Optimization of Ultrasonic-Assisted Enzymatic Extraction of Betalains from Pitahaya Peel: A Comparative Study with Conventional Methods

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Abstract
Ultrasonic-assisted enzyme extraction (UAEE) is an innovative extraction technique that has demonstrated good yields for the extraction of bioactive compounds because it combines the advantages of enzymatic and ultrasonic extraction. This study evaluated UAEE to obtain rich extracts of betalains and antioxidant compounds from the pitahaya peel. The effect of temperature (20, 30, 40, and 50 °C), extraction time (15, 30, 45, 60, 75, and 90 minutes), and enzyme/substrate ratio (100, 200, 300, and 400 mg/g) on the total betalains was analyzed. UAEE was compared with other conventional methods to assess its ability to extract betalains and antioxidant compounds. The concentration of total betalains decreases with the increasing temperature of the ultrasonic bath, resulting in a higher concentration at 20 °C. In the case of time, it can be observed that the highest concentration is at 45 minutes; after that (60, 75, and 90 minutes), a decrease is observed. The quantity of enzyme that extracted the most significant amount of betalains was 25 mg. The UAEE gave the highest total betalains and antioxidant compound values to compare with enzymatic hydrolysis and ultrasonic bath extraction ($p \leq 0.05$). UAEE is an innovative extraction technique that can obtain rich extracts of betalains from the pitahaya peel, potentially used to formulate functional foods.

Keywords: *antioxidant activity, betalains, digestive enzymes, hylocereus undatus, pitahaya peel, ultrasonic-assisted enzymatic extraction*

1. Introduction
Pitahaya is an exotic fruit belonging to the family of Cactaceae and is native to tropical and subtropical areas of the American continent (Oney-Montalvo et al., 2023). This fruit has been shown to have multiple gastronomic and nutraceutical uses, attracting recent attention due to its economic value and possible health benefits (Garbanzo et al., 2021). This has led to an increase in exports from the Yucatán Peninsula, one of the leading production areas of Mexico, reporting a production in 2023 of 2355.97 tons (SENASICA, 2024).

Pitahaya peel is rich in phytochemicals with good bioactivity, which can be reused to develop functional foods or serve as antioxidants to prolong the shelf life of food; this is why the food industry has used them for fortification or as nutritional ingredients (Jiang et al., 2021). New functional foods have also been developed, for example, wheat and bakery products, dairy products, meats, confectionery, and wines (Huang et al., 2021). Also, biodegradable food coatings with antioxidant and antibacterial activity have been developed from extracts obtained from the pitahaya peel (Jiang et al., 2021).

The leading group of compounds that confer these characteristics are betalains. These are water-soluble pigments present in the fruits of the cactus *Hylocereus* sp, and compared to other natural pigments such as anthocyanins, carotenoids, and chlorophylls, they have not been studied in depth (Slimen et al., 2017). Betalains are classified into two types: betacyanins and betaxanthins, both of which have been shown to have pharmacological properties, among which anti-cancer, antimicrobial, anti-lipid, and antioxidant activity stand out (Slimen et al., 2017). Because of this, it has been proposed for use in the food industry to give foods their antioxidant and pharmacological effects. It may also be used as a food additive to prevent the discolouration of foods, demonstrating its potential application in functional foods (Calva-Estrada et al., 2022).

In the literature, different methods have been reported that have allowed the production of betalains from various types of matrices, highlighting (1) ultrasonic-assisted extraction, (2) microwave-assisted extraction, and (3) enzyme-assisted extraction (Bastos et al., 2017, Da Silva et al., 2018, Lombardelli et al., 2021). In the quest to improve yields in bioactive compound production, a combination of these techniques has been proposed, resulting in ultrasonic-assisted enzyme extraction (UAEE), which has demonstrated good yields because it combines the advantages of enzymatic and ultrasonic extraction (Liao et al., 2015). The efficiency of UAEE (Figure 1) can be influenced by different factors, among which are (1) extraction time, (2) extraction temperature, (3) the amount of enzyme used, (4) ultrasound power, and (5) liquid-to-solid ratio (Li et al., 2017). So, these factors should be evaluated when implementing this technique.

