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Ultrasound-Assisted Extraction of Betalains from *Opuntia* Fruit Pulp of Different Color Varieties

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Abstract: Betalains are water-soluble pigments that have exhibited important pharmacological properties such as antioxidant, anticancer, antilipidemic and antimicrobial activity. These compounds have been isolated in numerous purple plants or fruits, as is the case of the wild species under the *Opuntia* genus. The fruits of these species are often disregarded because of their small size as well as the frequent presence of prickles. Based on this, this research has as its objective the optimization of a method based on ultrasound-assisted extraction to obtain extracts enriched with betalains from a wild *Opuntia* species (*Opuntia dillenii* (Ker Gawl.) Haw.). Four variables (%EtOH in the solvent, temperature of extraction, ultrasound amplitude and cycle) were selected using a Box–Behnken design. The quadratic interaction of %EtOH and the interaction of %EtOH–cycle have proven to be influential variables at 95% confidence. The conditions to obtain the highest betalain concentration were 100 mg of pulp with 20 mL (60%:40% EtOH:H_2O) solvent at 20 °C at 24% amplitude and 0.2 cycle for 10 min. The suitability and reliability of the method were evaluated with repeatability and intermediate precision tests obtaining CVs <5%. Finally, the developed method has been employed in the analysis of five *Opuntia* commercial samples and obtained significant antioxidant activity of the extracts, confirming its applicability.

Keywords: antioxidant activity; betalains; Box–Behnken; DPPH; optimization; *Opuntia dillenii*; ultrasound-assisted extraction

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1. Introduction

In recent years, increasing consumers' consciousness of the consumption of foods and additives and how they affect their health has triggered an increment in the use of natural pigments derived from plants or fruits. Some of these pigments are polyphenols, anthocyanins, or betalains, which have become rather commonly used in the food industry these days [1,2], mainly because they present certain pharmacological properties of great interest, such as antioxidant, anticancer, antilipidemic, or antimicrobial activity [1,3].

Betalains, in particular, are nitrogenous pigments soluble in water that contain the group betalamic acid in their basic structure [4]. These pigments can be organized into: (i) betacyanins (of a reddish-violet color) and betaxanthins (of a yellowish-orange color). The former include hydroxycinnamic acid derivatives or sugars in their structure, while the latter have incorporated amino acids or amine derivatives [5]. Betalains can be found in a multitude of plants, vegetables, and flowers [6,7], especially in those of a violet color. Betalains have exhibited certain important health-promoting properties. Firstly, they exhibit a free radical scavenging activity that represents a significant antioxidant capacity [8–10]. They have also been confirmed to protect the vascular endothelium from damage caused by inflammation alterations from oxidations induced by cytokines [11,12].

In summary, these compounds have shown a considerable antiradical activity that suggests their hepatoprotective capacity [13,14].

More than 200 species have been classified under the *Opuntia* genus within the *Cactaceae* family [8]. Their fruits are commonly known as prickly pear and have sizes that can vary from 20 mm to 50 mm and a weight of around 10–20 g. These fruits present a thick peel and pulp of red-purple color with numerous seeds of small size [15]. Furthermore, they present a pleasant acidic taste that, together with their characteristic purple color, makes them very attractive for consumers, either to be consumed as is or even as juice mixed with other fruits [16].

However, other species in this genus are considered wild invasive species, such as *Opuntia dillenii* (Ker Gawl.) Haw. that grows in the Canary Islands, Murcia and Andalusia [17]. These wild species usually produce small-size fruits, compared to those from other species of the *Opuntia* genus, sometimes covered with prickles, which is why they are often disregarded for any usage. Nevertheless, many researchers have investigated their content of bioactive compounds and their potential applicability. These studies have confirmed the presence of several compounds of interest in these wild species, such as phenolic compounds, polysaccharides and betalains, which are responsible for their characteristic purple color [18–20]. Multiple analytical techniques have been employed to extract betalains from *Opuntia* fruits, such as solid–liquid extraction [20], Soxhlet [11], and pressurized-liquid extraction [4].

Ultrasound-assisted extraction (UAE) is one of the non-traditional technologies that is most often employed to obtain enriched extracts in interesting analytes from natural matrices. This analytical technique uses the power of ultrasound to increase the extraction yield by generating cavities and localized hotspots that break the matrix's cell walls, thus favoring the transfer of the interesting compounds into the solvent [21,22]. Extraction yields can also be enhanced based on the polarity of the solvent used [23,24]. This technique has been extensively used to obtain enriched extracts in polyphenols, anthocyanins, or betalains from different fruits and plant matrices because of its multiple advantages, such as short extraction times, reduced amount of solvent and plant material and greater extraction yields [21,25].

