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Extraction of Anthocyanins and Total Phenolic Compounds from Açai (*Euterpe oleracea* Mart.) Using an Experimental Design Methodology. Part 2: Ultrasound-Assisted Extraction

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Abstract: Two optimized methods for ultrasound-assisted extraction were evaluated for the extraction of two types of acai bioactive compounds: Total anthocyanins (TAs) and total phenolic compounds (TPCs). For the extraction optimization, a Box Behnken factorial design of different variables in the following intervals was used: Methanol-water (25%–75%) for solvent composition, temperatures between 10 and 70 °C, amplitude in the range between 30% and 70% of the maximum amplitude −200 W), extraction solvent pH (2–7), the ratio for sample-solvent (0.5 g:10 mL–0.5 g:20 mL), and cycle between 0.2 and 0.7 s. The extraction kinetics were studied using different periods between 5 and 30 min. TA and TPC were analyzed by UHPLC and the Folin–Ciocalteu method, respectively. Optimized conditions for TA were: 51% MeOH in water, 31 °C temperature, pH 6.38, cycle 0.7 s, 65% amplitude, and 0.5 g:10 mL of sample-solvent ratio. Optimized conditions for the TPC were: 49% MeOH in water, 41 °C temperature, pH 6.98, cycle 0.2 s, 30% amplitude, and 0.5 g:10 mL of sample-solvent ratio. Both methods presented a relative standard deviation below 5% in the precision study. The suitability of the methods was tested in real samples. It was confirmed that these methods are feasible for the extraction of the studied bioactive compounds from different açai matrices.

Keywords: açai; anthocyanins; Box Behnken methodology; phenolic compounds; ultrasound-assisted extraction; UHPLC

1. Introduction

Açai (*Euterpe oleracea* Mart.) is a fleshy fruit from a tropical palm tree, which grows in wet forests near riverbanks in some Northern regions in South America. It produces small spherical dark drupes (1.0–1.8 cm), which have become very popular because of its healthy properties [1,2] related to its elevated content of carbohydrates, proteins, fats, and fibers. Açai has been part of the daily diets of the native people of these regions for years as complement of their main courses [3]. Different studies from the last few years have proved that açai is one of the richest fruits in natural antioxidant contents (anthocyanins, phenolic compounds, vitamins, etc.) [4–6]. Their large content in biological compounds is reflected in their antioxidant, anti-inflammatory, and anticancer properties [7–10]. In addition, several studies have shown the beneficial properties of açai against chronic diseases,

such as obesity [11,12], neurodegenerative [13], or cardiovascular diseases [14]. This fruit also has a significant effect on the treatment of these conditions because of its capacity to relieve pain thanks to its vasodilating capacities [15,16]. Several studies carried out in human beings and cell culture models have demonstrated the antioxidant and anti-inflammatory activities of açai pulp and açai juice [6]. These properties are mainly associated to the phenolic compounds present in this fruit [17–19].

Total phenolic compounds (TPCs) are usually related to the prevention of degenerative diseases among others, being also responsible for the antioxidant and anticancer properties of the berry [19–21]. Among others, syringic acid, protocatechuic acid, vanillic acid, *p*-coumaric, or caffeic are some of the most relevant phenolic compounds present in açai [22,23].

The deep purple color of the drupes is due to the high content of total anthocyanins (TAs), phenolic compounds that are also associated with potential therapeutic effects [24–26]. A total of 20 anthocyanidins have been identified in nature. These are the sugar-free counterparts of anthocyanins, based on flavylium ions [27].

Cyanidin and peonidin are among the six most abundant anthocyanidins in nature [10,25,28], which are also found in acai. Specifically, two major (cyanidin 3-*O*-rutinoside and cyanidin 3-*O*-glucoside) and two minor (peonidin 3-*O*-rutinoside and peonidin 3-*O*-glucoside) anthocyanins [4,29] have been recognized.

Despite the important properties of açai, this fruit is mainly consumed in the growing or nearby areas, since it is highly perishable and rather unknown in many countries. However, it is not difficult to find foods made from açai in the form of juices, pulps, concentrated extracts, freeze-dried açai, tablets, etc. [29–32] in different world regions.

Various extraction techniques like ultrasound-assisted extraction, maceration [17,33], soxhlet [5,34], or pressurized extractions [35,36] have been applied for both types of bioactive compounds (TPC and TA) in açai. However, an in-depth study on these extraction techniques has never been carried out for analytical purposes.

