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ECOLOGICAL EXTRACTION OF PROTEIN FROM BROCCOLI (*Brassica oleracea* VAR. *italica*) STEM RESIDUES

Extracción ecológica de proteínas a partir de residuos de tallo de brócoli (*Brassica oleracea* var. *italica*)

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Key words: plant waste, biomolecules, eutectic solvent, ultrasound-assisted extraction.

ABSTRACT

The search for innovative and ecological techniques to obtain high-value compounds from waste materials with a lower impact on the environment is prevailing. Particularly, in protein extraction, utilizing plant waste can reduce environmental impact by taking advantage of residues that, if not properly managed, contribute to soil and air pollution. Deep eutectic solvents (DES) emerge as a sustainable and efficient alternative to plant protein extraction, aiming to replace organic solvents, which are generally toxic. Similarly, employing eco-friendly techniques for the extraction of protein is crucial, with ultrasonication being widely recognized as a green technology. By combining these strategies, an important decrease in environmental impact can be feasible. In the present work, we compared ultrasound-assisted protein extraction (80% amplitude, 30 ºC, 15 min) from a dried broccoli stem sample using two different solvents: ethanol and a deep eutectic solvent consisting of sodium acetate and urea (NaOAc:urea). Total protein was determined by the Bradford technique, showing that using ethanol for the extraction makes it possible to obtain an average of 0.212 ± 0.07 mg/mL, while with the DES the total protein obtained was 0.778 ± 0.09 mg/mL. We optimized ultrasound-assisted protein extraction using NaOAc:urea, assessing time, temperature, and amplitude. Ultrasonic amplitude and extraction time were the most critical factors. The optimal condition was 15 min of sonication at 80% amplitude and 40 ºC, which maximized the protein yield. This demonstrates that broccoli stems, as plant waste, represent a valuable source of protein. Also, that using DES for protein extraction from broccoli stems could maximize its utilization and contribute to a more sustainable cycle in plant protein production, as it may reduce the environmental impact associated with traditional solvents, and is also more effective in protein extraction.

Palabras clave: residuo vegetal, biomoléculas, solvente eutéctico, extracción asistida por ultrasonido.

RESUMEN

La búsqueda de técnicas innovadoras y ecológicas para obtener compuestos de alto valor a partir de residuos que supongan un menor impacto en el medio ambiente es predominante. En particular, en el caso de la extracción de proteínas, el uso de residuos vegetales puede reducir el impacto ambiental al aprovechar residuos que, si no se

gestionan adecuadamente, contribuyen a la contaminación del suelo y el aire. Además, el uso de disolventes eutécticos profundos (DES, por su sigla en inglés) surge como una alternativa sostenible y eficiente para la extracción de proteínas vegetales, con el objetivo de sustituir el uso de disolventes orgánicos, que suelen ser tóxicos. Del mismo modo, el uso de técnicas ecológicas en la extracción de proteínas es crucial, siendo la ultrasonicación ampliamente reconocida como una tecnología verde. La combinación de estas estrategias puede contribuir a la disminución del impacto ambiental. En el presente trabajo, evaluamos el uso de dos solventes para la extracción asistida por ultrasonido de proteínas a partir de una muestra seca de tallo de brócoli. La proteína total fue determinada por la técnica de Bradford, demostrándose que, si se utiliza etanol para la extracción, es posible obtener un promedio de 0.212 ± 0.07 mg/mL, mientras que con el DES, compuesto por acetato de sodio y urea (NaOAc:urea), la proteína total obtenida fue de 0.778 ± 0.09 mg/mL. Posteriormente, optimizamos la extracción de proteínas asistida por ultrasonido con NaOAc:urea, evaluando diferentes condiciones de tiempo, temperatura y amplitud ultrasónica. Los resultados mostraron que la amplitud ultrasónica y el tiempo de extracción son los factores más significativos en el incremento de proteína total de un residuo de tallo de brócoli. Esto demuestra que el tallo de brócoli, un residuo vegetal, representa una buena fuente de proteína, y que el uso de DES para la extracción de proteína podría maximizar su utilización, contribuyendo a un ciclo más sostenible en la producción de proteína vegetal. Esto, debido a que puede reducir el impacto ambiental asociado con los disolventes tradicionales y también es más eficaz para la extracción de proteínas.