In fact, bioactive compounds (including betalains) have been previously extracted and identified in *Opuntia* fruits using UAE [26–28]. However, this research has been focused on the flowers and peel of the fruit, and not on its pulp, which is more commonly consumed directly and used in different commercial products such as jams, desserts, custards or ice creams. This study intends, therefore, to determine the variables that exert any significant influence on UAE efficiency and their subsequent optimization for efficient extraction of the betalains from the pulp of *Opuntia* commercial fruits. To the best of our knowledge, there are few previous studies of betalains in *Opuntia* fruit pulp. In most of these articles [29], the bioactivity of the aqueous extracts of the pulp of this fruit has been evaluated; however, the composition of these extracts has not been identified or quantified. As a result, betalain-enriched extracts, with their health-promoting properties and applicability to the food, medicine, pharmacy, or cosmetic industries, are expected to be obtained.

2. Materials and Methods

2.1. Samples

Opuntia dillenii (Ker Gawl.) Haw. was selected as the wild species to be taken as the reference to optimize the extraction procedure. Their fruits were collected from Vía Verde to Matagorda, Puerto Real (36°32′14.7″ N 6°12′11.0″ W), and the water of the fruit pulps (without seeds) was removed by means of a VirTis BenchTop Pro Freeze Dryer (SP Industries, Warminster, PA, USA) and subsequently ground using an electric MKM6003 coffee grinder (BSH Electrodomésticos España S.A., Zaragoza, Spain). The powder was kept at −20 °C until its analysis. Once the method had been optimized and its suitability had been confirmed, it was validated by comparing the extracts obtained from pulps from "garden" prickly pears without prickles (collected from Torrecera (36°36′43.6″ N 5°56′11.2″ W) and

four commercially available products purchased from "El Tio lo Chumbo", Coin, Malaga. These 4 products were named by their producer as Red, Pink, Malagueño (of a dark orange color) and White (green color).

2.2. Chemicals and Reagents

Water was obtained from a Milli–Q water purification system from Millipore (Bedford, MA, USA), and ethanol (EtOH, Scharlau S.L., Sentmenat, Spain) at HPLC grade was the solvent used to extract the betalains from the samples. For the chromatographic separation of the betalains to be identified and quantified, water, ethanol and formic acid (Scharlau S.L., Sentmenat, Barcelona, Spain) at HPLC grade were the solvents employed.

For the determination of the antioxidant activity of the extracts obtained, 6–hydroxy–2,5,7,8–tetramethylchroman–2–carboxylic acid (Trolox) (Sigma-Aldrich, Steinheim, Germany) was used as a standard, and DPPH (2,2–diphenyl–1–picrylhydrazyl) (Sigma-Aldrich, San Luis, MI, USA) was employed to determine radical scavenging.

2.3. Ultrasound-Assisted Extraction (UAE)

2.3.1. Ultrasound Equipment

The system employed for the UAE of betalains was a UP200S ultrasonic device (Hielscher Ultrasonics GmbH, Teltow, Germany) coupled to a water bath (Selecta, Abrera, Spain).

For the extraction procedure, 100 mg samples were weighed into the extraction vessel and mixed with 20 mL of solvent (at the corresponding percentage of ethanol). Then, the probe was placed into a flask while ensuring that it did not touch its walls, and the ultrasound waves were applied at the corresponding amplitude and cycle for 5 min. The invariable parameters (amount of sample, volume of solvent, and time of extraction) were selected according to previous experiments in comparable matrices and compounds [30]. Once the extraction process was completed, the resulting mixture was centrifuged at 6810 g for 5 min and at 4 °C by means of a centrifuge manufactured by J.P. Selecta, Abrera, Spain. The supernatant was separated and adjusted to 25 mL so that all the extractions had the same volume. Finally, nylon syringe filters (0.22 µm) were used to perform filtration prior to analysis through a UHPLC–QToF–MS and quantification by means of a UHPLC–Diode Array Detector (DAD).