Ultrasound-assisted extraction (UAE) uses the energy provided by ultrasounds and allows easiy extraction of the organic compounds from different matrices, such as plants. The efficiency of this technique is due to the phenomenon of cavitation together with the mechanical effect and enhanced mass transfer produced by ultrasounds [37,38]. Furthermore, the extraction efficiency can be increased by using a greater range of temperatures, since the number of bubbles formed in the cavitation phenomenon increases [39,40].

UAE has been earlier applied to extract bioactive compounds from different matrices, such as myrtle, [41], black chokeberry [42], perlette grapes [43], sesame [44], etc. Additionally, this technique is easy to use and cheaper than other extraction techniques. Some authors have already implemented this technique to identify and quantify biological compounds in açai but not to extract organic compounds from açai.

The techniques that are commonly used to analyze the TPC and TA are spectroscopic techniques and high-performance liquid chromatographic techniques, respectively [45]. The determination of TPC and TA has been successfully carried out by high-performance liquid chromatography (HPLC) in Chinese cabbage [46], raspberry [47], mango [48], and açai [31], among others, and by ultra-high-performance liquid chromatography (UHPLC) in wine [49], strawberry [50], black chokeberry [42], and açai [29].

Ultraviolet-visible spectroscopy is generally employed to elucidate several functional groups and perform their quantifications based on its absorption or reflectance in the UV-vis range. The Folin–Ciocalteu approach is commonly used to determine the TPC concentration in different types of samples, such as in vinegar [51], raspberry [47], aloe vera [52], rice [53], and açai [10] among others.

Therefore, the research carried out in this paper focuses on developing and validating an alternative UAE method for fast and accurate extraction of both individual anthocyanins and TPC in lyophilized açai. Thus, a fast and reliable extraction method for industries and laboratories to quantify the TA and the TPC in açai and other similar products made from açai is proposed.

2. Materials and Methods

2.1. Solvents and Reagents

For the chromatographic separation, methanol (Fisher Scientific, Loughborough, UK), and formic acid (Panreac, Barcelona, Spain) both HPLC grade were used. To adjust the pH of the extraction solvents, hydrochloric acid (Panreac, Barcelona, Spain; "for analysis" grade) was used. Ultra-pure water was obtained from a Milli-Q water purifier system from Millipore (Bedford, MA, USA). Standard of anthocyanin (cyanidin chloride, ≥ 95% purity) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

For TPC analysis, Folin–Ciocalteu reactive (EMD Millipore, Darmstadt, Germany), sodium carbonate anhydrous (Panreac, Barcelona, Spain), and gallic acid standard ≥99% (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) were used.

2.2. Biological Material and Commercial Foods

For the optimization of the extraction methods lyophilized açai (produced by ecological agricultural methods, MundoArcoiris, Besalú, Girona, Spain) was used. The methods developed were applied to various açai foods presentations: Three lyophilized, three juices, a tablet, and a jam.

2.3. Extraction Procedure

An UP200S sonifier (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 used for the UAE.

All the extractions were performed in duplicate using 0.5 g of sample in each experiment. The variables chosen for the optimization were: Solvent of extraction (25%–75% methanol in H_2O), the extraction temperature (10–70 °C), the amplitude (30%–70% of the maximum amplitude −200 W), the cycle (0.2–0.7 s), the pH of the extraction solvent (2–7), and the amount of sample (g):volume of the extraction solvent (mL) ratio (0.5:10–0.5:20). Methanol was used as the extraction solvent, due to the very good extractant qualities for this type of compound, such as its small size (easy penetration capacity), low density, and polarity related to the polarity of the studied compounds [38,41,42,44].

The procedure of the extraction was as follows: 0.5 g of sample was weighted and the solvent with the percentage of methanol corresponding to each experiment was added. The sonifier was inserted into the solution and the conditions of the extraction were set. The time used during the development of the extraction methods was 10 min. The obtained extracts were double centrifuged under the following conditions: 5 min and 7500 rpm (orbital radius 9.5 cm). The supernatant was introduced into a volumetric flask (25 mL). The extracts were filtered using a 0.20-µm nylon syringe filter (Membrane Solutions, Dallas, USA) prior to analysis.

2.4. Optimization Study

The optimization of the extraction conditions was performed by a Box-Behnken (BBD) design and by using as response variables the TA (amount of anthocyanins (mg) per amount of açai (g) carried out by ultra-high performance liquid cromatography) and TPC (mg gallic acid equivalents (GAE) per g of sample determined by the Folin–Ciocalteu method). The results of these designs for the 54 extractions (average values of each experience performed in duplicate for both types of compounds) considering the 6 extraction variables at the three different levels are shown in Table S1.