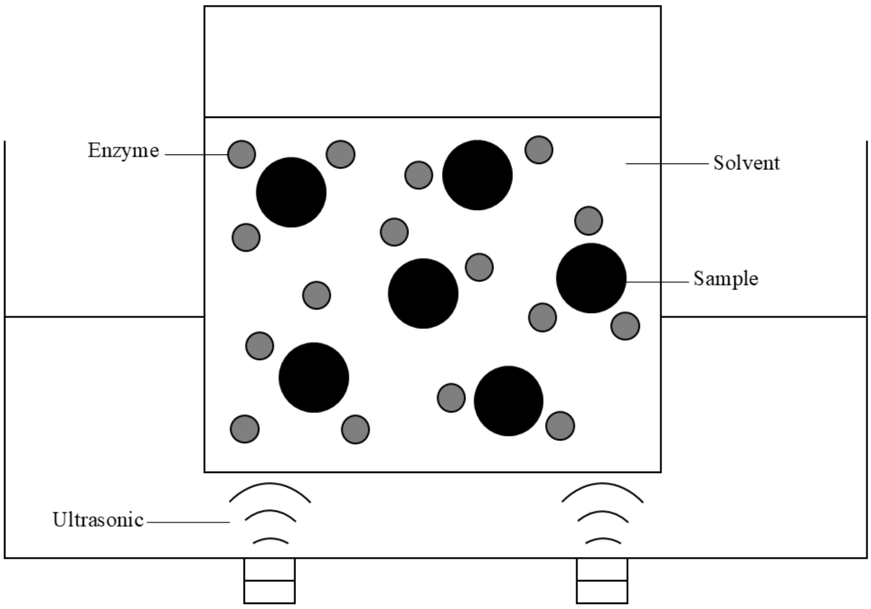

Figure 1: Schematic diagram of ultrasonic-assisted enzymatic extraction (UAEE)

Despite the technical advantages, UAEE for bioactive compounds from pitahaya peel has not yet been reported. Because of this, in the present research, UAEE will be evaluated as an innovative extraction technique that can be used to obtain rich extracts of betalains from the pitahaya peel. This will allow the use of the peel of this exotic fruit, which is considered a by-product discarded in industrial processes. Extracts obtained can also be used to formulate functional foods that help promote optimal health in consumers.

2. Objectives

1) To evaluate ultrasonic-assisted enzymatic extraction (UAEE) in obtaining betalain-rich and antioxidant-rich extracts from the pitahaya peel.
2) To analyze the effect of temperature, time, and enzyme quantity on the yield of total betalains from pitahaya peel using UAEE.
3) To compare the efficiency of UAEE with conventional extraction techniques in terms of total betalains yield and antioxidant compound extraction from pitahaya peel.

3. Materials and Methods

3.1 Materials

The solvents used were ethanol and methanol grade reagents (≥99 %) of the brand Sigma Aldrich® (St. Louis, Missouri, United States). The water used in this work was distilled type II, brand B. Medina® (Mexico). 2,2-diphenyl-1-picrylhydrazil (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were Sigma Aldrich® (St. Louis, Missouri, United States) with purity of ≥98 %.

The enzyme used was a blend of vegetarian digestive enzymes of the brand Life extension® composed of protease SP (*Aspergillus oryzae, Bacillus subtilis*) 25000 FCC, bromelain (*Aspergillus comosus*) 800000 FCC, lactase (*Aspergillus oryzae*) 2000 FCC, cellulase (*Aspergillus niger*) 2000 FCC, lipase (*Candida rugosa*) 2500 FCC, alpha-galactosidase (*Aspergillus niger*) 150 FCC, hemicellulase (*Aspergillus niger*) 4000 FCC, pectinase (*Aspergillus niger*) 25 FCC, protease 3.0 (*Aspergillus niger*) 10 FCC, xylanase (*Trichoderma longibrachiatum*) 500 FCC.