2.3.2. Optimization Procedure

The effect of the UAE variables was determined using a Box–Behnken Design–Response Surface Methodology (BBD–RSM). This method uses a type of quadratic design which employs three-level incomplete factorial methodology. This means that three levels are assigned to each independent variable, where −1 is the lowest level, 0 is the intermediate level, and 1 is the highest level. This results in comprehensive data sets that are suitable for analysis [31], where the axial points are discarded, i.e., design points are spherically distributed. Hence, in contrast with the more commonly used orthogonal design, the amount of experiments is lower [32], as extreme experimental values are excluded. These mild conditions represent a substantial advantage, since highly power-demanding or otherwise conditions, which could produce betalain degradation, were disregarded. For our particular case, four independent variables to be optimized were selected as follows: percentage of ethanol in the solvent (10–35–60%), extraction temperature (10–35–60 °C), amplitude (10–30–50%), and cycle (0.2–0.5–0.8 s^{-1}). The variables and the range to be studied were selected according to the group experience in comparable matrices and compounds [30,33]. The BBD–RSM comprised a total of 27 experiments (Table 1) that were performed randomly.

Table 1. Box–Behnken design obtained from the experiments completed to optimize the procedure to extract betalains from *Opuntia dillenii*.

Experiment	%EtOH	Temperature (°C)	Amplitude (%)	Cycle	Total Betalains Observed (mg/g)	Total Betalains Adjusted (mg/g)	Error (%)
1	35	10	30	0.2	2.48	2.90	14.47
2	35	35	50	0.8	2.58	2.38	8.61
3	10	35	30	0.8	3.34	3.90	14.31
4	35	35	30	0.5	2.33	2.10	11.05
5	35	35	30	0.5	2.39	2.10	13.69
6	35	10	50	0.5	2.32	1.95	18.85
7	60	35	10	0.5	2.56	2.65	3.16
8	35	10	30	0.8	2.40	2.37	1.40
9	35	60	50	0.5	1.63	1.68	2.96
10	60	60	30	0.5	2.52	2.78	9.43
11	60	35	30	0.8	2.52	2.37	6.36
12	10	35	30	0.2	2.49	2.25	10.77
13	10	10	30	0.5	3.20	3.02	5.76
14	60	35	30	0.2	4.42	4.47	1.11
15	35	60	10	0.5	1.22	1.20	2.14
16	60	35	50	0.5	2.42	2.98	18.76
17	35	35	10	0.2	2.15	2.44	11.99
18	35	35	10	0.8	1.94	1.89	2.86
19	35	10	10	0.5	2.16	2.11	2.00
20	10	35	10	0.5	2.73	2.47	10.28
21	10	60	30	0.5	2.21	2.10	5.17
22	35	60	30	0.8	2.20	2.09	5.53
23	10	35	50	0.5	2.24	2.46	8.99
24	35	35	30	0.5	2.08	2.10	0.95
25	60	10	30	0.5	2.85	3.04	6.46
26	35	35	50	0.2	2.12	2.27	6.26
27	35	60	30	0.2	1.66	2.00	16.93

A mathematical model fits a second-order polynomial function Equation (1) according to the response variable generated from the data resulting from the chromatographic analysis:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4 + \beta_{11}{X_1}^2 + \beta_{22}{X_2}^2 + \beta_{33}{X_3}^2 + \beta_{44}{X_4}^2 \quad (1)$$

where Y is the aforesaid response, and β_0 corresponds to the ordinate, whereas X_1 (%EtOH in the solvent), X_2 (extraction temperature), X_3 (amplitude) and X_4 (cycle) are independent variables. For last, βi correspond to the linear coefficients, βij to the cross-product coefficients and βii to the quadratic coefficients. The influence of each of the independent variables selected and their interactions on the resulting extraction of betalains, the second-order mathematical model, the surface graph, the optimal levels of the influential variables and the variance analysis were calculated using the software application Statgraphic Centurion (version XVII) (Statgraphics Technologies, Inc., The Plains, VA, USA)

2.3.3. Repeatability and Intermediate Precision

Once the method had been optimized, its suitability was evaluated according to repeatability and intermediate precision tests. For the repeatability evaluation, nine experiments were conducted on the same day, whereas to determine the intermediate precision of the method, nine additional extractions were performed on two consecutive days (a total of 27 extractions), all under optimal conditions. The coefficients of variation obtained were the statistical parameters selected to determine the suitability and accuracy of the developed method.