INTRODUCTION

The growing world population, estimated to exceed 10 billion people by 2050, is generating significant pressure on agricultural production, especially in terms of protein demand. This increase in demand has led to the exploitation of various protein sources, but has also resulted in a growing environmental footprint that contributes to climate change (Aimutis 2025).

Due to the high level of greenhouse gas emissions associated with animal protein production, alternative sources are being explored, focusing on plant proteins, specifically utilizing plant waste products, such as those from broccoli (*Brassica oleracea* var. *italica*). Broccoli production has increased by 32.1% in the last decade, reaching 37.2 × 106 t in 2018 (production values combined with cruciferous), with China and India as the main producers. These countries accounted for 81.1% of the total world production, followed by the United States, Mexico, and Spain. The global increase in the production of broccoli residues is mainly due to its relevance as a beneficial health food (de Evan et al. 2020). In Mexico, the state of Guanajuato produces the largest amount of broccoli (420 770 t in 2018), and is estimated to produce a similar amount of broccoli stems. Agricultural production of broccoli involves soil treatment, greenhouse germination of seeds, tillage, harvesting activities, and transportation (Shinali et al. 2024).

Once the agricultural cycle is accomplished, broccoli is transported approximately 20 km to the processing plant, where 50 % of the total material is transformed into broccoli stems. The remaining biomass is processed in order to obtain broccoli florets, which are frozen, packaged, and exported. Finally, the broccoli stems, without any stabilization treatment, are transported 60 km to the processing plant (Quintero-Herrera et al. 2021).

Previous research has shown that 70% of the total weight of broccoli plants is wasted in fields, whereas 45-50% of the edible broccoli florets harvested are wasted during processing and transportation (Berndtsson et al. 2020). In this regard, the carbon footprint of broccoli has been reported to be about 0.81 kg CO_2 eq/kg (Rasines et al. 2023, Hernández-Jiménez et al. 2025).

Similar to other plant residues, the use of broccoli wastes in ruminant feed can decrease agricultural costs and environmental pollution caused by their accumulation, as broccoli residues have a high water content and are easily perishable (de Evan et al. 2020). On the other hand, several parts of broccoli are not used as food nowadays, but are interesting sources for new food products (Berndtsson et al. 2020, Shinali et al. 2024). However, their production has been limited due to the low yields of protein that can be obtained from these sources (Kumar et al. 2022). Additionally, the extraction and purification of bioactive compounds such as proteins often rely

on traditional organic solvents. Nevertheless, most of these solvents are flammable, environmentally damaging, and toxic to workers involved in the processing. Although they are not effective at extracting both polar and non-polar components, these solvents continue to be commonly used in the food industry. Consequently, there is an urgent need to develop alternative extraction solvents that can protect the environment and reduce their negative impacts on human health (Suthar et al. 2023).

Other conventional techniques for obtaining proteins from plant sources include alkaline extraction (Sun and Bandara 2019, Hewage et al. 2024), which has generated environmental concerns due to its use involving the production of residual water and high consumption of chemical products. This, in addition to environmental issues, increases production costs. Therefore, environmentally friendly extractions of compounds have been adopted because they involve a reduction in the number of unit operations, raw materials, and waste and ecological footprint, as well as the promotion of energy-saving methods (Chemat et al. 2012, Awad et al. 2021). For this reason, the search for green methodologies has been focused on green solvents such as deep eutectic solvents (DES). These mixtures have attracted the attention of researchers, not only because of their high efficiency in obtaining proteins, but also because they represent a considerable decrease in the negative environmental impact (Hewage et al. 2024).

