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Optimization of an Enzyme-Assisted Extraction Method for the Anthocyanins Present in Açai (*Euterpe oleracea* Mart.)

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Abstract: Several investigations have proven the presence of anthocyanins in different parts of açai plants. These compounds are responsible for the notable therapeutic properties of açai such as antioxidant, antimicrobial, anti-inflammatory, anticancer, and anticonvulsant. We have therefore optimized an enzyme-assisted extraction method for the anthocyanins found in açai, to be subsequently applied in many fields such as agrifood, medicine, or cosmetics. A Plackett–Burman design with seven variables (time of extraction, pH, temperature, agitation, percentage of ethanol in the solvent, amount of sample, and units of enzyme) was employed to determine the predominant extraction variables, of which four were categorized as influential. Subsequently, a Box–Behnken design–response surface methodology made it possible to determine the degree of influence from these variables and their optimal values. The optimal conditions were established as 0.1 g of açai heated up to 60 °C and extracted using 15 mL of solvent with pH 4 and 40% ethanol, 500 units of enzyme per gram of sample, and agitation at 150 rpm for 15 min. The repeatability and intermediate precision of the developed method were confirmed by variation coefficients below 5%. Finally, the developed method was compared against the extensively used maceration and ultrasound-assisted extraction methods.

Keywords: açai; anthocyanins; Box–Behnken; Enzyme-assisted extraction; Euterpe Oleracea Mart.; optimization; Plackett–Burman

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1. Introduction

The increasing food awareness of consumers–regarding its composition, the presence of additives and the different ways the compounds present in food can have an influence on health–has led to a notable growth in fruit and vegetables consumption. This is the case of numerous purple fruits [1,2], the characteristic color of which typically indicates the presence of certain kinds of polyphenols such as anthocyanins [3], whose consumption has skyrocketed in recent years because of their multiple beneficial properties.

Açai is the fruit of a tropical palm tree (Euterpe Oleracea Mart.) native to Northern and South America [4]. This rounded dark purple fruit with a diameter of about 1–1.8 cm [5] has been consumed for years by the people living in the Amazon estuary for its nutritional properties (high in carbohydrates, proteins, fats, and fibers) [6,7]. However, certain advances in food processing and transport have allowed a remarkable distribution of this fruit at a global level, with outstanding acceptance and an increasing demand because of its characteristic flavor and texture [8].

In addition, this has triggered a considerable growth in the number of research studies on the composition of this fruit, which has led to the discovery that it is one of the richest foods in natural antioxidants. About 90 bioactive substances have been described in açai,

among which flavonoids, lignoids, and anthocyanins; in addition, fatty acids, quinones, terpenes, and norisoprenoids stand out [6,9,10].

Anthocyanins are bioactive compounds with significant pharmacological properties such as antimicrobial, antioxidant, or even anticancer [11,12]. In fact, açai has exhibited its high impact potential on the treatment of Alzheimer's disease, the regulation of hypertension, the reduction of aging in mice, and the treatment of breast cancer, among others [13–19]. In addition, good results have been obtained from investigations on microcirculation or cardiovascular diseases [20,21], pulmonary emphysema [22], obesity [23], neurodegenerative diseases [24], or malaria [25]. It also provides chemoprotection against saxitoxins [26], ulcerative colitis, and intestinal damage [27–29], and enhances tissue regeneration [30]. In general, the treatment of diseases by means of açai biocompounds is mainly based on its ability to cause vasodilatation, the regulation of intracellular signal cascades, and its capacity to neutralize free radicals such as reactive oxygen species (ROS) [17], which prevents damage to the DNA and to macromolecules, thus mitigating the cumulative genetic damages that might trigger certain diseases [31].

Enzyme-assisted extraction (EAE) is based on the capacity that certain enzymes have to degrade or alter cell walls and thus allow the release of intracellular compounds, including certain bioactive compounds such as anthocyanins [32]. One of the main advantages of enzymes is that they can work effectively even under adverse conditions, in contrast with other chemical catalysts. Enzymes also have the capacity to act in synergy with other solvents. For this reason, certain combinations of chemical solvents with EAE processes enhance the efficiency and benefits of both methods, while being more environmentally friendly than many other conventional approaches to anthocyanin extraction. EAE, in particular, presents significant advantages in terms of anthocyanin extractions, since it is a process that can be conducted under mild conditions. Accordingly, moderate temperatures and shorter extraction times result in reduced consumption of energy while achieving substantial extraction yields [33]. Pectinases are the most frequently used enzymes for the extraction of natural compounds from plant matrices because of their broad substrate specificity and high stability under extreme extraction conditions [34,35].

EAE has been used for the extraction of phenolic compounds and anthocyanins from multiple matrices, such as pumpkin [35], residues from the production of blackberry wine [36], dragon fruit skin [37], or *Akebia trifoliata* [38]. However, to the best of the authors' knowledge, EAE has not been previously applied to açai samples. For this reason, this research study intends to optimize an EAE method for the efficient extraction of anthocyanins from açai samples because of their bioactive properties as antioxidants and their potential applications in multiple fields.

2. Materials and Methods

2.1. Samples

The lyophilized açai powder (0% water content) used for this research was acquired from NaturaleBio® (Monterotondo, Italy). This form of presentation ensures an improved contact between the sample and the solvent surfaces that enhances extraction yields. After the method had been optimized, it was applied to three different commercially available lyophilized açai products: the first one was acquired from Mundo Arcoiris Sin Límites, S.L., Besalú, Girona, Spain and the other two were purchased at a local supermarket (El Corte Inglés) in Cadiz, Spain.