2.4. Identification of Betalains by UHPLC–QToF–MS

The betalains present in *Opuntia dillenii* fruits were identified by means of ultra-performance liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer, (UHPLC–QToF–MS) (Xevo G2, Waters Corp., Milford, MA, USA) according to the method described by Carrera et al. [30]. This represents a total of 13 min per experiment, including 3 min to return to the initial conditions. The retention time and exact molecular weight were employed in the identification of individual betalains. Three betalains were identified: betanin ([M–H$^+$]: 551), isobetanin ([M–H$^+$]: 551) and neobetanin ([M–H$^+$]: 549).

2.5. Betalain Separation

Once betalains obtained from the optimized procedure were identified, they were quantified using a Chromaster UltraRS (VWR Hitachi, Tokyo, Japan) with a diode array detector. The methodology employed was the same described by Carrera et al. [30], which required a total of 10 min, including the time allowed to return to the initial conditions. Figure 1 shows a typical chromatogram (λ = 540 nm) of the characterized anthocyanins. The relative proportions of the betalains obtained were: betanin (57.62%), isobetanin (40.51%) and neobetanin (1.87%).

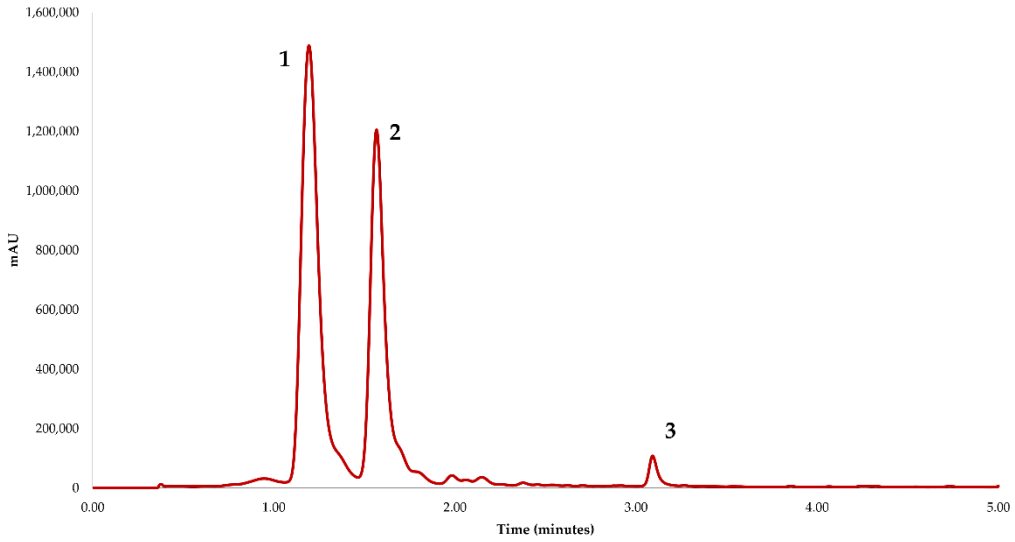

Figure 1. Chromatogram of the betalains identified in *Opuntia dillenii* extracts (λ = 540 nm). 1. Betanin; 2. Isobetanin; 3. Neobetanin.

2.6. Betalain Quantification

The amount of total betalains was determined by colorimetric analysis. The extracts were diluted in deionized water to determine their absorption within the appropriate range. Their absorbance was measured at 540 nm using a Cary 60 UV–Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The total betacyanin content was calculated according to Equation (2):

$$\text{Betalains content (mg/g)} = \frac{A \times V \times DF \times MW \times 10^{-3}}{\varepsilon \times L \times W} \quad (2)$$

where A is the absorbance at 540 nm, V is the volume of extracts (L), MW is the molecular weight of betanin (550 g·mol^{-1}), Ɛ is the molar extinction coefficient of betanin (65.000 L·mol^{-1} cm^{-1}), L is the path length of the cuvettes (1 cm) and W is the weight of the samples. The limits of detection and quantification were established after running the extraction of a blank six times. The LOD values (n = 6) and LOQ values (n = 6) were 0.01 and 0.04 mg·g^{-1}, respectively.

2.7. Antioxidant Activity

The antioxidant activity of the extracts obtained from the *Opuntia* species in the commercial samples was evaluated by DPPH assays based on the method described by Brand-Williams et al. [34] with some modifications by Miliauskas et al. [35].