3.2 Raw Material

The pitahayas (*Hylocereus undatus*) were purchased at a market in the town of Calkiní, Campeche, Mexico. The fruit peels were separated from the pulp and dried at 45 °C for 72 hours in a convection oven. Subsequently, these were ground and sieved at a size of 40-mesh (0.4 mm) to homogenize the particle size. Finally, the obtained powder was stored in Falcon tubes and refrigerated at -4 °C until it was analyzed.

3.3 Extraction Methods

3.2.1 Extraction by Ultrasonic Bath

Ultrasound-assisted extraction was performed using a reference to the work done by Singh et al. (2021), with some modifications; this consisted of the conditions shown below. 250 mg of the sample was weighed, and 8 mL of distilled water was added. The samples were then sonicated for 30 minutes at 25 °C.

3.2.2 Extraction by Enzymatic Hydrolysis

The extraction was carried out by weighing 250 mg of the sample and 25 mg of the digestive enzyme blend, and then 8 mL of distilled water as a solvent was added. The mixture was continuously stirred with a vortex for 60 minutes.

3.2.3 Ultrasonic-assisted Enzyme Extraction

An ultrasonic bath (Creworks, USA) of 3L capacity with a sonication energy of 120 W and a frequency of 40 kHz was used. The extraction was performed by weighing 250 mg of the sample and adding 8 mL of water as solvent. The extraction conditions were a) temperatures of 20, 30, 40, and 50 °C; b) extraction times of 15, 30, 45, 60, 75, and 90 minutes; and c) enzyme/substrate ratios of 100, 200, 300, and 400 mg/g. The ultrasonic bath temperature was controlled using ice packs to prevent it from increasing due to sonication. After extraction under the abovementioned conditions, the samples were centrifuged at 4500 rpm for 10 minutes, and the supernatant was filtered with a nylon membrane disc filter with a pore size of 45 µm. The extracts obtained were analyzed using the methodology shown below.

3.4 Determination of Total Betalains

Total betalains were determined using the work of Shakir & Simone (2024) as a reference with some modifications. Samples were placed in cells, and readings were taken at 538 nm and 480 nm for betacyanins and betaxanthins, respectively. The concentration of total betalains was calculated from the sum of the concentrations of betacyanins and betaxanthins. This was done with a UV-Vis spectrometer (PerkinElmer®). The absorbances obtained were analyzed using the following formula to get the concentrations of betacyanins and betaxanthins in the extracts:

$$\text{Betacyanins or betaxanthins } (\mu g/mL) = \frac{(A)(DF)(MW)}{(e)(l)}$$

Whereas A is Absorbance at 538 nm for betacyanin and 480 nm for betaxanthin; DF is dilution factor; MW is molecular weight (betacyanin: 550 g/mol; betaxanthin: 308 g/mol); e is Molar extinction coefficient (betacyanin: 60,000 L/mol cm; betaxanthin: 48,000 L/mol cm); and l is cell length (1 cm).

3.5 Determination of Antioxidant Activity by DPPH Radical Scavenging Assay

The antioxidant activity was determined by the 2, 2-diphenyl-1-picrylhydrazil (DPPH) radical removal method following the methodology described by Brand-Williams et al. (1995), with some modifications. In 15 mL Falcon tubes, 3800 µL of DPPH solution (0.1 mM) was added, and 200 µL of extract was subsequently added. The mixture is homogenized using a vortex and incubated in the dark at room temperature for 60 minutes. After, the reading was done at 515 nm absorbance with a UV-Vis spectrophotometer (PerkinElmer®), and the percentage of inhibition of DPPH for each sample was calculated with the following formula:

$$\text{\% of DPPH inhibition} = \frac{A_{t0} - A_{t60}}{A_{t0}} \times 100$$

Where: At_0 is the absorbance at 0 minutes; At_{60} is the absorbance at 60 minutes. The calibration curve uses Trolox at different concentrations as a reference standard (100, 150, 200, 300, 400, 500, 600, 700, and 800 µM).