2.2. Chemicals and Solvents

The solvents used for the extraction were composed of Milli Q water obtained from a Millipore water purification system (Bedford, MA, USA), absolute ethanol (Scharlau, Sentmenat, Spain), and methanol (Fisher Scientific, Loughborough, UK) of HPLC purity. The pHs of the extractions solvents were adjusted using HCl 1 M and NaOH 0.5 M solutions (Panreac, Barcelona, Spain).

For the chromatographic separation for the anthocyanin analysis and its quantification, formic acid (Panreac, Barcelona, Spain) and methanol (Fisher Scientific, Loughborough, UK), both of HPLC grade, were employed. Cyanidin chloride (95% purity, Sigma–Aldrich Chemical Co., St. Louis, MO, USA) was the reference standard selected for the quantification of the anthocyanins. Finally, a pectinase from *Aspergillus niger* (P4716–25KU, Sigma–Aldrich Chemical Co., St. Louis, MO, USA) was the enzyme chosen to carry out the enzyme-assisted extractions. A citric/phosphate buffer was added to ensure the stability of the enzyme against changes in pH. For that, different mixtures of citric acid 0.1 M (CH_8O_7) and sodium disodium phosphate dibasic dodecahydrate hydrate 0.2 M ($Na_2HPO_4 \cdot 12H_2O$) were employed. For pH 4, the mixture used was 62% CH_8O_7/38% $Na_2HPO_4 \cdot 12H_2O$; for pH 5, the mixture was 49% CH_8O_7/51% $Na_2HPO_4 \cdot 12H_2O$; and for pH 6, the mixture was 37.5% CH_8O_7/6.5% $Na_2HPO_4 \cdot 12H_2O$.

2.3. Enzyme-Assisted Extraction

2.3.1. Extraction Procedure

The general procedure followed for the extraction of the anthocyanins from the açai powdered samples was as follows: the corresponding amount of sample was mixed in an Erlenmeyer flask with both 15 mL of solvent (with the corresponding percentage of ethanol (%EtOH) and at the right pH) and the corresponding volume of buffer solution (according to the solvent pH). After that, the corresponding grams of the enzyme were added to the mixture and placed inside a temperature-controlled orbital shaker incubator (Nahita 640/1, 580 W, I.C.T., S.L., Lardero, La Rioja, Spain). The temperature, agitation, and time of the extractions were set and the incubator was turned on. After the incubating period was completed, the samples were centrifuged using a refrigerated Centrofriger BLT (JP Selecta, Abrera, Spain) and the resulting supernatants were filtered through 0.20 µm nylon syringes. Finally, the extracts were analyzed by UHPLC–UV–Vis, and the sums of the anthocyanins detected were expressed as total anthocyanins (mg/g of sample).

2.3.2. Optimizing the Enzyme-Assisted Extraction Method Experimental Design

First of all, a screening study was conducted to determine the variables associated with EAE that had a relevant influence on the extraction of the anthocyanins from açai. For this, a Resolution III 2–level Plackett–Burman (PB) design (Table 1), where the main effects are aliased with 2-factor interactions, was employed. This method allows the most important factors to be successfully identified early on in the experimentation phase [39]. For this specific study, seven variables were selected: % ethanol (10–60%), pH (4–6), temperature (40–60 °C), agitation (50–200 rpm), ratio (0.1–0.2 g/15 mL), time (10–60 min), and units of enzyme per gram of sample (100–1000 U). These potentially influential variables and their ranges to be considered were selected based both on the bibliography and on the previous experience of our research team [40,41]. Once these variables were entered into the PB design, a total of 12 experiments were scheduled (Table 1), which, as previously explained, were randomly performed once under their corresponding conditions. Finally, the resulting extracts were analyzed by UHPLC–UV–Vis, and the sum of the anthocyanins detected was expressed as total anthocyanins (mg/g of sample) to be used as the response variable.

Once these variables had been identified, the next step consisted in delving into their influence on the efficiency level of the anthocyanin extraction method, and its subsequent optimization for a maximum yield. For this purpose, a Box–Behnken response surface (BBD–RSM) design was used. This type of response surface design does not use a factorial or fractional factorial design to determine the optimal conditions and the most influential variables. BBD–RSM designs are based on different combinations of three levels per factor: (−1) low, (0) medium, and (1) high. It does not generate axial points, which results in a more spherical layout of the design points than other statistical designs. Consequently, not only is a smaller total number of experiments required, but the experiments that would be conducted under extreme conditions and that might either cause the degradation of the anthocyanins or represent an excessive economic expense are also excluded.

Table 1. Experimental extraction conditions and total anthocyanins values obtained and adjusted to the 12 Plackett–Burman tests with seven variables.

Experiment	%EtOH [1]	pH	Temperature (°C)	Agitation (rpm)	Ratio (g/15 mL)	Time (min)	Enzyme Units (U/g)	Total Anthocyanins Observed (mg/g Sample)	Total Anthocyanins Adjusted (mg/g Sample)	Error (%)
1	10	4	60	200	0.2	60	100	3.90	3.64	7.01
2	10	6	40	50	0.2	10	1000	0.91	1.00	9.10
3	10	4	60	50	0.1	60	1000	4.98	4.99	0.15
4	60	6	60	50	0.2	60	100	6.78	6.32	7.21
5	60	6	60	50	0.1	10	100	5.97	6.43	7.13
6	10	4	40	50	0.1	10	100	4.89	4.08	19.79
7	10	6	60	200	0.2	10	1000	0.77	0.68	13.86
8	60	4	40	200	0.2	10	100	5.82	5.83	0.18
9	60	4	60	200	0.1	10	1000	6.50	6.85	5.09
10	60	6	40	200	0.1	60	1000	4.64	4.58	1.35
11	10	6	40	200	0.1	60	100	1.42	1.48	4.13
12	60	4	40	50	0.2	60	1000	6.35	7.06	10.03

[1] Percentage of ethanol in the solvent.