The search for efficient techniques has led to the implementation of ultrasound-assisted methodologies, which have been used to extract proteins by DES. This process has been considered environmentally friendly and has been investigated with the aim of substituting those in which organic solvents are used (Kingwascharapong et al. 2021, Liu et al. 2023). Additionally, ultrasound-assisted extraction (UAE) has been widely used to obtain superior yields in protein extraction, considering that it is not only more efficient, but also an economical and energy-efficient process, as it employs high-energy-density acoustic waves that produce the cavitation effect, which favors the rupture of plant cell walls, promoting mass transfer and collision between particles (Chemat et al. 2017, Liu et al. 2023). Additionally, it has been reported that UAE has the capability to reduce or eliminate the requirement for organic solvents, mitigating environmental impact, while significantly enhancing the yield of target bioactive compounds, establishing it as a valuable industrial method (Shen et al. 2023).

Nevertheless, it is essential to adapt the experimental conditions focused on samples such as plant residues in order to obtain the best extraction conditions. Therefore, the aim of this work was to evaluate the extraction of total protein from dried broccoli stem waste obtained with a DES in comparison to a conventional organic solvent (ethanol), employing ultrasonic sonication under the following conditions: 80% amplitude, 30 ºC, and 15 min. Additionally, the influence of these parameters was analyzed to optimize extraction efficiency.

MATERIALS AND METHODS

Obtention of broccoli stem

The broccolis were purchased in a local market in Santiago de Querétaro, Querétaro, Mexico. They were washed with a solution of hypochlorite (2 ppm v/v) and water. Then, the florets were removed to obtain stems, which were cut into slices of approximately 0.5 cm. The stems were weighed and subsequently dried under sun conditions from 9:00 to 16:00 LT, and finally dried in an oven at 60 ºC. The total drying time was about 24 h until the loss of humidity (Kumar et al. 2017, Babu et al. 2018). After drying, the weight of the stems was measured; then, they were pulverized in a mill at 30-s intervals and stored in hermetic glass containers until use.

Preparation of DES sodium acetate:urea (NaOAc:urea)

The DES was prepared in a ratio of 1:2, using a solution of sodium acetate (0.1 M) and urea (0.1 M). The mixture was heated at 80 ºC for 2 h under stirring conditions until the formation of a homogeneous transparent liquid (Cui et al. 2021). Finally, the solvent was tempered and stored at 4 ºC until use.

Obtention of broccoli stem extracts

For extraction, 10 mL of the respective solvent (NaOAc:urea or ethanol 96%) was placed in a 25 mL beaker, and 0.2 g of a dried broccoli stem sample was added. This mixture was transferred to an ultrasonic homogenizer (Huxi, model JY88-IIN). The exploratory experimental condition for both extractions was 80% amplitude, temperature of 30 ºC, and time of 15 min, considering the parameters reported for vegetable protein extraction (Ivanović et al. 2020, Çelik et al. 2022, Inguanez et al. 2023, Yin et al. 2024). Afterwards, the extracts were vacuum filtered using 1 μm pore size filter paper, and then they were kept at 4 ºC under dark conditions for further analysis.

Total protein determination

By using the Bradford technique, the total protein content in each extract was determined. For each extract, 50 µL was added to 500 µL of Bradford reagent, and the mixture was stirred using a vortex for 10 s. After the samples were incubated at room temperature for 10 min, we measured their absorbance at 595 nm using a UV-Vis spectrophotometer model Genesys (Bose et al. 2021). To quantify the protein, a calibration curve using bovine serum albumin (BSA) was prepared within a range of 0.1 to 1 mg/mL.

Optimization of protein extraction using NaOAc:urea

The best solvent for extracting total protein was used to prepare an experimental design to improve the extraction conditions. This analysis considered conditions such as amplitude, time, and temperature. Using the statistical software Design-Expert 13, we performed an optimal custom design, considering three numeric factors: ultrasound amplitude (20, 40, 60, and 80%); temperature (20, 30, 40, and 50 ºC); and time (5, 15, and 25 min). As a response, the protein content (mg/mL) was evaluated using the Bradford technique.

Statistical analyses

A one-way analysis of variance (ANOVA) was conducted to evaluate the effects of sonication time, temperature, and ultrasonic amplitude. The Tukey test was used for mean comparisons between treatments (ethanol vs. NaOAc:urea), with both analyses performed using Minitab 19. Furthermore, the coefficient of determination (R^2) was calculated to quantify the variance explained by the model.

