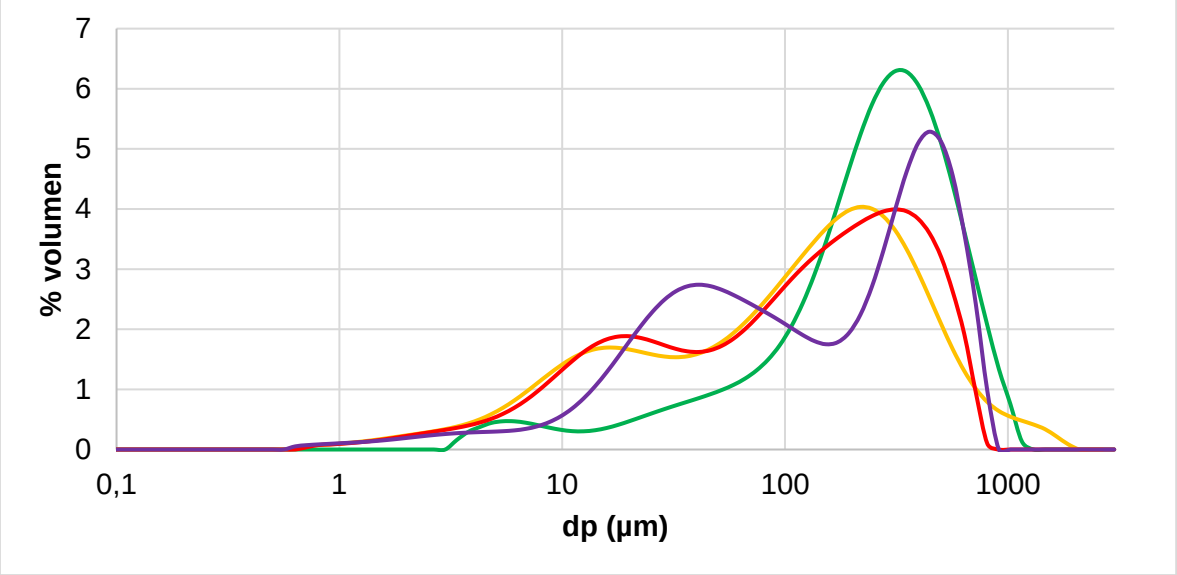

Figure 4. The particle size of the raw material (green) and solid after trials under the next ultrasound conditions: Trial 1 (yellow) and 2 (red) time set of 5 min, S-F 20:1 v/w and temperature 20 ºC. Trial 3 time of 25 min, S-F 20:1 v/w, T 40ºC (purple).

4.2.2 Extraction performance: variables affect

Effect of time

Initially, the effect of ultrasound treatment duration was studied at T = 40ºC and S/F of 20:1 v/w for three different times as shown in Table III (Annex). Figure 5 demonstrates the impact of time on extraction yield and protein content during ultrasound-assisted extraction from the raw material, which is rich in protein (19.4%), chitin (16.9%), and ash (39.6%) (Table 1). In the first 5 minutes, an increase in extraction yield (8.9%) and protein content (3.63%) occurs, likely due to the release of water-soluble proteins. Based on the research of Negi et al. (2024) and Bendicho and Lavilla (2017), ultrasound enhances the diffusion of solutes by breaking cell walls, facilitating the fast extraction of soluble proteins in the initial stage (Lavilla & Bendicho, 2017, p. 11; Negi et al., 2024).

At 15 minutes, the increase in extraction yield and protein content is limited, likely because the remaining proteins are more embedded in the matrix, particularly within the chitin structure. According to the study of Vallejo-Domínguez et al. (2020), chitin acts as a barrier to protein release, and while ultrasound can disrupt it, the extraction becomes more difficult as time progresses (Vallejo-Domínguez et al., 2020). Further, according to Ismail et al. (2022), the high ash content (primarily inorganic components like calcium carbonate) could reduce protein solubility, further complicating the extraction process (Ismail et al., 2022).

After 15 minutes, the extraction yield plateaus, suggesting that most of the easily extractable proteins have already been released. Given that the experiments were conducted at 40ºC, protein denaturation is unlikely to have played a role in limiting further extraction, as denaturation typically occurs at temperatures of 60ºC or higher. The slowing of extraction efficiency may instead be attributed to the presence of more embedded proteins, which are harder to extract due to their strong interaction with the chitin-protein matrix (Suchintita Das et al., 2022).