Based on the results obtained from the PB analysis, three variables were discarded, and thus the variables selected and the range studied for the BBD were: percentage of EtOH (20−50−80), pH (4−5−6), agitation (50−100–150 rpm), and units of enzyme (100−300–500 U/g sample). The ranges of study were based on the results obtained by our research group in previous studies. According to the number of variables and their values, a total of 27 experiments were estimated to be required to generate an appropriate design (Table 2). Such experiments were randomly performed following the previously explained procedure. Then, the total anthocyanins obtained were quantified, as this was the reference value to be employed as the response variable for the optimization process.

Table 2. Experimental extraction conditions and total anthocyanins obtained and adjusted to the 27 BBD–RSM tests with four variables.

Experiment	%EtOH	pH	Agitation (rpm)	Enzyme Units (U/g)	Total Anthocyanins Observed (mg/g Sample)	Total Anthocyanins Adjusted (mg/g Sample)	Error (%)
1	20	4	100	300	6.14	6.03	1.79
2	80	4	100	300	5.57	5.49	1.43
3	20	6	100	300	5.02	4.76	5.25
4	80	6	100	300	5.67	5.43	4.13
5	50	5	50	100	6.19	5.89	4.97
6	50	5	150	100	5.80	5.58	3.73
7	50	5	50	500	5.90	5.77	2.16
8	50	5	150	500	6.30	6.27	0.57
9	50	5	100	300	6.19	5.96	3.71
10	20	5	100	100	4.89	5.24	7.16
11	80	5	100	100	5.00	5.43	8.58
12	20	5	100	500	5.86	5.65	3.63
13	80	5	100	500	5.72	5.59	2.33
14	50	4	50	300	5.98	5.97	0.27
15	50	6	50	300	5.33	5.60	5.16
16	50	4	150	300	6.42	6.36	0.91
17	50	6	150	300	5.16	5.39	4.50
18	50	5	100	300	5.79	5.96	2.95
19	50	4	100	100	6.12	6.06	0.96
20	50	6	100	100	5.96	5.76	3.29
21	50	4	100	500	6.39	6.71	5.06
22	50	6	100	500	5.49	5.68	3.39
23	20	5	50	300	5.08	5.22	2.81
24	80	5	50	300	5.16	5.19	0.65
25	20	5	150	300	5.12	5.22	1.83
26	80	5	150	300	5.40	5.39	0.29
27	50	5	100	300	5.90	5.96	1.00

The estimated response Y of the total anthocyanins obtained from each test can be fitted to a second degree polynomial equation, as follows:

$$Y = \beta_0 + \beta_{1\times1} + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2$$

where β_0 corresponds to the ordinate, whereas X_1 (%EtOH in the solvent), X_2 (pH), X_3 (agitation), and X_4 (enzyme U/g) are the independent variables, β_i are the linear coefficients, β_{ij} are the coefficients of the cross products, and β_{ii} are the quadratic coefficients.

Finally, the developed method was compared against ultrasound-assisted extraction (UAE), an analytical technique that uses the energy of ultrasound waves to facilitate the extraction of organic compounds from different matrices, such as plants. This technique has been used by many food industries for the extraction of anthocyanins from different matrices. A UP200S sonifier unit (200 W, 24 kHz) (Dr. Hielscher. GmbH, Berlin, Germany) with a water bath coupled to a temperature controller (FRIGITERM–10, J.P. Selecta, S.A., Barcelona, Spain) was employed for these extractions under the optimized conditions that had been reported by Aliaño–González et al. [42] for açai samples.

2.4. Anthocyanins Identification

The anthocyanins present in açai were identified by means of an Ultra-High-Performance Liquid Chromatography system coupled to a photodiode array detector and to a Quadrupole Time-of-Flight Mass Spectrometer (UHPLC–PDA–QToF–MS) model Xevo G2 (Waters Corp., Milford, MA, USA). For that purpose, a reverse phase C18 column (100 Å, 2.1 mm, 1.7 µm, Acquity UPLC BEH C18, Waters) was employed. The procedure was based on the expertise of our research group on the analysis of anthocyanins [42]. Water with 2% formic acid was used as phase A, while phase B was 100% MeOH. A flow rate of 0.4 mL/min was applied according to the following gradient: 5% B, 0 min; 20% B, 3.30 min; 30% B, 3.86 min; 40% B, 5.05 min; 55% B, 5.35 min; 60% B, 5.64 min; 95% B, 5.94 min; 95% B, 7.50 min. The total analysis time was 12 min, including the return time to the initial conditions.

Regarding the mass analyses, a positive ionization method was applied. The desolvation gas and source temperatures were 500 and 150 °C, respectively. The capillary cone was set at 700 V and the cone voltage was 20 V. Finally, the desolvation gas and the cone gas flow were set up at 700 L/h and 10 L/h, respectively. The collision energy of the trap used was 4 eV. Full scan mode in a range from 100 to 1200 *m/z* was employed for the identification of the anthocyanins.