3.6 Determination of Antioxidant Activity by ABTS Radical Scavenging Assay

The determination of antioxidant capacity by ABTS methodology was carried out using the work prepared by Nenadis et al. (2004) as a reference, with some modifications. For the preparation of ABTS+ solution, 19.2 mg of the reagent was weighed and dissolved in 5 mL of water, then 88 uL of $K_2S_2O_8$ (37.8 mg/mL) was added and incubated at room temperature for 16 hours. ABTS+ was diluted with ethanol to absorb 0.7 0.02 at 734 nm. In a 15 mL Falcon tube, 2970 µL of ABTS+ solution was added, and 30 µL extract was subsequently added. Homogenized and left to stand in the dark at room temperature for 8 minutes. Absorbances were read at 734 nm in a UV-vis spectrophotometer (PerkinElmer®). To calculate the percentage of ABTS inhibition for each sample, use the formula shown below:

$$\text{\% of ABTS inhibition} = \frac{A_{to} - A_{t8}}{A_{t0}} \times 100$$

Where: At_0 is the time absorbance at 0 minutes; At_8 is the absorbance at 8 minutes. The calibration curve was prepared using different concentrations of Trolox (100, 150, 200, 300, 400, 500, 600, 700, and 800 µM).

3.7 Statistical Analysis

Analysis of variance (ANOVA) was used with $\alpha = 0.05\%$ to analyze all data. The statistical test used to separate means was the last significant difference test with a confidence level of 95%. All statistical analyses were performed with the software Statgraphics Centurion XVII.II-X64 (Statgraphics Technologies Inc., the Plains, VA, USA).

4. Results and Discussion

4.1 Evaluation of the Effect of Temperature

The effect of temperature on extractions is one of the most studied because this factor has been shown to affect yield due to its ability to change sample porosity, mass transfer, surface tension, and viscosity of extracts (Dzah et al., 2020). The temperature increase correlated with a better extraction due to the rise in the abovementioned characteristics. However, working at too high temperatures instead of improving extraction performance results in the compounds being readily hydrolyzed and oxidized, mainly when extracted for extended periods (Dzah et al., 2020).

The results of the evaluation of the effect of temperature on total betalains extraction by the ultrasonic-assisted enzyme extraction technique are shown in Figure 2. The concentration of total betalains presented in the results was calculated from the sum of the concentrations of betacyanins and betaxanthins. The concentration of total betalainas decreases with increasing temperature of the ultrasonic bath, resulting in a higher concentration of the compound of interest at 20 °C (475.3 ± 26.5 µg/g). On the other hand, the lowest concentration of betalains was obtained when extraction was carried out at a temperature of 50 °C, resulting in a concentration of 385.5 ± 12.0 µg/g. This result may be because high temperatures lead to degradation of the betalains. This phenomenon has been observed in other pigments where the sonication temperature affects the extraction yields (Maran et al., 2015). In addition to the report by Da Silva (2018), the concentration of betalains decreases when the extraction temperature increases.

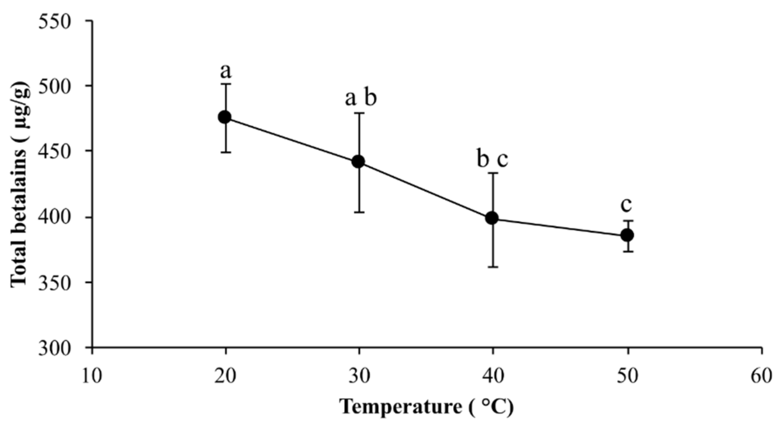

Figure 2: Effect of temperature on the extraction of total betalains by ultrasonic-assisted enzyme extraction (UAEE). Different letters (a, b, c) are used to demonstrate statistically significant differences.