Trolox was used as the standard, and a six-point linear regression model was generated from 0 until 1.4 mM in triplicate. The regression equation obtained (y = 88.94x + 0.75) exhibited a regression coefficient of R^2 = 0.9959. The antioxidant activity was expressed as mg of Trolox equivalents (TE) per gram of sample (mg TE/g).

3. Results and Discussion

3.1. UAE Optimization

As previously mentioned, BBD was selected as the method to determine the influence of the UAE variables on the extraction procedure of betalains from *Opuntia dillenii* fruit pulps. A total of four variables were selected to be evaluated, for which a total of 27 experiments (Table 1) were randomly performed. Based on the previous experience of our research team, 100 mg samples, 20 mL solvent and 5 min extraction time remained invariable for all the experiments. Finally, the extracts were analyzed by HPLC–UV–Vis, and the total betalains in the extracts were quantified to be used as the response variable. The results were analyzed by BBD–RSM at 95% confidence.

It was observed that the interaction between the percentage of EtOH and the cycle (*p*-value: 0.0005), and the quadratic interactions of the percentage of EtOH (*p*–value: 0.006), all with *p*-values lower than 0.05 (Table 2), were influential variables in the betalains extraction procedure.

Table 2. Results from the BBD–RSM analysis on the betalains extracted from *Opuntia dillenii* fruits.

Variable	Sum of Squares	*F*-Value	*p*-Value
X_1:%EtOH	0.19	0.64	0.439
X_2: Temperature	0.13	0.44	0.519
X_3: Amplitude	0.01	0.02	0.880
X_4: Cycle	0.07	0.24	0.632
AA	3.17	10.88	0.006
AB	0.11	0.37	0.555
AC	0.03	0.10	0.754
AD	3.50	12.01	0.005
BB	0.10	0.34	0.573
BC	0.10	0.36	0.561
BD	0.10	0.34	0.573
CC	0.29	0.99	0.340
CD	0.11	0.37	0.553
DD	0.74	2.55	0.136
Total Error (%)	3.50		

These results are graphically represented by means of a Pareto chart (Figure 2), where it can be detected that the interaction between the percentage of EtOH and the cycle value had a negative effect, whereas the quadratic interaction of the percentage of EtOH had a positive one, which suggests that the highest percentage values evaluated favored the extraction of the betalains. The quantified total betalains were correlated with the predicted values (Table 1), obtaining an average error of 8.16% that ranged from 0.95% to 18.85%. The model developed showed an R-squared statistic value of 0.85. Finally, a lack-of-fit analysis was used to verify the linearity properties of the model, obtaining a value of 0.08 (F = 1.69), confirming the linearity of the developed method and that no relevant differences between the predicted and the measured values were found.

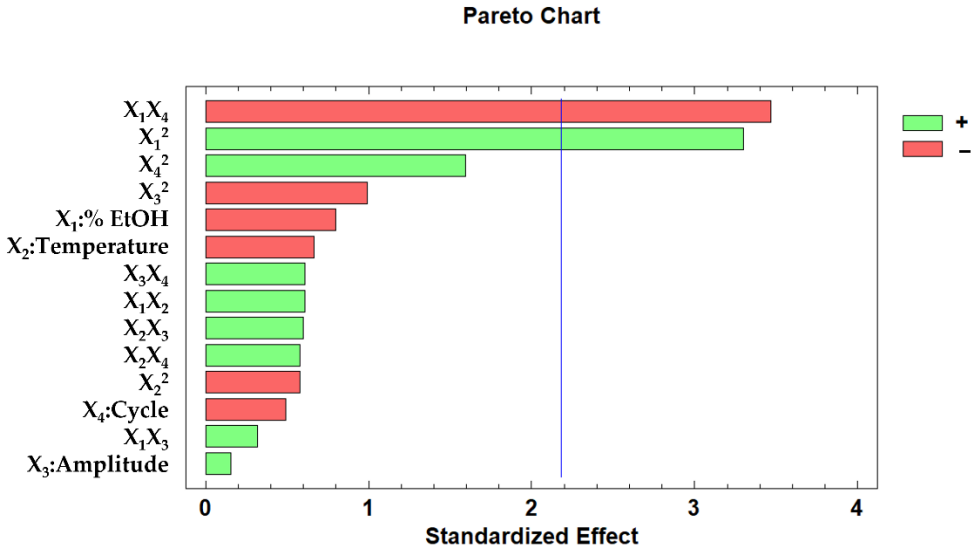

Figure 2. Pareto chart of the BBD–RSM analysis used to optimize the procedure for the extraction of betalains.