The following anthocyanins were identified $[M]^+$: cyanidin 3-*O*-glucoside (*m/z* 449), cyanidin 3-*O*-rutinoside (*m/z* 595), peonidin 3-*O*-glucoside (*m/z* 463), and peonidin 3-*O*-rutinoside (*m/z* 609). The identified anthocyanins were in agreement with those described by other authors and with those detected by our research group in previous studies [43].

2.5. Anthocyanins Quantification

Once the anthocyanins present in the açai samples had been identified, they were quantified by means of an Elite UHPLC LaChrom Ultra liquid chromatography system (Hitachi, Tokyo, Japan). This system is formed by an automatic sampler (L–2200 U), a column oven (L–2300), two pumps (L–2160 U), and a UV–Vis detector (L–2420 U). The conditions set for the analysis were based on previous experiments by our research group. Consequently, the temperature was adjusted to 50 °C, an injection volume of 15 µL was established, and the detector was set up to a length of 520 nm since this is the maximum absorption level that has been observed in anthocyanins. A Kinetex EVO C18 column (2.6 µm, 2.1 × 100 mm, Phenomenex, Torrance, CA, USA) was used for this purpose. For the chromatographic separations, the following phases were employed: methanol (Phase B) and a 5% aqueous solution of formic acid (Phase A). The flow rate was fixed at 1.0 mL min^{-1}, while the gradient used was the following: 0 min, 15% B; 1.50 min, 20% B; 3.30 min, 30% B; 4.80 min, 40% B; 5.40 min, 100% B; 8.40 min, 100% B; 9 min, 15% B. Under these conditions and in a short period of time (less than 5 min), a total of four anthocyanins were identified (Figure 1).

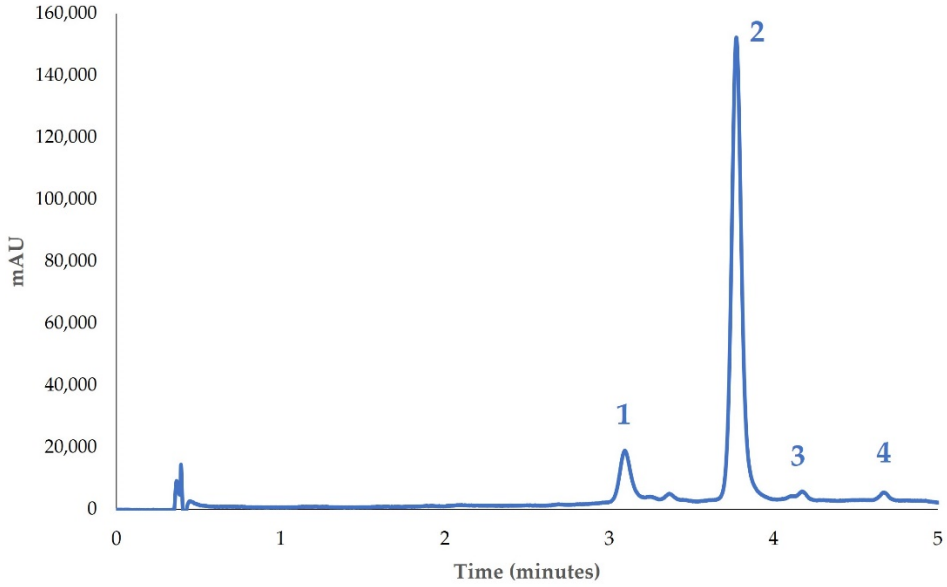

Figure 1. Chromatogram of the anthocyanins in açai extract (λ = 520 nm). 1. Cyanidin 3-*O*-glucoside; 2. Cyanidin 3-*O*-rutinoside; 3. Peonidin 3-*O*-glucoside; 4. Peonidin 3-*O*-rutinoside.

The anthocyanins were quantified based on the conditions described by Aliaño-Gonzalez et al. [5], who used the UHPLC–UV–Vis method to generate a calibration curve of cyanidin chloride ($y = 300568.88x - 28462.43$, $R^2 = 0.9999$) as the standard for anthocyanins. The detection and quantification limits were 0.198 mg L^{-1} (LOD) and 0.662 mg L^{-1} (LOQ). The four anthocyanins present in açai (cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, peonidin 3-*O*-glucoside, and peonidin 3-*O*-rutinoside) were quantified based on the calibration curve of cyanidin chloride, assuming that the different anthocyanins have similar absorbances, and taking into account their individual molecular weights. The results have been expressed in milligrams of total anthocyanins per gram of lyophilized açai.

2.6. Statistical Analysis

The statistical software Statgraphic Centurion (version XVII) (Statgraphics Technologies, Inc., The Plains, VA, USA) was used to generate and analyze the PB and BBD–RSM designs. A *t*-test was performed at 95% confidence level to work out the *p*-values corresponding to each of the variables studied. Accordingly, the variables that presented *p*-values below 0.05 were considered influential.

3. Results and Discussion

3.1. Determining the Variables' Influence

As previously mentioned, a 2-level PB design was used to determine the influence of certain variables in the EAE of anthocyanins from açai. This design was based on 12 experiments (Table 1), which were randomly performed.