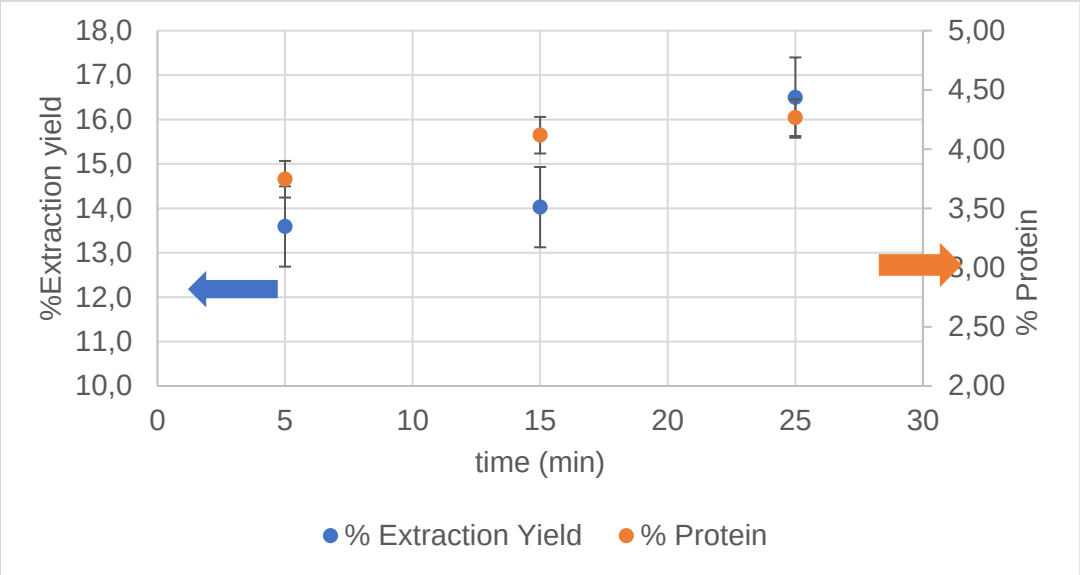

Figure 5. Effect of Time on Extraction Yield and Protein Content at constant extraction conditions: temperature of 40ºC, and the solvent-to-feed ratio of 20:1 (Table III Annex).

Effect of solvent-to-feed ratio

The solvent-to-feed ratios affect the extraction yield and protein content based on the research by (Chemat et al., 2017) where low solvent-to-feed ratios (10:1) tend to limit the availability of solvent for solubilizing proteins, which explains the relatively low protein extraction efficiency at the 10 and 20 mL/g ratios (Table IV. Annex).

As shown in Figure 6, increasing the solvent-to-feed ratio from 10 to 20 mL/g does not significantly affect the extraction yield, which remains at about 14%, while the protein content stays close to 2%.

This suggests that, at lower ratios, the solvent is insufficient to fully penetrate the chitin-protein matrix, limiting the efficiency of the process. Such as the study (C. Yang et al., 2024) discusses, that a higher solvent volume improves the mass transfer of solutes, which is crucial for extracting proteins from complex matrices like shrimp shells.

As said before, solvent-to-feed ratio (S/F) plays a crucial role in both extraction yield and protein content. At an S/F of 20 mL/g, the protein content reaches its maximum of 3.75%, while the extraction yield is 13.6%. As the S/F ratio increases to 40 and 60 mL/g, the extraction yield continues to rise to 16.1% and 15.7% respectively, but the protein content decreases to 3.05% and 2.62%. This confirms that, while higher S/F ratios improve global extraction, they limit protein extraction efficiency as noted by (Matouri et al., 2024) who found that chitin acts as a significant barrier to protein release, and while a higher solvent volume can disrupt the matrix to some extent, the complexity of the chitin structure and its interaction with other components, such as calcium carbonate, limits further extraction.

Based on the research of Ismail et al., 2022**,** the high ash content (primarily calcium carbonate) in shrimp byproducts further complicates protein extraction, as the inorganic materials reduce protein solubility. Even with higher solvent volumes, this mineral barrier limits protein recovery, suggesting that other extraction techniques, such as ultrasound, may be needed to improve the disruption of the chitin-ash matrix (Ismail et al., 2022).