Then, the coefficients for a second-order polynomial equation used to estimate total betalains extracted according to the different variables were determined, which resulted in Equation (3) below:

$$Y = 5.46 \times 10^{-3} - 3.70 \times 10^{-5}X_1 - 3.08 \times 10^{-5}X_2 + 3.84 \times 10^{-5}X_3 - 1.57 \times 10^{-3}X_4 + 2.03 \times 10^{-6}X_1X_2 + 4.77 \times 10^{-2}X_1X_3 + 3.14 \times 10^{-3}X_1X_4 - 2.40 \times 10^{-4}X_2X_3 - 8.48 \times 10^{-2}X_2X_4 + 1.09 \times 10^{-6}X_3X_4 + 4.40 \times 10^{-5}{X_1}^2 - 1.82 \times 10^{-6}{X_2}^2 + 4.99 \times 10^{-5}{X_3}^2 + 6.30 \times 10^{-3}{X_4}^2 \quad (3)$$

In this equation, X_1 represents the percentage of EtOH in the solvent, X_2 the extraction temperature, X_3 the amplitude, and X_4 the cycle. Finally, the optimal conditions for UAE were calculated based on the previous data, and the results were as follows: 100 mg of samples extracted with 20 mL of solvent (60% EtOH) at 20 °C, with 22% amplitude and a cycle of 0.2. The optimal value of the cycle was at the lowest end of the evaluated range. No lower cycle values were tested, since 0.2 is already a rather low value for ultrasounds. In addition, numerous researchers have already proven that the application of this cycle value to UAE processes effectively enhances yield extractions [36]. On the contrary, the percentage of ethanol was at the top end of the evaluated range and, consequently, an extension of such range was to be considered.

In general, the optimal conditions obtained were similar to those described by other authors. This is, for example, the case of Gómez-López et al. [4], who determined that the best extraction conditions were 50% ethanol in water at 25 °C (cycle and amplitude were not optimized), which are very similar conditions to those determined by our research group. Brahmi et al. [26] established 36% ethanol at 53 °C for 60 min extraction time as the optimal conditions (cycle and amplitude were not optimized). Nevertheless, even if this temperature level would not cause any degradation of the bioactive compounds, the lower temperature requirements of our method represent an important advantage from an economic and environmental point of view.

3.2. Ethanol Percentage Optimization

The optimal percentage of ethanol for the extraction was at the high end of the evaluated range, which means that a wider range had to be examined. For this purpose, different extractions were performed at 50, 60, 70, 80, 90, and 100% EtOH. The rest of the conditions remained invariably at the optimal values established, i.e., 100 mg of the sample with 20 mL of the corresponding solvent at 20 °C with an amplitude of 24% and a cycle of 0.2 for 5 min. All the experiments were performed in duplicate. The extracts

were analyzed by HPLC–UV–Vis and the total betalains were calculated according to the method previously mentioned. The average total betalains for each ethanol percentage were calculated and are graphically represented in Figure 3.