RESULTS

Total protein determination

The Bradford technique demonstrated that, by performing the extraction with ethanol, it is possible to obtain a total protein content of 0.212 ± 0.07 mg/mL in the extract, whereas extraction with the eutectic solvent yielded a total protein content of 0.778 ± 0.09 mg/mL in the extract. **Figure 1** shows photographs of the appearance of both extracts, which, after sonication, exhibited different physical characteristics; **figure 1a** presents a yellow and crystalline extract, while **figure 1b** presents a brown and more turbid extract.

Improvement of extraction using NaOAc:urea

Based on the experimental design, the results (**Fig. 2a**, **b**) showed the differences between the effects of each factor on protein content. **Figure 2a** illustrates the difference in protein content due to different times (5, 15, 25 min) of ultrasonic extraction, demonstrating through statistical analysis that significant differences exist between the measurements; at 5 min, the obtention of protein is statistically different compared to 15 and 25 min; however, at 15 min, there is a tendency that suggests that with this condition, a better protein extraction was obtained. The results presented in **figure 2b** indicate that at a higher ultrasonic amplitude (80%), there is a tendency for protein increase compared to the other amplitudes evaluated. Temperatures between 20 and 50 ºC (**Fig. 2c**) did not show statistically significant differences in protein content. The values of these results are shown in **table I**.

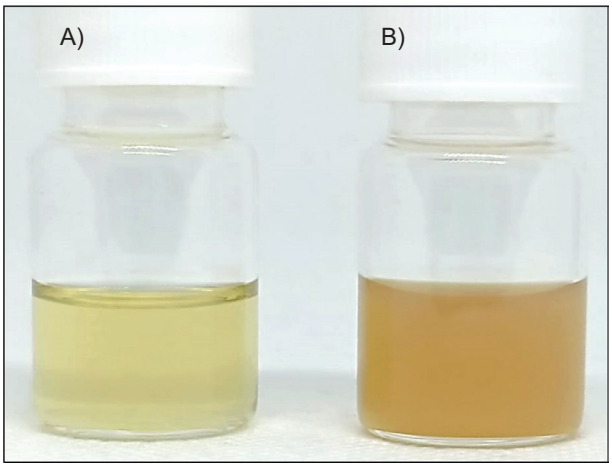

Fig. 1. Photography of extracts obtained by using (a) ethanol and (b) NaOAc:urea, after vacuum filtration.

The analysis of variance (**Table II**) showed that sonication amplitude and time are the factors that affect protein extraction, also indicating that the interaction between sonication amplitude and temperature has a significant effect on the response.

Response surface analysis of protein extraction with NaOAc:urea

The response surface plot in **figure 3A** shows the interaction between the sonication amplitude and time, indicating an effect on the increase of protein content by increasing the sonication amplitude and working in extraction times between 15 and 20 min, which is confirmed in **figure 3C**. On the other hand, the interaction between sonication amplitude and temperature (**Fig. 3B**) shows a tendency indicating that, by increasing the sonication amplitude and considering temperatures above 30 ºC, a higher amount of protein can be obtained.