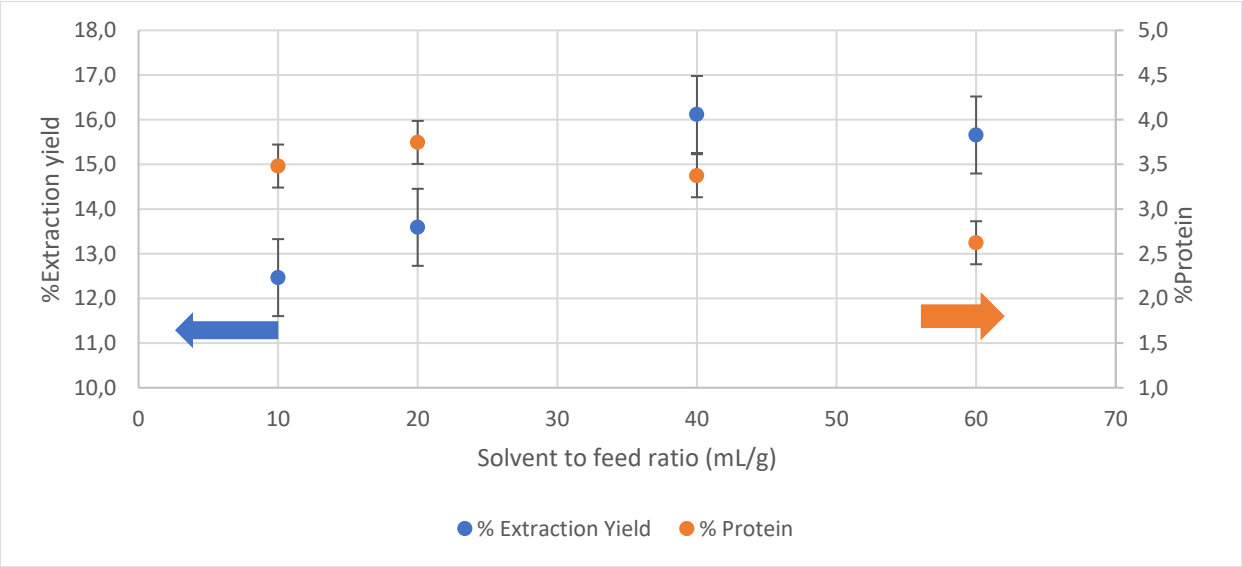

Figure 6. Effect of solvent-to-feed ratio for extraction yield and protein content at 5 minutes treatment (Table IV. Annex).

Effect of temperature

The impact of temperature on protein extraction was studied at an S/F ratio of 20 with a 5-minute extraction time, as shown in Figure 7. Protein extraction increased linearly from 3.5% at 20°C to 4.05% at 50°C, consistent with findings by (Sharayei et al., 2021). that higher temperatures enhance molecular movement and diffusion, improving protein solubility and extraction. However, the global extraction yield reached a plateau at 40°C with 13.6%, indicating diminishing returns beyond this temperature.

As the temperature rises to 40°C, both the extraction yield and protein content increase significantly. Such as the study of Orellana-Palacios et al. (2022) suggests that this increase is driven by the enhanced solubility of proteins and improved mass transfer at moderate temperatures, allowing the ultrasound to more effectively break down the chitin matrix and release trapped proteins (Orellana-Palacios et al., 2022). Ultrasound-assisted extraction is particularly efficient at these temperatures, as cavitation and thermal effects work synergistically to disrupt cell walls and improve protein release. As shown in Figure 1, the acoustic power displayed from 20ºC to 40ºC is the highest in the studied range and approximately constant.

At temperatures above 40°C, the extraction yield remains stable, but the protein content shows minimal further increase. This could be attributed to the high chitin and ash content, which act as physical barriers to protein release. Such as the study of Kanani et al., 2023 points out, that while ultrasound can disrupt the chitin matrix, higher temperatures may lead to protein denaturation, reducing solubility and limiting further

extraction. The high ash content, primarily consisting of calcium carbonate, may also contribute to this stabilization by hindering protein release (Kanani et al., 2023).