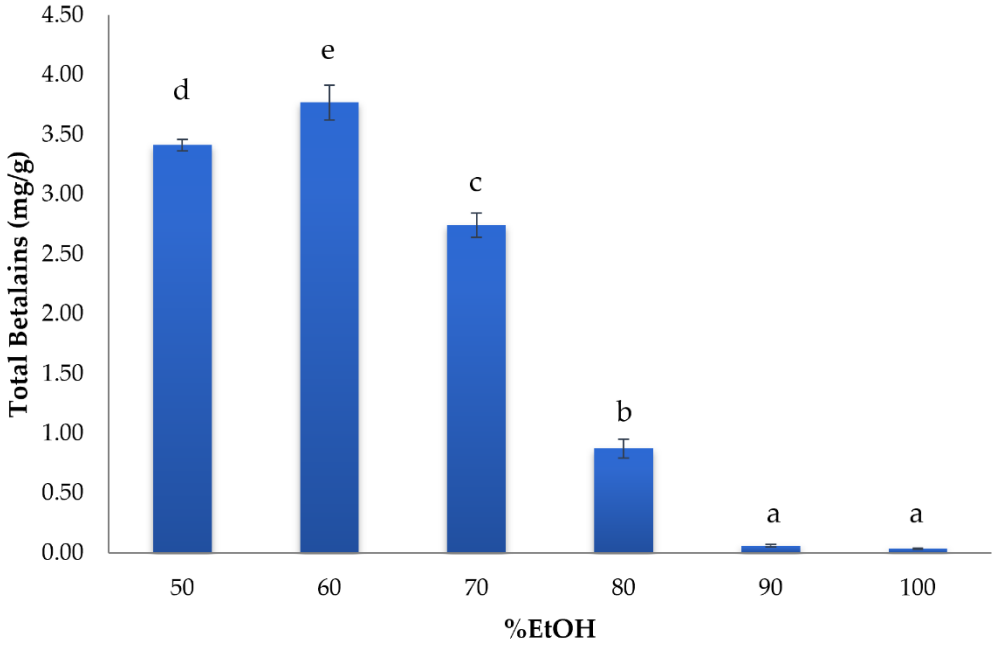

Figure 3. Total betalains extracted (mg/g sample) according to the percentage of EtOH in the solvent. Different letters indicate significant differences with 95% of confidence according to Duncan's post hoc analysis.

As can be seen from the graph, 60% EtOH achieved the highest total betalains concentrations, which could be related to the fact that solvents with an intermediate polarity facilitate the extraction of intermediate polarity compounds, like betalains [37]. Consequently, 60% was selected as the optimal value for ethanol percentage.

3.3. Optimal Extraction Time

The following step was to optimize the extraction time. For that purpose, multiple extractions were carried out at different extraction times (2, 5, 10, 15, 20, 25, and 30 min), whereas the rest of the variables were fixed at optimal values. All of them were performed in duplicate and the extracts were analyzed by HPLC–UV–Vis to determine the total betalains (mg/g sample) obtained. The average concentrations for each extraction time have been graphically represented in Figure 4.

It can be observed that the concentrations obtained increased as time was extended up to 10 min, when the maximum concentration was achieved. When longer times were applied, a reduction in the concentration could be noticed, although no significant differences between 15 and 30 min extraction time were registered. This concentration reduction was associated with the degradation of the betalains due to a longer exposure to ultrasound [30,38]. With regard to the 10 min extraction time, the results obtained were similar to those described by other authors with respect to the extraction of betalains [4,26].

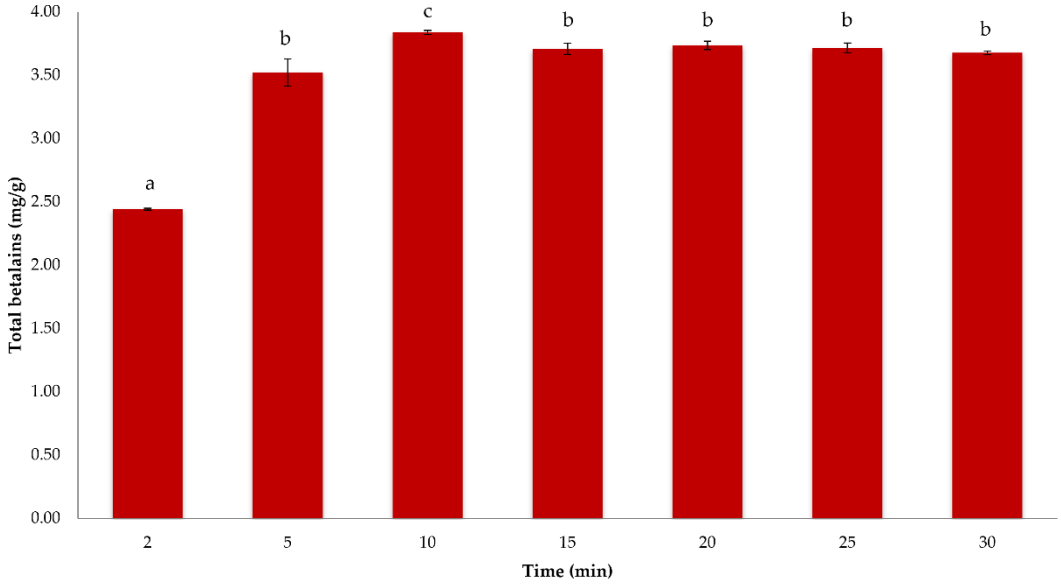

Figure 4. Total betalains (mg/g) obtained from *Opuntia dillenii* fruits according to the different extraction times tested. Different letters indicate significant differences with 95% of confidence according to Duncan's post hoc analysis.