The total anthocyanins were assessed and used as the response variable. The R^2 obtained was 0.93, which confirmed a good adjustment of the developed model. In addition, the predicted values were correlated with the actual measured values, which revealed a 7.09% average error that ranged from 0.15% to 19.80% and confirmed a satisfactory correlation. This percentage error was calculated as the difference in absolute value between the predicted value and the observed value and divided by the observed value.

A *t*-test was performed at 95% confidence and both %EtOH (*p*-value: 0.0037), and pH (*p*-value: 0.0266) exhibited *p*-values below 0.05, which suggests that they are influential factors in the extraction of anthocyanins. The results were graphically represented by means of a Pareto Chart (Figure 2).

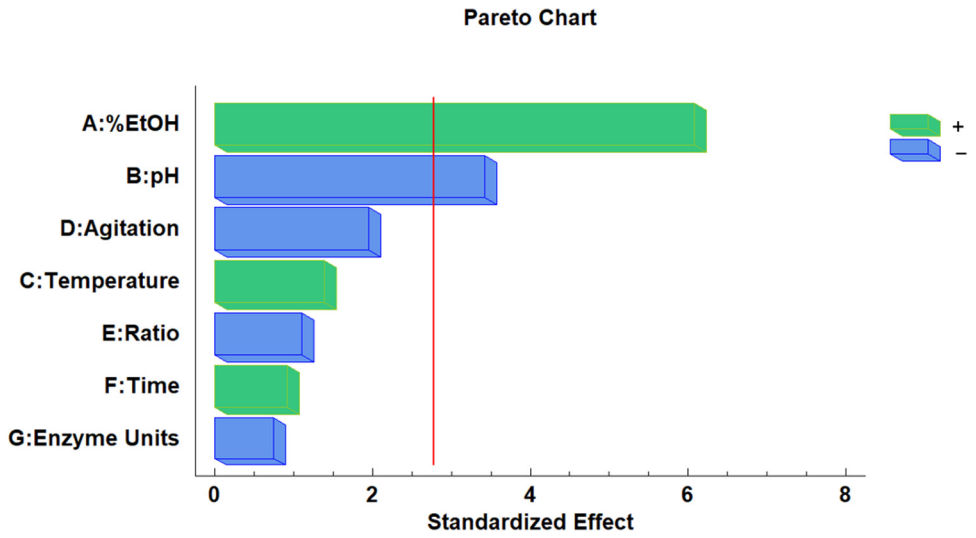

Figure 2. Pareto Chart of the PB design corresponding to the extraction of anthocyanin. A: %EtOH in the solvent; B: solvent pH; C: temperature; D: agitation; E: ratio; F: time of extraction; G: units of enzyme per gram of sample.

It could be observed that the percentage of EtOH exhibited a positive effect, which means that the extraction yields were greater when the highest percentage in the range studied was employed. For this reason, the range of the study was increased to 80% for the last BBD–RSM design. On the contrary, pH exhibited a negative effect, which means that the yields were larger when the lowest value of the range was used. However, a very acidic medium might promote the degradation of the enzyme and affect its extraction efficiency [44]. For this reason, it was decided to keep the same range for future optimization. Finally, regarding the values of the rest of the variables, which had been determined as non–influential according to the PB design, they were set up based on the expertise of our research team in EAE processes [41]. For that purpose, the solvent ratio was established at 0.1 g/15 mL, the temperature at 60 °C, and 20 min was established as the extraction time.

3.2. Box–Behnken Optimization

The variables that had already been optimized by PB still had to be optimized by means of a BBD–RSM design with four variables. Overall, 27 randomly performed experiments (Table 2) were required to generate this design, where the total anthocyanins in the extracts were quantified and used as the response variable.

The results were statistically analyzed following the BBD–RSM design, and the model obtained exhibited an R^2 of 0.80. In addition, the adjusted concentrations were related to the actual measurements, with 3.05% average error and diversions ranging from 0.27% up to 8.58%. The error was calculated as previously mentioned for the PB design. The *p*-value for Durbin–Watson's method was 0.141; since being greater than 0.05, it indicates that there are no relevant differences between the predicted and the measured values. The sum of squares, the *F* values, and the *p*-values of the four variables can be seen in Table 3.

Table 3. Results from the BBD–RSM of the anthocyanins extracted from the açai samples.

Variable	Sum of Squares	*F*-Value	*p*-Value
A: %EtOH	0.01	0.15	0.706
B: pH	1.34	1.45	0.003
C: Agitation	0.03	0.30	0.594
D: U/g	0.24	2.66	0.129
AA	1.63	1.77	0.001
AB	0.37	4.01	0.068
AC	0.01	0.12	0.740
AD	0.02	0.17	0.685
BB	0.00	0.04	0.851
BC	0.09	1.00	0.337
BD	0.13	1.45	0.252
CC	0.12	1.32	0.273
CD	0.16	1.72	0.214
DD	0.03	0.29	0.599

It was observed that pH (*p*-value: 0.0025) and the quadratic interaction of %EtOH (*p*-value: 0.0012) presented *p*-values below 0.05, which indicates that they are influential variables. The results were graphically represented by means of a Pareto Chart (Figure 3), where it could be seen that both pH and the quadratic interaction of %EtOH had a negative effect.