In conclusion, maintaining a moderate temperature of around 40°C is optimal for balancing both extraction yield and protein content. Higher temperatures, such as 50°C, may extract unwanted compounds, reducing the overall extraction specificity. Although protein extraction increases similarly between 20-40°C and 40-50°C, it is more practical to operate at 40°C due to the convenience of ambient temperature conditions in the water bath to keep isothermal conditions; the need to heat the bath further, which is necessary for extraction at 50°C, is avoided (Maher et al., 2021; Orellana-Palacios et al., 2022)

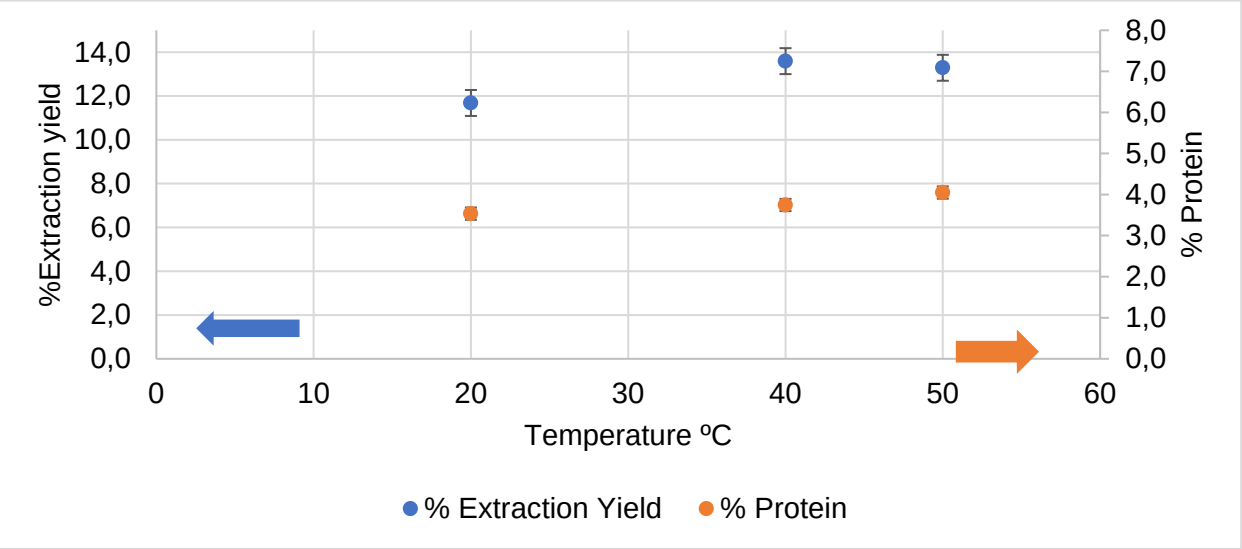

Figure 7. Effect of temperatures on extraction yield and protein content at a solvent-to-feed ratio of 1:20 and 5 minutes (Table V. Annex)

4.2.3 Free Amino Acid Content Determination

The ninhydrin method is widely recognized for detecting free amino acids due to its reaction with primary amines, which forms a colored complex that can be measured spectrophotometrically. In the current experiments, the percentage of free amino acids ranged from 0.23% to 0.46% as shown in Table II (Annex), with higher values observed under specific conditions, such as a 1:40 solvent-to-feed (SF) ratio, where the percentage reached 0.46%. This is in line with research showing that an increased SF ratio improves extraction efficiency by enhancing solute dissolution and making the amino acids more available for reaction (Nollet & Toldra, 2012).

Additionally, experiments demonstrated that higher temperatures (40°C) and longer extraction times (e.g., 28 minutes) resulted in higher free amino acid percentages compared to lower temperatures (20°C) and shorter extraction times. This observation aligns with studies indicating that elevated temperatures accelerate protein denaturation

and peptide bond breakdown, leading to the release of more free amino acids (Masson & Lushchekina, 2022)

However, under the conditions used in this study, it was verified that the degradation of proteins to free amino acids during extraction was minimal. The free amino acid content remained approximately constant, around 0.28 ± 0.07% after 5 minutes of extraction and 0.32 ± 0.02% after 25 minutes. This suggests that while some extraction conditions (like higher temperatures and extended times) can increase the release of free amino acids, the overall degradation of proteins to amino acids in these specific trials was relatively limited.

Overall, the literature suggests that optimal free amino acid extraction is influenced by multiple factors, including temperature, SF ratio, and extraction time, which affect protein solubility and hydrolysis (Belitz et al., 2009).