A second-order polynomial equation, where all the variables were considered, was applied to the responses obtained from all these extractions. For this design, the polynomial equation is the following:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_5X_5 + \beta_6X_6 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{15}X_1X_5 + \beta_{16}X_1X_6 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{25}X_2X_5 + \beta_{26}X_2X_6 + \beta_{34}X_3X_4 + \beta_{35}X_3X_5 + \beta_{36}X_3X_6 + \beta_{45}X_4X_5 + \beta_{46}X_4X_6 + \beta_{56}X_5X_6 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2 + \beta_{55}X_5^2 + \beta_{66}X_6^2. \quad (1)$$

Briefly, Y is the aforesaid response, β_0 is the ordinate; X_1 (% of methanol in the UAE solvent), X_2 (extraction temperature), X_3 (ultrasound amplitude), X_4 (cycle), X_5 (pH of the solvent), and X_6 (ratio solid sample (g): extraction volume (mL)) are the independent variables; β_i are the linear coefficients; β_{ij} are the cross product coefficients; and β_{ii} are the quadratic coefficients.

The optimization study was carried out by using Statgraphic Centurion software (version XVII) (Statgraphics Technologies, Inc. The Plains, Virginia, USA) This software allows the estimation of the effects of the variables on the final response, the second order mathematical model, the surface graphs, the optimum levels of the significant variables, as well as the variance analysis.

2.5. UHPLC-QToF-MS

The identification of the anthocyanins was performed by UHPLC-QToF-MS as described in our previous article [54]. Briefly, water (2% formic acid) and methanol were used as solvent A and solvent B, respectively. The mobile phases were set at a flow rate of 0.4 mL min^{-1}. The gradient selected was the following: 15% B at 0 min; 20% B at 3.30 min; 30% B at 3.86 min; 40% B at 5.05 min; 55% B at 5.35 min; 60% B at 5.64 min; 95% B at 5.94 min; 95% B at 7.50 min. 12 min was the total run time, including the re-equilibration (4 min). Electrospray source (positive ionization mode) with the following settings were used: 700 L h^{-1} (desolvation gas flow), 500 °C (desolvation temperature), 10 L h^{-1} for (cone gas flow), 150 °C (source temperature), 700 V (capillary voltage), 20 V (cone voltage), and 4 eV (trap collision energy). Full-scan mode in the range of *m/z* = 100–1200 was used.

The following anthocyanins were identified in the extracts of açai: 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).

2.6. UHPLC-UV-vis Analysis

With respect to anthocyanins, the most common way to analyze them is by HPLC-UV-vis. Lately, UHPLC is displacing HPLC chromatography, as the chromatographic separations are achieved with greater sensitivity and in shorter analysis times [20,35,38]. The analysis of anthocyanins was performed by UHPLC-UV-vis as described in our previous article [54].

Briefly, acidified water (5% formic acid, solvent A) and methanol (solvent B) working at a flow rate of 1.0 mL min^{-1} was employed as a gradient method for the chromatographic separation. The gradient used was: 15% B (0 min); 20% B (1.50 min); 30% B (3.30 min); 40% B (4.80 min); 55% B (5.40 min); 60% B (5.90 min); 95% B (6.60 min); 95% B (9.30 min); 15% B (10 min).

To obtain a calibration curve by the UHPLC method, cyanidin chloride (y = 300568.88 x − 28462.43) was chosen since it is the anthocyanidin standard commercially available. The regression equation and the determination coefficient (R^2 = 0.9999) were obtained. The normal distribution of residuals was evaluated with the Shapiro–Wilk test. The W value obtained was 0.8514, very close to 1. The *p*-value obtained was 0.803 (above 0.05), and proved a normal distribution of the residuals. The detection limit (LOD) (0.198 mg L^{-1}) and the quantitation limit (LOQ) (0.662 mg L^{-1}) were determined as described in our previous article [54].

The calibration curve for cyanidin chloride was used to quantify the four anthocyanins present in açai. To do so, it must be assumed that the absorbances of the different anthocyanins were similar, and considered the molecular weight of each one. TA were determined as the sum of the individual ones. The analyses were performed in triplicate.