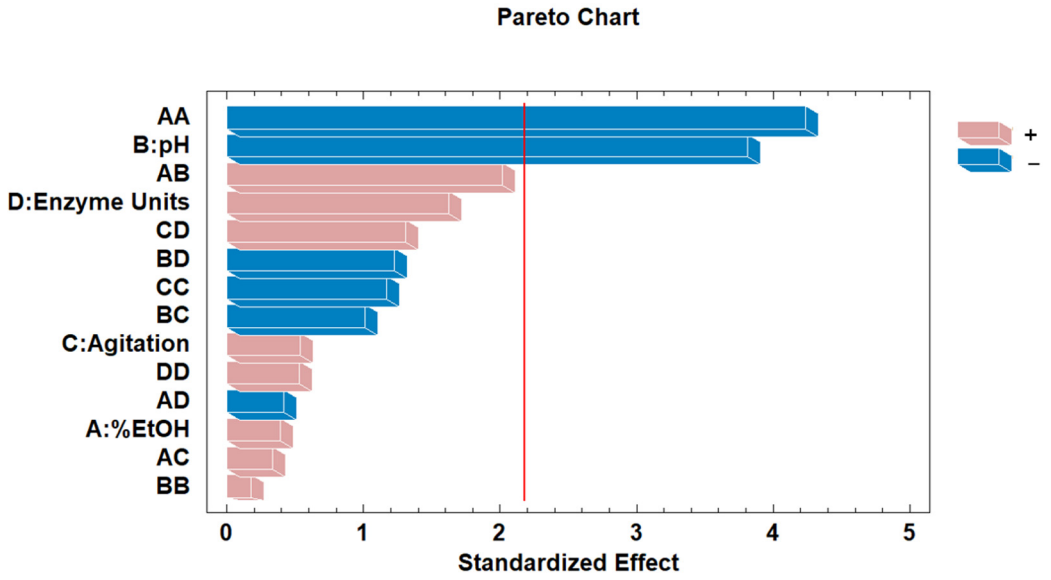

Figure 3. Pareto Chart from the BBD–RSM design corresponding to the extraction of the anthocyanin. A: %EtOH in the solvent; B: solvent pH; C: agitation; D: units of enzyme per gram of sample.

A separate response surface graph of these two variables—%EtOH and pH—that had been determined as influential can be seen in Figure S1. It could be observed in this graph that the maximum total anthocyanin concentrations were achieved when the percentage of EtOH was around the intermediate values of the range, whereas pH was at the lowest values in the range studied. The coefficients of a second-order polynomial equation to calculate the concentration of the extracted anthocyanins were taken from the BBD–RSM design and resulted in the following formula:

$$Y = 5.957 + 0.033\,X_1 - 0.334\,X_2 + 0.048\,X_3 + 0.143\,X_4 + 0.303\,X_{1\times2} + 0.051\,X_{1\times3} - 0.063\,X_{1\times4} - 0.1514\,X_{2\times3} - 0.182\,X_{2\times4} + 0.199\,X_{3\times4} - 0.552\,X_1^2 + 0.025\,X_2^2 - 0.151\,X_3^2 + 0.071\,X_4^2$$

where Y is the aforementioned response; X_1 (percentage of EtOH in the extraction solvent), X_2 (pH), X_3 (agitation), and X_4 (units of enzyme/g).

Lastly, the optimal variable values to achieve the greatest yields from the açai anthocyanin extractions were acquired from the BBD–RSM design as follows: 40% of EtOH, 4 pH, 150 rpm agitation, and 500 units of enzyme per gram of sample. It was therefore observed that an intermediate percentage of ethanol (40%) was required for efficient extraction of the anthocyanins in açai by means of EAE, which is in agreement with the data previously reported by the relevant bibliography. For example, Nicolescu et al. [45] optimized a method based on EAE combined with UAE for the extraction of the anthocyanins in *Rosa canina* L. and concluded that the optimal conditions were 40% of EtOH, 50 °C, and 81.23 min of exposure time when employing a 40 kHz ultrasonic bath. In addition, Granato et al. [46] observed that 50% of EtOH allowed the largest anthocyanins extraction at real scale from blackcurrant (*Ribes nigrum* L.). Kumar et al. [47] also affirmed that 50% of EtOH was the most suitable concentration for the successful extraction of the anthocyanins that are found in the seed coat of black soybeans (*Glycine max* L.).

Furthermore, it has been observed that the optimal pH value is located at the lower end of the interval studied. This was in consonance with the values that had been previously determined by our research group regarding the extraction of anthocyanins from blackcurrant samples [41]. Nevertheless, no experiments at lower pH values were conducted given the range of action of the enzyme (pH 4–6) and, as mentioned above, the potential risk of enzyme degradation. On the other hand, the optimal values for both agitation and U/g were found to be at the upper end of the range studied, although since they had not been ascertained as influential, no further optimization experiments would be required.

To summarize, the optimal conditions were established at 0.1 g of açai heated up to 60 °C and mixed with 15 mL of solvent at 40% ethanol with pH 4, using 23.31 units of enzyme per gram of sample and agitated at 150 rpm.

3.3. Time of Extraction

Once the rest of the variables had been optimized for the enzymatic extraction of the anthocyanins present in açai samples, the optimal extraction time was still to be determined. To that end, different extraction times were tested (5, 10, 15, 20, 30, and 60 min) under the optimal conditions that had been already established. Similarly to the previous procedure, the extracts were analyzed using a UHPLC–UV–Vis method, and the total anthocyanin concentrations were determined to be used as the variable response. Each experiment was performed in duplicate. The results have been graphically represented in Figure 4.