4.3 Ultrasonic vs Conventional extraction

The comparison between ultrasound-assisted extraction and non-ultrasound extraction at 40ºC and S/F 20 mL/g (Figure 8) shows a significant improvement in protein extraction when using ultrasound. In the experiment without ultrasound 2.10% ± 0.09 protein, while the one with ultrasound yielded 3.75% ± 0.01 protein at 5 minutes process. This marked increase highlights the efficiency of ultrasound-assisted extraction (UAE) in breaking down complex matrices, such as shrimp byproducts, and enhancing protein release (Sert et al., 2022).

As described by Badjona et al., 2024, ultrasound-assisted extraction enhances the protein yield due to the cavitation effect, which creates shear forces that disrupt cellular structures, improving the mass transfer between the solvent and the matrix (Badjona et al., 2024, 2024). This disruption allows the release of proteins that are otherwise trapped by rigid biopolymers like chitin. In comparison to traditional methods, UAE significantly increases extraction efficiency, as seen in this study with shrimp byproducts, which often contain high levels of chitin and calcium carbonate that act as barriers to protein release (Jahan et al., 2022).

Moreover, the data shows that the sample treated with ultrasound not only had a higher protein yield but also exhibited a lower variance (±0.01 vs. ±0.09), indicating that ultrasound treatment produces more consistent results. This improved reliability is critical in industrial applications, where consistent yields are necessary for process optimization and cost efficiency (Orellana-Palacios et al., 2022; Sert et al., 2022).

In conclusion, the substantial increase in protein extraction observed in the ultrasound-treated sample supports the growing body of evidence suggesting that ultrasound power, along with optimized extraction parameters such as solvent composition and temperature, can significantly enhance protein recovery from complex matrices like shrimp byproducts.

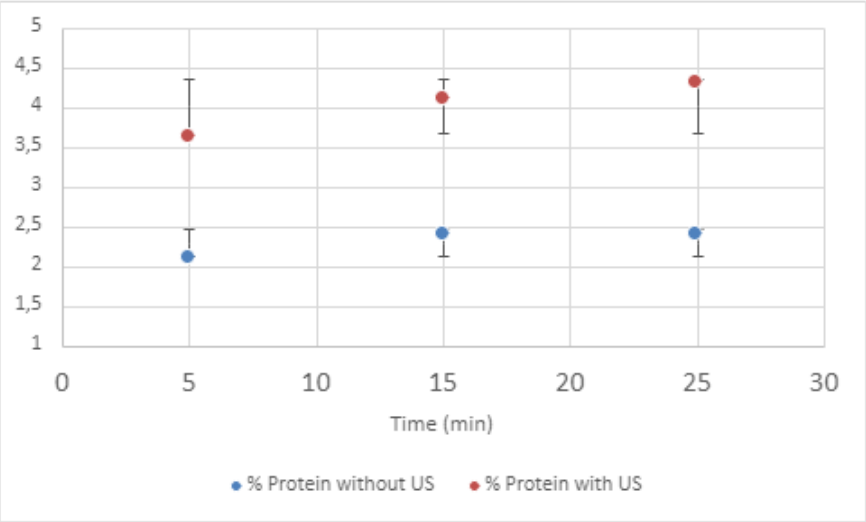

Figure 8. Comparison between the US treatment and the Conventional using the same parameters (Time of 5 minutes, temperature 40ºC and SF ratio 20:1 v/w)

4.3.1 Extraction yield

The extraction yield (%Extraction Yield) from two different processes: one without ultrasound and the other using ultrasound-assisted extraction for 25 minutes. The data demonstrates a significant increase in extraction yield when ultrasound is applied. The non-ultrasound method resulted in a yield of approximately 14.4%, while the ultrasound-assisted process achieved a higher yield of about 15.7%.

This increase in yield can be attributed to the cavitation effect generated during ultrasound treatment, which enhances mass transfer by creating microbubbles that implode, releasing localized energy. This energy helps break down the cellular structures of the shrimp byproduct, allowing more proteins and other valuable compounds to be extracted. Studies, such as the one by Sert et al. (2022), confirm that ultrasonic treatment significantly improves the extraction of bioactive compounds by disrupting cellular walls and improving solvent penetration (Sert et al., 2022).

The improvement in extraction yield with ultrasound is consistent with findings from Eze et al., (2022); Negi et al., (2024); Vallejo-Dominguez et al. (2020), and Quiterio et al., (2022) who also noted that optimizing ultrasound power, frequency, and time can enhance the release of bioactive compounds from complex matrices like shrimp shells.