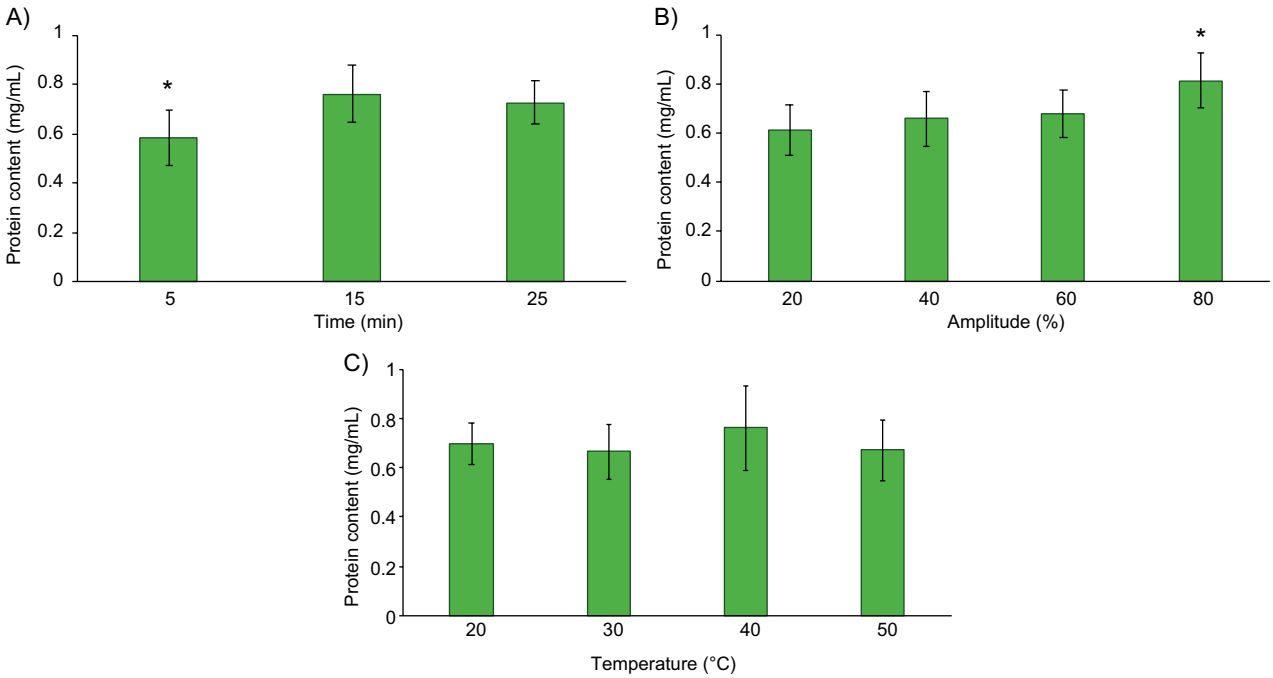

Fig. 2. Extraction of protein with NaOAc:urea based on (a) time, (b) sonication amplitude, and (c) temperature, based on the Bradford method. Error bars represent the standard deviation of triplicate measurements. (Means with an asterisk are statistically different).

TABLE I. RESULTS ONE-WAY-ANOVA AND POST HOC (TUKEY) TEST OF CONTENT OF PROTEIN INFLUENCED BY TIME, ULTRASONIC AMPLITUDE, AND TEMPERATURE, CONSIDERING A CONFIDENCE LEVEL OF 95%.

Factor	Value	Mean	Grouping*
Time	15	0.7599	A
	25	0.6974	A
	5	0.6708	B
Amplitude	80	0.8123	B
	60	0.6804	A
	40	0.6581	A
	20	0.6108	A
Temperature	40	0.7599	A
	20	0.6974	A
	50	0.6708	A
	30	0.6638	A

Validation of the statistical model

To verify the proposed model, its validation was conducted. This model presents an adequate normality adjustment since the residuals plot (**Fig. 4a**) shows a trend along a straight line, which satisfies the normality assumption. On the other hand, the residuals plotted vs. the predicted response (**Fig. 4b**) show a random distribution in the graph, demonstrating that the model fits the data satisfactorily.

DISCUSSION

The difference in the values of protein extracted using ethanol and NaOAc:urea was attributed to the chemical nature of the solvents, mainly because ethanol is more volatile, tends to precipitate proteins and has affinity for extracting polar compounds (Yoshikawa et al. 2012, Ferraris and Qian 2021), whereas the chemical species forming the DES (sodium acetate and urea) act as hydrogen bond acceptor and donor, respectively, generating through this type of bonds, a higher extraction yield, as well as protein solubilization (Ling et al. 2020, Rico et al. 2021, Gallego et al. 2023). Ethanol is also used in protein extraction due to its ability to precipitate proteins, as well as its effect on solubilization (Hu et al. 2016, Tai et al. 2020). However, it has been shown that the impact on protein denaturation using DES is lower compared to other systems, such as alkaline extraction, as well as organic solvents such as

TABLE II. ANALYSIS OF VARIANCE FOR PROTEIN DETERMINATION.