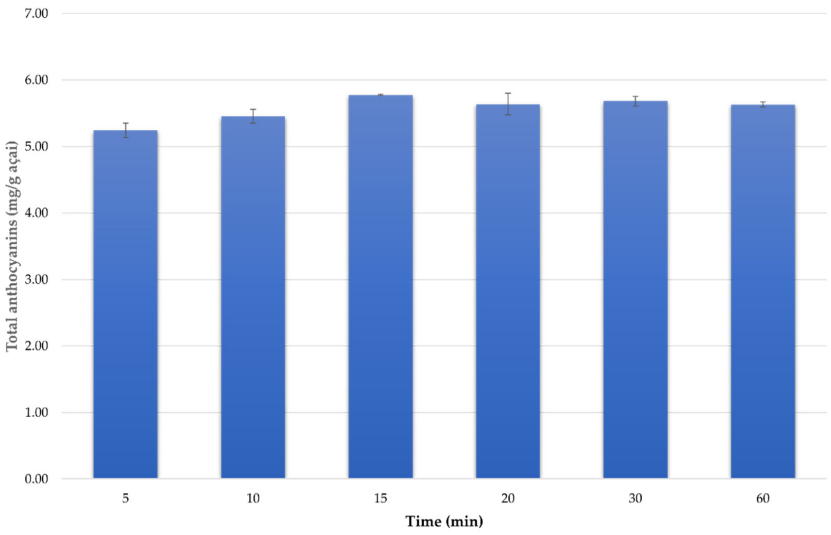

Figure 4. Total anthocyanin concentrations (mg/g açai) according to extraction times (n = 12).

It could be observed in Figure 4 that there was an increment of the concentration of total anthocyanins extracted as time was increased up to 15 min. This extraction time

resulted in the maximum extractions of total anthocyanins per gram of açai, reaching 5.78 ± 0.10 mg average concentration. In addition, it could be observed that times longer than 15 min did not lead to any significant differences with regard to the amount of total anthocyanins extracted. A reduced extraction time (15 min) represents a considerable advantage regarding the practicability of the developed method.

3.4. Repeatability and Intermediate Precision Analysis

Once all of the optimal extraction conditions had been determined, a repeatability and intermediate precision study was to be conducted in order to validate the optimized extraction method. For the repeatability study, 12 experiments were carried out under optimal conditions on the same day. For the intermediate precision study, six daily extractions were performed on three different days. The coefficient of variation (C.V.) was the statistical parameter used as the reference to determine the suitability of the optimized method. The results can be seen in Table 4.

Table 4. Repeatability (n = 12) and intermediate precision (n = 6 + 6 + 6) of the optimized method.

	Repeatability	**Intermediate Precision**
Average (total anthocyanins mg/g açai)	5.882	5.769
Standard deviation	0.186	0.197
Coefficient of variation (%)	3.16	3.42

In view of the C.V. results it could be concluded that the developed method presented a good repeatability (C.V. 3.16%) and intermediate precision (C.V. 3.42%), since both values were below 5%.

In addition, the standard deviation and the C.V. for each of the four individual anthocyanins extracted were determined. Thus, the chromatograph peaks corresponding to each of the four anthocyanins, i.e., cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, peonidin 3-*O*-glucoside, and peonidin 3-*O*-rutinoside, presented standard deviations of 0.007, 0.028, 0.005, and 0.007, respectively. Regarding their respective C.V., 2.21%, 3.06%, 1.95%, and 3.85% were obtained. Therefore, the accuracy of the analytical data was confirmed to be good.

3.5. Comparison of Enzyme-Assisted Extraction (EAE) against Maceration and Ultrasound-Assisted Extraction (UAE)

Finally, the optimized method was compared against two of the most commonly used methods in the food industry for the extraction of anthocyanins, i.e., ultrasound-assisted extraction and maceration. Specifically, four experiments under the conditions optimized for the EAE method and four experiments under these optimized conditions without the use of enzymes (maceration) were conducted. For the comparison with the UAE method, four additional experiments according to the optimal conditions reported by Aliaño–Gonzalez et al. [42] were conducted, i.e., 0.5 g of sample in 10 mL of solvent with 51% methanol with pH 6.38 at 31 °C in 0.7 s cycles at 20% amplitude. The extracts were analyzed by UHPLC–UV–Vis and their concentration of total anthocyanins was determined and then used as the response variable. The results are shown in Figure 5.