2.7. Total Phenolic Content

Regarding the phenolic compounds, fruits in general and açai in particular have hundreds, if not thousands of different phenolic compounds. Addressing the study of individual phenolic compounds is therefore virtually impossible. In this sense, the TPC was determined by the Folin–Ciocalteu methodology as described by Aliaño-González et al. [54]. So, in order to produce a standard curve, a solution of gallic acid in methanol (1000 mg L^{-1}) was diluted (ranging from 0.1 up to 500 mg L^{-1}) in methanol.

Briefly, in a 25-mL volumetric flask, 0.25 mL of the sample or the standard was first added followed by 12.5 mL of distilled water, 1.25 mL of Folin–Ciocalteu reagent, and 5 mL of aqueous sodium carbonate solution 20% (w/v). Distilled water was added until the volumetric flask was full and then, it was manually agitated for 30 s. The volumetric flask was kept away from the light and maintained at 25 °C (room temperature) for 30 min before the analysis.

A calibration curve (y = 0.0024 x − 0.0031) was obtained for the analytical standard (gallic acid). Other analytical parameters were as follows: R^2 = 0.9999; limit of detection = 1.649 mg L^{-1}; limit of quantitation = 5.498 mg L^{-1}. Normal distribution of residuals was also evaluated for gallic acid standard with the Shapiro–Wilk test (W = 0.9201). The *p*-value obtained was 0.762 (above 0.05) that proves the normal distribution of the residuals.

3. Results

3.1. Optimization of the Extraction Method

For the optimization of the extraction conditions for both, TA and TPC from a freeze-dried açai, a BBD was used. A total of six extraction variables were evaluated in the following intervals:Methanol in water (25%–75%), temperature (10–70 °C), ultrasound amplitude (30%–70%) of the maximum amplitude −200 W, ultrasound cycle (0.2–0.7 s), solvent pH (2–7), and sample-to-solvent ratio (0.05–0.025 g mL^{-1}). For the experimental design, an extraction time of 10 min and 0.5 g of sample were used.

Under the specified conditions, a total of 54 experiments (Table S1) were carried out in duplicate. TA and TPC were measured according to the previously explained procedures and the average values were employed to obtain the optimum conditions for each method.

3.2. TA Optimization

3.2.1. Extraction Method

In order to determine the TA content, individual anthocyanins were added together. BBD was carried out for the determination of the most influential variables and the optimal conditions for anthocyanin extraction. For this design, the average of each duplicate analysis was used. Subsequently, the real values of TA and the values predicted by the BBD were correlated. Table S1 shows the BBD matrix with the values of the six variables for each experiment and the measured and predicted responses. A percentage of 4.84% was the resulting average difference. These values ranged from 0% until 12.93%; therefore, the extraction conditions influence the amount of TA extracted.

The *p*-values were calculated according to the t-test by the Statgraphic Centurion software considering the 95% confidence level, which means that variables with *p*-values less than 0.05 were considered influential. The calculated *p*-values (Table 1) support the variables that have a predominant influence on the extraction of TA, the percentage of methanol in the water used as the extraction solvent (*p*-value: 0.0011) and the ratio of solvent:solvent (*p*-value: 0.0000).

Table 1. Box Behnken Design (BBD) coefficients for total anthocyanins (TA) and total phenolic compounds (TPC).

	Total Anthocyanins Coefficient	Total Anthocyanins *p*-Value	Total Phenolic Compounds Coefficient	Total Phenolic Compounds *p*-Value
β_0	4.4667		9.4717	
Solvent (β_1)	−0.2233	0.0011	0.1871	0.33
Temperature (β_2)	0.0521	0.39	0.1400	0.47
Amplitude (β_3)	0.0154	0.80	0.0346	0.86
Cycle (β_4)	0.0638	0.30	0.2075	0.28
pH (β_5)	−0.0150	0.81	0.0821	0.67
"Ratio" (β_6)	−0.0154	0.80	−0.0200	0.92
Solvent:Solvent (β_{11})	−1.7110	0.00	−1.2765	0.0002
Solvent:Temperature (β_{12})	−0.0175	0.87	−0.2500	0.45
Solvent:Amplitude (β_{13})	0.1775	0.10	0.5000	0.14
Solvent:Cycle (β_{14})	0.0575	0.45	−0.1156	0.62
Solvent:pH (β_{15})	0.0025	0.98	0.3175	0.34
Solvent:"Ratio" (β_{16})	−0.1425	0.19	0.2062	0.54
Temperature:Temperature (β_{22})	−0.1672	0.083	−1.2040	0.0003
Temperature:Amplitude (β_{23})	−0.0550	0.61	−0.4488	0.18
Temperature:Cycle (β_{24})	0.0762	0.48	−0.0388	0.91
Temperature:pH (β_{25})	0.0369	0.62	0.1069	0.65
Temperature:"Ratio" (β_{26})	0.2100	0.057	0.6463	0.060
Amplitude:Amplitude (β_{33})	−0.1085	0.25	0.5051	0.093
Amplitude:Cycle (β_{34})	0.1125	0.30	0.5375	0.11
Amplitude:pH (β_{38})	0.0638	0.55	−0.4225	0.21
Amplitude:"Ratio" (β_{36})	0.0394	0.60	0.3494	0.14
Cycle:Cycle (β_{44})	0.0103	0.91	0.1022	0.73
Cycle:pH (β_{45})	0.0888	0.41	0.0538	0.87
Cycle:"Ratio" (β_{46})	−0.1425	0.19	0.0863	0.80
pH:pH (β_{55})	−0.1185	0.21	0.1093	0.71
pH:"Ratio" (β_{56})	−0.0512	0.63	−0.3050	0.36
"Ratio":"Ratio" (β_{66})	−0.0085	0.93	−0.1136	0.70