4.2 Evaluation of the Effect of Time

Extraction time is an essential factor to assess because it is reported to be one of the most effective factors in the ultrasonic extraction of bioactive compounds; very short times do not allow complete extraction of the compounds found in the matrix, and very extended times could cause the degradation of some molecules of interest (Da Silva et al., 2018). In addition, time will be related to the extraction stages, which will be two main ones. These are (1) extraction of surface compounds, which occurs between the first 10 to 20 minutes; this phenomenon consists of the dissolution of the compounds found on the surface of the matrix; (2) slow extraction, in which the transfer of the solute from the matrix to the solvent is carried out by diffusion; this stage may last longer times, which can last more than 60 minutes (Medina-Torres et al., 2017).

The results obtained from the time evaluation in the ultrasonic-assisted enzyme extraction are shown in Figure 3. In this one, it can be observed that as the extraction time passes, the amount of betalains extracted from the pitahaya shell begins to increase, extracting the highest concentration of this compound in 45 minutes (710.6 ± 7.2 µg/g). At extraction time after 45 minutes (60, 75, and 90 minutes), a decrease is observed, which may be associated with the degradation of betalains. This is consistent with what Maran et al. reported (2015), in which times of extraction greater than 45 minutes resulted in a reduction in total betalains.

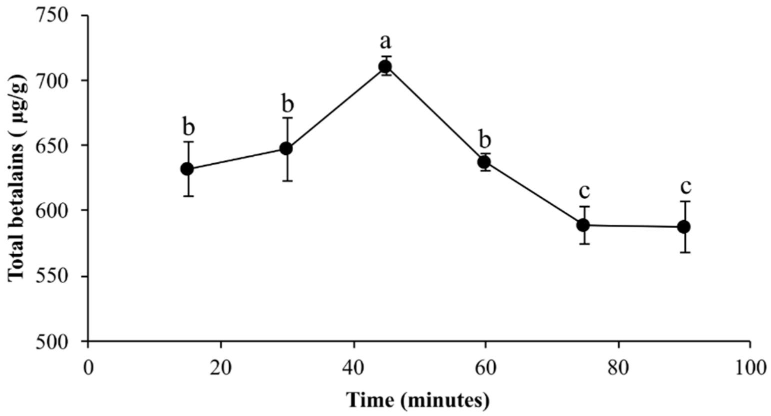

Figure 3: Effect of time on the extraction of total betalains by ultrasonic-assisted enzyme extraction (UAEE). Different letters (a, b, c) are used to demonstrate statistically significant differences.

4.3 Evaluation of Enzyme Quantity

Figure 3 shows the results of evaluating the quantity of enzymes in extracting total betalains from pitahaya peel by the UAEE. The results showed that the lowest extraction yield was obtained using a 400 mg/g of enzyme/substrate ratio (242.0 ± 24.5 µg/g). The highest yields were achieved at 100, 200, and 300 mg/g, which showed no significant differences ($p \leq 0.05$) in total betalains extraction (521.3 – 536.4 µg/g). Although there are no significant differences between the concentrations of 100, 200, and 300 mg/g ($p \leq 0.05$), a tendency is observed that extraction yield begins to decrease when the enzyme/substrate ratio is increased; this may be attributed to inhibition of final production caused by too rapid hydrolysis (Huynh et al., 2023).

The phenomenon observed in Figure 4 is because the enzymatic hydrolysis will be influenced to a large extent by the concentration of the enzyme used in the reaction, that the higher amount of enzyme evaluated has given the lower yield of hydrolysis can be explained by the enzymatic aggregation that occurs when a high concentration of the enzyme is used, causing the amount of enzyme in contact with the substrate to be reduced, and therefore the catalytic activity.