This is due to the ultrasound waves' ability to break down rigid structures such as chitin, allowing for more efficient protein release (Eze et al., 2022; Negi et al., 2024; Quiterio et al., 2022; Vallejo-Domínguez et al., 2020).

5. Conclusions

The study shows the results of ultrasound-assisted extraction (UAE), as an innovative, eco-friendly technology, for extracting proteins from shrimp waste. Compared to traditional methods, UAE significantly improves protein yield by 1.6-fold. However, despite this improvement, the highest protein extraction achieved was 4.26 +/- 0,47 at 40ºC, solvent-to-feed ratio (S/F) of 20 mL/g, and 25 minutes of ultrasounds at 100% amplitude.

This represents only 19.9% of the total protein content in the residue, indicating that while the method is effective, it may not be sufficient to fully release proteins due to the strong binding of proteins within the chitin matrix. Adjustments in key ultrasonic parameters—such as time, temperature, and solvent-to-feed (SF) ratio—allow for the sustainable extraction of proteins from shrimp shells, which are otherwise considered waste. Further research should focus on varying amplitude and cycle times, as these are critical factors in ultrasound applications.

Although UAE presents a promising solution for sustainable protein extraction, the moderate yields observed suggest that operating under the current conditions, especially at low temperatures, may be insufficient to break down the complex chitin-protein interactions. Future research should explore the potential use of ultrasound as a pretreatment in conjunction with other extraction processes, such as microwaves, which may further enhance the release of proteins and other bioactive compounds. Additionally, studies on the energy consumption of the UAE are necessary to determine its feasibility for large-scale food production and industrial applications.

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ANNEX

Acoustic Power Measurement

Table I. Measurement of the different temperatures with the acoustic power in 6-minute treatment.

time (s)	Temperature 1 (ºC)	Acoustic Power 1 (W)	Temperature 2 (ºC)	Acoustic Power 2 (W)
15	23,5	110,4	23,1	120,5
30	25,7	110,4	25,4	115,5
45	27,9	110,4	27,7	115,5
60	30,1	110,4	30	115,5
75	32,2	105,4	32,3	115,5
90	34,3	105,4	34,4	105,4
105	36,4	105,4	36,7	115,5
120	38,5	105,4	39,4	135,6
135	40,6	105,4	41,3	95,4
150	42,6	100,4	43,2	95,4
165	44,4	90,4	45,1	95,4
180	46,5	105,4	47,1	100,4
195	48,4	95,4	48,9	90,4
210	50,2	90,4	50,7	90,4
225	51,9	85,3	52,5	90,4
240	53,7	90,4	54	75,3
255	55,4	85,3	55,6	80,3
270	57,1	85,3	57,2	80,3
285	58,7	80,3	58,7	75,3
300	60	65,3	59,9	60,2
315	61,6	80,3	61,3	70,3
330	62,8	60,2	62,6	65,3
345	64,1	65,3	63,8	60,2
360	65,2	55,2	64,9	55,2

Summary of results

The table below summarizes the results of all experiments evaluating the efficiency of ultrasound-assisted extraction UAE for protein recovery from shrimp by-products. The sample codes represent the specific experimental conditions, denoted as "Residue-Solid Mass-Cycle-Amplitude-Time-Temperature.". Some of them have been repeated to re-evaluate the parameters mark with “repeat” word. The ones with the “WUS” letters mean “Without Ultrasound”

Table II. Results of the experiments assessed.

Experiment Code	Initial mass (g)	S/F ratio (mL/g)	time (min)	Initial T (ºC)	Final T (ºC)	Average acoustic power (W)	Energy (J)	% Extraction Yield	% Protein BCA	% Protein AA
GF-9-1-100-5-20	9,0004	20	5	19,9	65,2	87,5	26250	20,0	4,49	0,36
GF-9-1-100-5-20A	9,0000	20	5	15,8	41	106	31800	9,1	3,75	0,30
GF-9-1-100-5-20B	9,0057	20	5	17,8	40,4	106	31800	22,2	3,25	0,32
GF-9-1-100-5-40A	9,0014	20	5	37	40,1	113	33900	10,1	3,66	0,32
GF-9-1-100-5-40B	9,0005	20	5	26,3	38,5	109	32700	7,7	3,60	0,23
GF-9-1-100-15-40.A	9,0089	20	15	39,1	42,9	105	94500	12,4	3,95	0,32
GF-9-1-100-15-40B	9,0039	20	15	40	41,4	107	96300	15,7	4,29	
GF-9-1-100-28-40	9,0025	20	28	35,7	41,7	100	168000	17,2	4,60	0,33
GF-9-1-100-25-40	9,0040	20	25	25,7	41,5	107	160500	15,7	3,93	0,31
GF-4,5-1-100-5-40A	4,5024	40	5	40,3	39	115	34500	15,6	3,34	0,46
GF-4,5-1-100-5-40B	4,5057	40	5	40,3	39	113	33900	16,6	3,41	0,39
GF-18-1-100-5-40A	18,0040	10	5	32,7	39,2	112	33600	11,5	3,55	