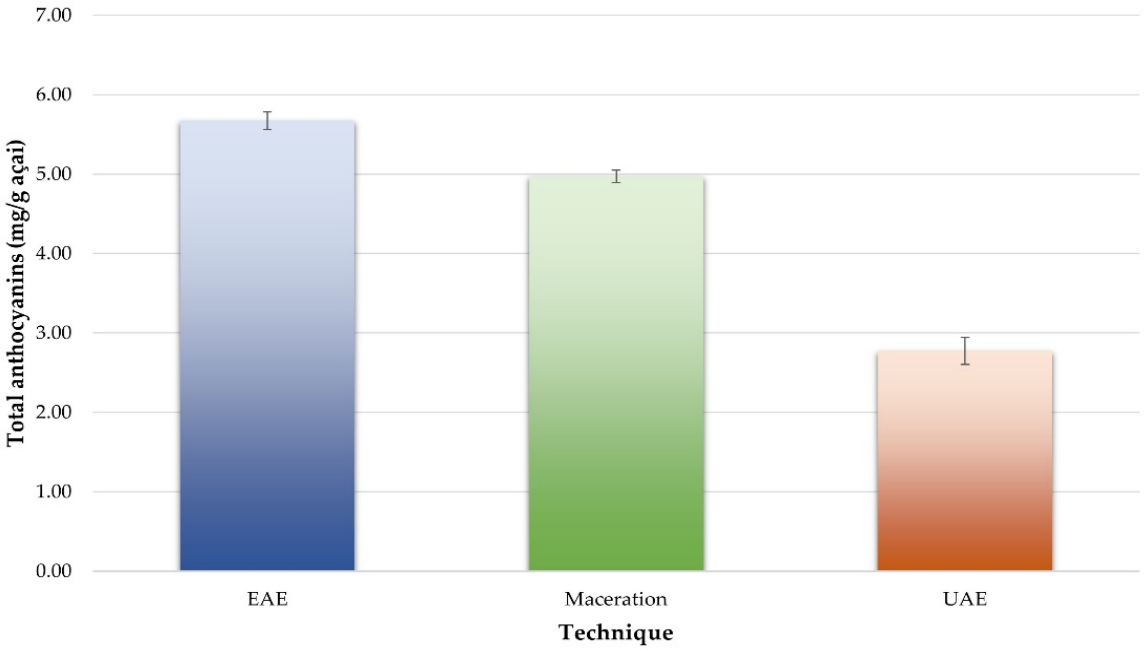

Figure 5. Total anthocyanin concentrations (mg/g açai) obtained by EAE, maceration and UAE (n = 4).

The average total anthocyanins and their standard deviation obtained by EAE, maceration and UAE were 5.67 ± 0.17, 4.97 ± 0.15, and 2.77 ± 0.27 mg anthocyanins per g of açai, respectively. It was concluded that the use of the enzyme significantly increased anthocyanin extraction yields. In fact, the total anthocyanin concentrations obtained were higher by 14% and 104% for maceration and UAE, respectively. Therefore, the effectiveness of the use of enzymes to improve the efficiency of extraction of açai anthocyanins was verified. Given that, according to previous studies, ultrasound may cause the degradation of phenolic compounds, the authors hypothesize that the considerable data differences between UAE and the two other extraction methods could be the result of such degradation caused by ultrasound [48].

We could consequently conclude that the reliability and efficiency of the developed method based on enzymes for the extraction of total anthocyanins from açai has been demonstrated.

3.6. Applying the Method to Real Samples

Finally, the lyophilized sample used in this research and three commercially available lyophilized açai products were analyzed under the optimal conditions that had been established. Then, each individual anthocyanin was quantified. Each extraction was performed in triplicate and the average concentrations obtained can be seen in Table 5. It was detected that the sample that had been used for the optimization process (lyophilized 1) exhibited the highest anthocyanin concentration, followed by the product acquired from Mundo Arcoiris, whereas the açai samples acquired from a local supermarket presented the lowest concentration. Furthermore, cyanidin 3-*O*-rutinoside was the anthocyanin found at the highest concentrations in all cases, ranging from 3.31 up to 4.62 mg/g açai. Finally, peonidin 3-*O*-rutinoside was found at the lowest concentration levels in all cases (0.22–0.31 mg/g açai).

Table 5. Individual anthocyanin concentrations (mg/g) in real samples. Lyophilized 1: used for the method optimization; Lyophilized 2: acquired from Mundo Arcoiris; Lyophilized 3 and 4: acquired from a local supermarket in Cadiz, Spain.

Concentration (mg/g Açai)	Cyanidin 3-*O*-glucoside	Cyanidin 3-*O*-rutinoside	Peonidin 3-*O*-glucoside	Peonidin 3-*O*-rutinoside	Total
Lyophilized 1	0.63	4.62	0.28	0.31	5.84
Lyophilized 2	0.59	4.28	0.26	0.28	5.42
Lyophilized 3	0.55	3.99	0.25	0.27	5.05
Lyophilized 4	0.45	3.31	0.20	0.22	4.19

4. Conclusions

During this research, four anthocyanins were identified in açai and a method based on enzyme-assisted extraction were optimized to obtain maximum total concentrations. Firstly, the Plackett–Burman design indicated that ethanol percentage and solvent pH were the variables that most influenced the extraction of anthocyanins from açai, while the Box–Behnken design allowed the optimization of these variables. The resulting optimal conditions were 0.1 g of açai heated at 60 °C and extracted in 15 mL of solvent with 40% ethanol and pH 4, using 500 units of enzyme per gram of sample and 150 rpm agitation for 15 min. In addition, the developed method exhibited good repeatability and intermediate precision with a C.V. below 5% for each of the anthocyanins identified during this study. Finally, EAE was compared against maceration and UAE, and it was confirmed that the enzymatic extraction method was more efficient regarding the concentration of total anthocyanins in the resulting extracts. It was therefore concluded that the presence of the pectinase enzyme considerably improved the extraction yields. Finally, the individual anthocyanin concentrations obtained from the lyophilized açai samples and from several commercially available samples confirmed the suitability of the development method for this kind of sample. It was therefore concluded that EAE is a rapid and efficient alternative for the extraction of anthocyanins from açai that presents several significant advantages that could be described as follows: higher efficiency than other traditional extraction methods (104% > UAE), and mild conditions that not only preserve the bioactive compounds of interest but also require lower energy and solvent consumption.

Supplementary Materials: The following supplementary information can be downloaded from: https://www.mdpi.com/article/10.3390/agronomy12102327/s1, Figure S1: Response Surface of the two variables selected as influential (pH and %EtOH) according to the BBD–RSM design of the extraction of anthocyanins from açai samples.

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