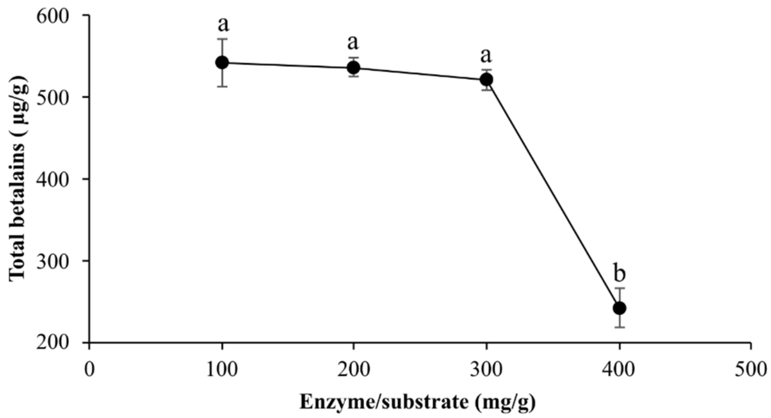

Figure 4: Effect of enzyme quantity on the extraction of total betalains by Ultrasonic-assisted enzyme extraction (UAEE). Different letters (a, b, c) are used to demonstrate statistically significant differences.

4.4 Comparison with Other Extraction Methods

Table 1 shows the results of comparing ultrasonic-assisted enzyme extraction with ultrasonic bath extraction and enzymatic hydrolysis. UAEE was carried out at a temperature of 20 °C, time of 45 minutes, and enzyme/substrate ratio of 100 mg/g. Total betalains were analyzed using the spectrophotometric method, and DPPH and ABTS used antioxidants. The results showed that the ultrasonic-assisted enzyme extraction gave the highest values of total betalains (710.6 ± 7.2 µg/g), followed by enzymatic hydrolysis (492.2 ± 24.8 µg/g), and the ultrasonic bath (369.2 ± 41.3 µg/g) gave the lowest values. In the case of antioxidant activity, the DPPH technique showed no significant difference between hydrolysis and ultrasonic-assisted enzyme extraction ($p \leq 0.05$). Finally, the antioxidant activity measured by ABTS again positioned the ultrasonic-assisted enzyme extraction as the technique that gave the best results (264.4 ± 13.7 mg of Trolox/100g).

Table 1 Concentration of betalains and antioxidant compounds extracted from the pitahaya peel using different extraction techniques

Extraction method	Total betalains (µg/g)	DPPH – Antioxidant activity (mg of Trolox/100g)	ABTS – Antioxidant activity (mg of Trolox/100g)
Ultrasonic bath	369.2 ± 41.3^{c}	100.7 ± 16.1^{b}	101.6 ± 32.4^{c}
Enzymatic hydrolysis	492.2 ± 24.8^{b}	205.4 ± 11.8^{a}	215.9 ± 29.2^{b}
Ultrasonic-assisted enzyme extraction	710.6 ± 7.2^{a}	208.3 ± 5.9^{a}	264.4 ± 13.7^{a}

Note: Values presented are the means ± standard deviation; a, b, c means groups of responses. Values followed by different letters in the same row indicate statistically significant differences using the least significant difference (LSD) test at $p \leq 0.05$.

5. Conclusion

Ultrasonic-assisted enzyme extraction showed better results for the extraction of betalains and antioxidant compounds than enzymatic hydrolysis and ultrasonic baths. The factors evaluated significantly affected the extraction of betalains, with the best results at a temperature of 20 °C, an extraction time of 45 minutes, and an enzyme/substrate ratio of 100 mg/g. This indicates that ultrasonic-assisted enzyme extraction is a technique that can be applied to extract betalains and antioxidant compounds from pitahaya peel, with the potential to produce extracts for the formulation of functional foods.

6. Acknowledgements

The authors thank the Instituto Tecnologico Nacional de México (TecNM) for funding project No. 20401.24-PD.

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