The effects of the different variables in anthocyanins extraction are observed in Figure 1 (standardized Pareto chart). The Pareto chart was obtained with a 95% confidence level. In this diagram, the cumulative influence of each variable on the total amount of anthocyanins is represented in descending order, i.e., the first variable is the most influential variable on the response. The percentage of methanol in the solvent extraction was expected to be an important factor for extracting anthocyanins. The reason is that the solvents or mixtures thereof must have a polarity similar to the compounds that are intended to be extracted.

As expected, the most influential factors were the individual effect of the % of MeOH and the quadratic interaction of the % of MeOH. With respect to this variable, 51% of MeOH in H_2O was the optimum value for TA. Table 2 shows the optimal values (TA) for each of the variables.

Table 2. Optimum conditions for TA and TPC by UAE.

Variable	Optimum Values for TA	Optimum Values for TPC
Solvent (% MeOH)	51	49
Temperature (°C)	31	41
pH	6.38	6.98
Cycle (s)	0.7	0.2
Amplitude (%)	65	30
"Ratio" (mL)	10	10

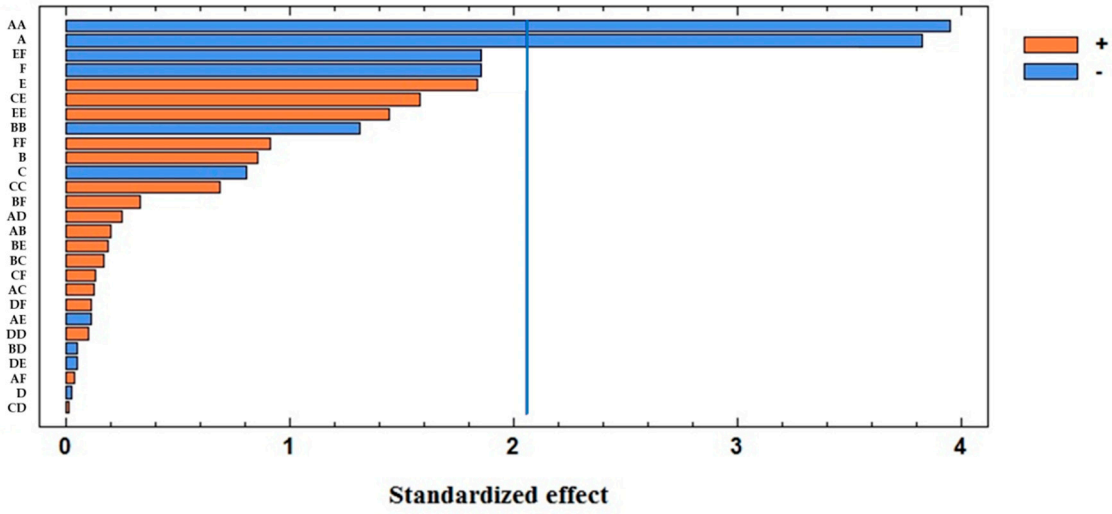

Figure 1. Standardized Pareto chart for TA extraction. A: Solvent (% MeOH); B: Temperature (°C); C: Amplitude (%); D: Cycle (s); E: pH; F: Ratio (mL).