3.4. Suitability of the Developed Method

The suitability and accuracy of the developed method were evaluated according to the evaluation of repeatability and intermediate precision. For this reason, 18 extractions were conducted under the optimal conditions established: 9 extractions were carried out on the first day, and 9 more extractions were carried out on each of the two following days.

Once the extracts had been obtained under the optimal conditions, they were analyzed by HPLC–UV–Vis and the total betalains were expressed as mg/g sample. The repeatability of the method was determined by the CV of the nine experiments performed on the first day, which was 1.05% (Table 3). The intermediate precision was calculated as the CV of the 27 experiments that were carried out on three consecutive days, with a value of 1.56%. In both cases the CVs were less than 5%, which confirms the repeatability and intermediate precision of the developed method. In addition, these values were expected according to the influence of the ultrasound power and temperatures used in our study on the betalain stability [26].

Table 3. Repeatability and intermediate precision of the developed method.

	Repeatability (*n* = 9)	**Intermediate Precision (*n* = 27)**
Average (mg/g)	3.57	3.59
Standard deviation	0.04	0.06
C.V.	1.05	1.56

3.5. Application to Real Samples

Finally, the developed method was applied to different samples (one cultivated sample and four commercial samples) in order to verify its suitability for other *Opuntiia* species. For that purpose, five commercial samples of *Opuntiia* genus, denominated as Garden, Red, Pink, Malagueño, and White, were analyzed. To this effect, 100 mg of each sample type were processed under the optimal conditions established (20 mL of solvent (60% EtOH) at 20 °C at an amplitude of 22% and a cycle of 0.2 for 10 min). Then, the extracts were analyzed by HPLC–UV–Vis. The total betalains were calculated and expressed as mg of betalains per gram of sample. Two replica were performed under the same conditions and the results are represented in Table 4. It could be observed that the Garden sample

(purple color) exhibited the greatest concentration with a total of 4.121 ± 0.040 mg of total betalains per gram of sample, followed by the Red sample with an average concentration of 0.153 ± 0.002 mg/g sample, whereas the rest of the samples presented significantly lower concentrations of betalains. These results were consistent with the color of the samples.

Table 4. Applicability of the developed method to commercial *Opuntiia* products.

Sample	Total Betalains (mg/g)	Antioxidant Activity (Trolox eq./g Sample)
Garden (purple)	4.121 ± 0.040	2.695 ± 0.039
Red	0.153 ± 0.002	0.773 ± 0.019
Pink	0.077 ± 0.001	0.743 ± 0.022
Malagueño	$0.013 \pm 3 \times 10^{-4}$	0.655 ± 0.006
White	$0.001 \pm 1 \times 10^{-5}$	0.570 ± 0.005

Furthermore, the antioxidant activity of these extracts was also measured according to the DPPH methodology and expressed in Table 4 as mg of Trolox per gram of sample. It was observed that the Garden sample exhibited the highest antioxidant activity, which is in agreement with previous observations. The rest of the samples exhibited good antioxidant activity, which would suggest a high antioxidant capacity of the betalains extracted. Therefore, its applicability to commercial products has been proven.

4. Conclusions

In this study, the influence from certain variables in UAE processes applied to betalain extraction from *Opuntia dillenii* (Ker Gawl.) Haw. has been evaluated. Consequently, it has been confirmed that the quadratic interaction of the percentage of EtOH and the interaction of the percentage of EtOH–cycle are influential variables in the process, with $p < 0.05$. In addition, the optimal conditions to obtain maximum concentrations of betalains have been established as follows: 100 mg of the sample with 20 mL (60% EtOH) solvent at 20 °C at an amplitude of 24% and a cycle of 0.2 for 10 min. Furthermore, the repeatability and intermediate precision properties of the method have been evaluated and CVs lower than 5% have been obtained. Finally, the suitability of the developed method to commercial products was studied by its application to five different products (Garden, Red, Pink, Malagueño and White). The resulting data confirm the suitability of the developed method for different *Opuntia* species, since the antioxidant activity exhibited by the betalains in the extracts was satisfactory. We can, therefore, conclude that a practicable method that benefits from UAE advantages was successfully developed to obtain betalains enriched extracts from *Opuntia* fruit species. The extraction method could be employed in the wild species that are currently disregarded, which would represent a valuable contribution to a circular economy that would benefit from the usage of the betalains that can be found in this fruit, or as a quality control method for *Opuntia* products derived from other currently available commercial products.

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