Source	Sum of squares	df	Mean square	F-value	p-value
Model	0.3854	10	0.0385	6.27	0.0002
A-Amplitude	0.1287	1	0.1287	20.95	0.0001*
B-Temperature	0.0016	1	0.0016	0.26	0.6119
C-Time	0.0738	1	0.0738	12.02	0.0022*
AB	0.0388	1	0.0388	6.32	0.0197*
AC	0.0052	1	0.0052	0.85	0.3654
BC	0.0037	1	0.0037	0.60	0.4456
A^2	0.0083	1	0.0083	1.34	0.2588
B^2	0.0021	1	0.0021	0.34	0.5602
C^2	0.0675	1	0.0675	10.98	0.0032*
Residual	0.1351	22	0.0061		
Lack of Fit	0.1247	19	0.0066	1.89	0.3319
Pure Error	0.0104	3	0.0035		
Cor Total	0.5205	32			
R^2	0.7404				
Adjusted R^2	0.6224				

*P-values less than 0.0500 indicate model terms are significant.

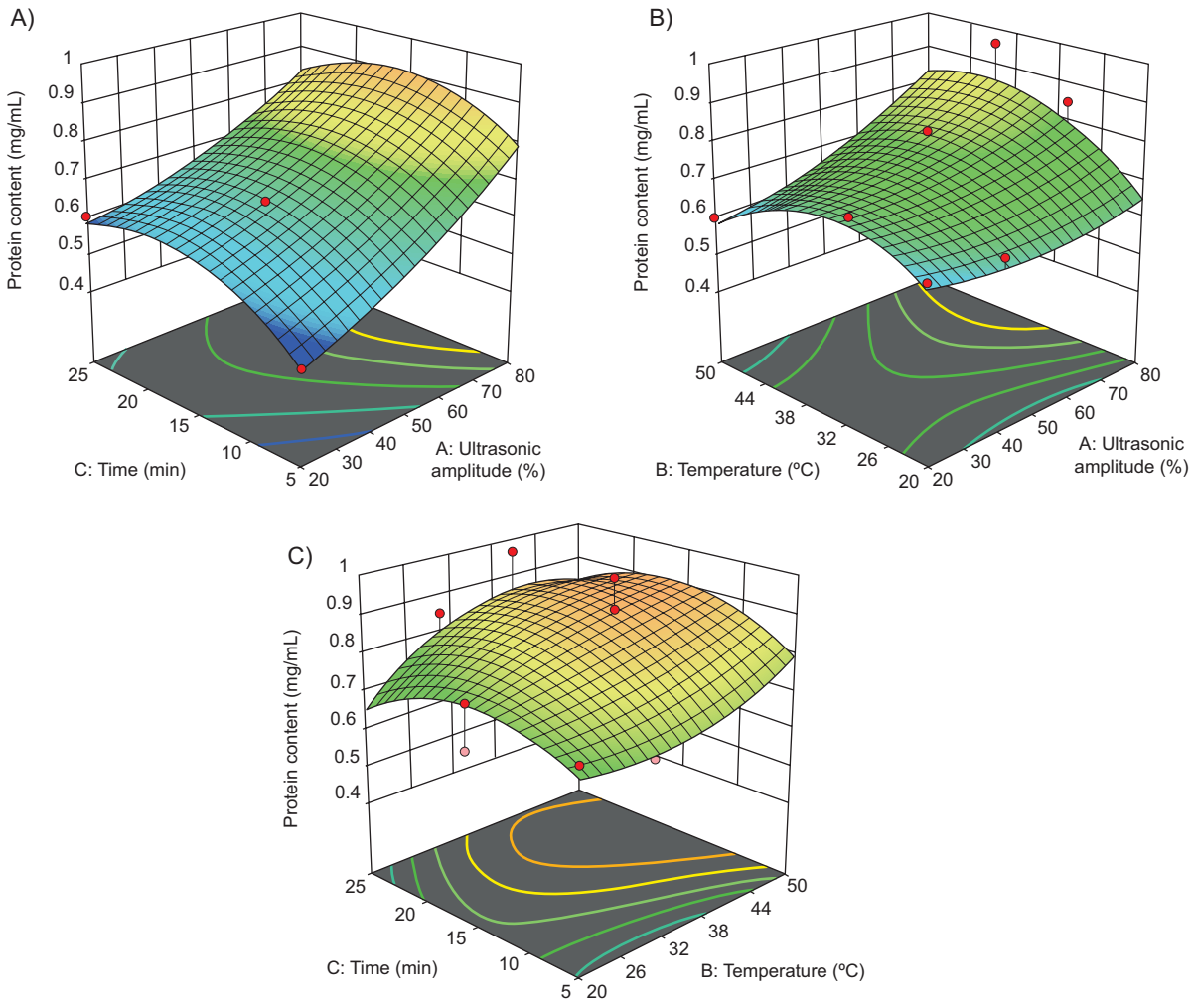

Fig. 3. Surface response diagram of interaction of (a) time and amplitude, (b) temperature and amplitude, and (c) time and temperature on protein determination.

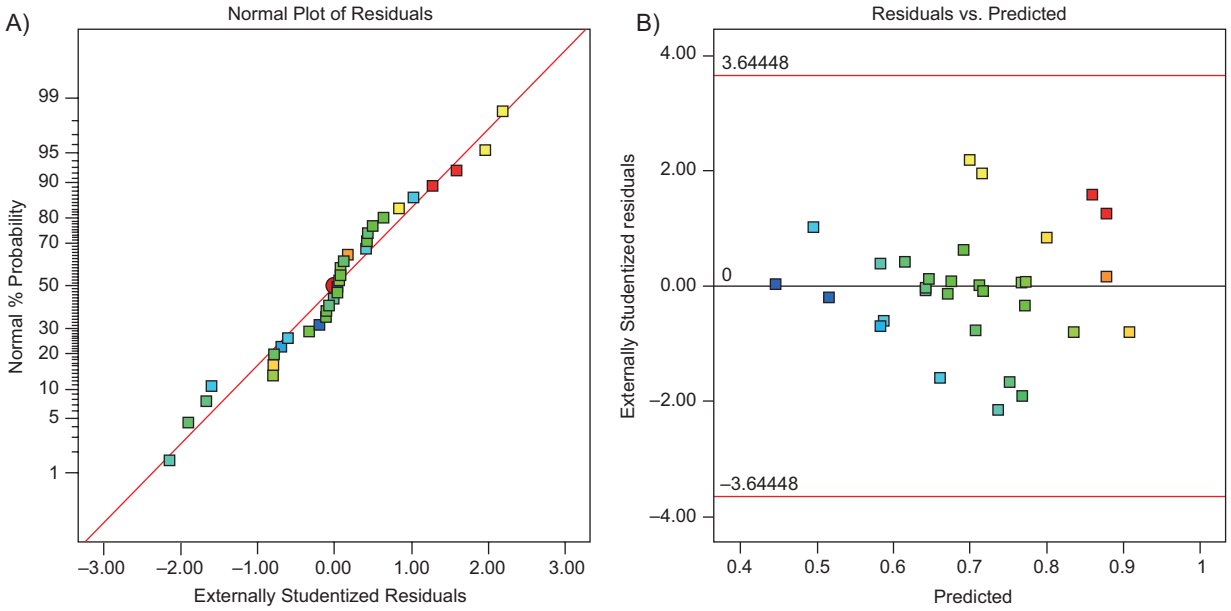

Fig. 4. Graphic of (a) normal plot of residuals and (b) residuals vs predicted response.

ethanol (Bowen et al. 2022, Zhou et al. 2022). Based on previous findings (Farooq et al. 2020), it has been demonstrated that urea-based DES can extract 79% of protein, compared to conventional extractions (alkalis or organic solvents), which, under the same experimental conditions, can achieve extractions in the range of 40-50%.

The graph of the extraction time (**Fig. 2a**) shows that when extracting for 25 min there was a decrease in the amount of protein extracted, which is attributed to the fact that, depending on the characteristics of the mass-solvent ratio, an excess in the extraction time promotes the aggregation of proteins as well as the saturation of the system, causing less mass transfer and, consequently, lower yields (Kumar et al. 2022, Mirón-Mérida et al. 2024). Additionally, it has been reported that, for extracting plant proteins, the best time conditions range is from 15 to 20 min, which can be attributed to the destruction of the plant cell wall due to the duration of exposure to the cavitation effect (Dabbour et al. 2018, Fatima et al. 2023).