GF-18-1-100-5-40B	18,0041	10	5	35	38,8	112	33600	13,4	3,41	
GF-9-1-100-5-20A repeat	9,0064	20	5	12,4	33,9	111	33300	12,0	3,69	
GF-9-1-100-5-20B repeat	9,0036	20	5	11,1	29,4	111	33300	11,4	3,38	
GF-9-1-100-5-50A	9,0015	20	5	48	47,2	101	30300	12,4	4,04	
GF-9-100-5-50B	9,0031	20	5	48	49,6	95	28500	14,2	4,19	
GF-9-1-100-50Arepeat	9,0034	20	5	52	53,6	89	26700		4,07	
GF-9-1-100-50Brepeat	9,0056	20	5	52	53,1	89	26700		4,03	
GF-9-WUS-5[a]	9,0090	20	5	40	39,5	105	31500		2,16	
GF-9-WUS-5B	9,0463	20	5	38,5	38	105	31500		2,03	
GF-9-WUS-15A	9,0090	20	15	40	40	105	94500		2,42	
GF-9-WUS-15B	9,0463	20	15	38,5	36	105	94500		2,36	
GF-9-WUS-25A	9,0090	20	25	40	40	105	157500		2,30	
GF-9-WUS-25B	9,0463	20	25	38,5	36	105	157500		2,46	
GF-9-WUS-25CA	9,0090	20	25	40	40	105	157500		2,52	
GF-9-1-100-5-40A Repeat	9,0091	20	5	40,6	40,6	105	31500	13,9	3,80	
GF-9-1-100-5-40B Repeat	9,0514	20	5	40,6	42,4	105	31500	13,3	3,70	

Extraction performance: variable affect

Effect of time

Table III presents the results of ultrasound-assisted extraction UAE of proteins from shrimp waste, specifically focusing on how the extraction time influences the overall extraction yield and protein content.

Table III. Effect of Extraction Time on Yield and Protein Content during Ultrasound-Assisted Extraction at 40°C.

Code	Time (min)	% Extraction Yield			% Protein		
GF-9-1-100-5-40	5	13,6	±	0,4	3,75	±	0,07
GF-9-1-100-15-40	15	14.0	±	2.3	4,1	±	0,2
GF-9-1-100-25-40	25	16,5	±	1,1	4,3	±	0,5

Effect of S/F ratio

Table IV summarizes the results of experiments designed to assess the effect of varying the solvent-to-feed S/F ratio on the extraction yield and protein content during ultrasound-assisted extraction UAE.

Table IV. Effect of Solvent-to-Feed S/F Ratio on Extraction Yield and Protein Content at 40°C

Code	S/F (mL/g)	% Extraction Yield			% Protein		
GF-18-1-100-5-40	10	12,5	±	0	3,5	±	0,1
GF-9-1-100-5-40	20	13,6	±	0,4	0,00	±	0,01
GF-4.5-1-100-5-40	40	16,1	±	0,7	3,37	±	0,05
GF-3-1-100-5-40	60	15,7	±	0,2	2,62	±	0,20

Effect of temperature

Table V presents the results of experiments conducted to evaluate the impact of temperature on the extraction yield and protein content during ultrasound-assisted extraction in UAE.

Table V. Effect of Temperature on Extraction Yield and Protein Content during Ultrasound-Assisted Extraction

Code	T (°C)	% Extraction Yield			% Protein		
GF-9-1-100-5-20	20	11,7	±	0,4	3,5	±	0,2
GF-9-1-100-5-40	40	13,6	±	0,4	0,00	±	0,01
GF-9-1-100-5-50	50	13,3	±	1,3	4,05	±	0,02