On the other hand, the different temperatures evaluated did not show significant differences (**Fig. 2b**); however, it is important to consider using temperature values that maintain the innocuity of the proteins (less than 70 ºC), since, if higher temperatures are used, denaturation of the proteins can occur (Wiesner et al. 2021, Ma et al. 2022, Sajib et al. 2023). The response surface analysis (**Fig. 3**) shows the influence of each independent variable on the protein content obtained, suggesting that increasing both sonication amplitude and temperature yields higher protein extraction. It has been demonstrated that increasing the sonication amplitude can play a role as one of the factors that allows a higher quantity in the extraction of vegetable protein, since the bubbles formed in the medium collapse due to cavitation, generating greater power and intensity of the waves, resulting in a higher protein yield (Kingwascharapong et al. 2021, Mirón-Mérida et al. 2024). However, it has been reported that protein yield may be affected by excessive amplitude increases, which can cause protein denaturation due to the cavitation effect. This phenomenon can be explained by the mass transfer principle and ultrasonic energy distribution in the solution, as well as the smaller ratio having a higher concentration gradient, leading to a higher diffusion rate and extraction yield (Phongthai et al. 2017).

Other factors that influence the protein extraction process using DES include solvent concentration, the type of salt used for its preparation, and the mass-solvent ratio (Tang et al. 2024). On the other hand, it has been demonstrated that the influence of ultrasonication in the extraction of protein from plant samples, such as fruit peels, stems and rough structures, can suffer partial erosion due to the penetration impacts of the eutectic solvent in the cell wall, and when an ultrasonic pulse is applied (García-Villegas et al. 2022, Cao et al. 2023). Fragmented structures have

been observed as a result of exposure to a greater amount of compounds, which promotes the release of intracellular components, resulting in extraction with higher yield (Fu et al. 2021).

It has been considered that, in a homogenization treatment, material pulverization and solvent extraction are completed in one step. Therefore, ultrasonic sonication extraction is a clean production process, with short operation time, absence of dust contamination, low extraction temperature, and high efficiency (Cao et al. 2019). Additionally, considering its use with DES, the protein yield obtained demonstrates its potential application in various industries (Hewage et al. 2024).

Due to the great potential of DES in the extraction of bioactive compounds, it is crucial to adapt these methodologies to extract proteins and other bioactive compounds from different sources. Each sample has unique characteristics, and the solvent properties can be adapted depending on the biomolecule to be extracted. In addition, it is important to consider that ultrasound-assisted and DES-based extraction methodologies can be improved by considering different factors such as treatment duration, temperature, sample composition, solvent ratio, size and shape of the sonicator, etc. (Rahman and Lamsal 2021). In this context, the combination of conditions for protein extraction with NaOAc:urea was crucial, as the evaluated circumstances played a significant role in optimizing the process. Therefore, it is recommended to focus on this extraction method as it increases protein yield and provides bioactive compounds with various industrial applications (Tang et al. 2024). In addition, the advantages of using DES over organic solvents lead us to consider further studies on the potential toxicity of these solvents, their stability, and their environmental impact (Hewage et al., 2024).

CONCLUSIONS

Our results show that the ultrasound sonication technique enables higher protein extraction from a broccoli stem residue when sodium acetate and urea are used as a DES, compared to ethanol. By performing the protein extraction under various conditions, we demonstrated that the optimal condition for extraction was 15 min of sonication at 80% ultrasonic amplitude and 40 ºC. Under these conditions, DES not only showed high extraction efficiency but also facilitated process optimization. Based on the observed results for protein extraction, ultrasound-assisted deep eutectic solvents could be an excellent alternative for obtaining other biomolecules, although specific conditions require further testing.

These findings highlight that broccoli stems, a plant waste product, represent a promising source of protein, and that utilizing DES for protein extraction could maximize their use while contributing to a more sustainable cycle in plant protein production by reducing the environmental impact associated with traditional solvents and enhancing extraction efficiency.

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