3.2.2. Optimum Extraction Time

For the evaluation of the kinetics of the extraction process, it was necessary to run several extractions in triplicate at different times ranging from 5 until 30 min. The extractions were performed at the optimal conditions determined for each parameter (Table 2). The results for the recovery of TA are displayed in Figure 2.

As it can be observed, the maximum amount of anthocyanins was obtained when the extraction time was 5 min, so this was established as the optimum period. Longer periods caused a decrease in the recoveries, which could be explained due to high temperatures degrading the anthocyanins [55].

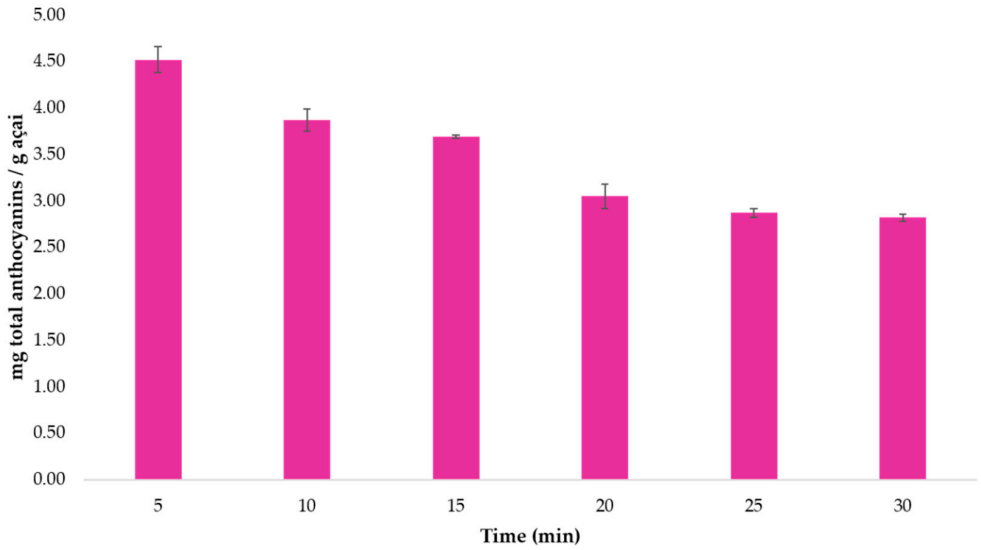

Figure 2. Mg TA recovery/g açai over the kinetic study (n = 3).

3.2.3. Precision Study

Once the optimal conditions were determined, a repeatability and intermediate precision was evaluated for the developed UAE method. To do so, 30 extractions were performed on three different days (12 extractions the first day, and 9 extractions on the two following days). The results (Table 3) showed good values with an intra-day and inter-day residual relative standard deviation (RSD) of

2.7% and 1.2%, respectively. Thus, the developed method proved its high precision for the extraction of anthocyanins using UAE.

Table 3. Results obtained from the precision study for TA and TPC.

	Repeatability [1]	Intermediate Precision [2]
Average (mg TA/g açai)	4.517	4.492
SD *	0.122	0.054
RSD ** (%)	2.7	1.2
Average (mg TPC/g açai)	10.374	10.465
SD *	0.440	0.180
RSD ** (%)	4.2	1.7

[1] Repeatability (n = 12); [2] Intermediate precision (n = 27); * Standard deviation; ** Relative standard deviation.

3.3. TPC Optimization

3.3.1. Optimization of the Extraction Method

Similarly, the 54 extractions in duplicate performed to determine the extraction of TA were also employed for the extraction of TPC (Table S1). The analyses were carried out using the Folin–Ciocalteu method that has been previously described. Based on the results, the BBD was used to calculate the most significant variables when extracting the TPC and to determine their optimal extracting conditions. The average difference between the real and predicted values was 5.79%, with values ranging from 0.00% until 20.80%. These results showed a very good correlation between the real values and those predicted by the design. In this case, the model could be applied to predict the amount of TPC that would be obtained under specific experimental conditions.

The effects of the different variables on phenolic compound extraction were represented in a standardized Pareto chart (Figure 3). As with anthocyanins, the Pareto chart was obtained with a 95% confidence level. Likewise, the cumulative influence of each variable on the total amount of phenolic compounds is again represented in descending order. As it could be observed in the Pareto chart, similarly to what happened with the extraction of anthocyanins, the quadratic interaction of the percentage of methanol in water as the solvent for the extraction was the most influential variable for the extraction of TPC from açai. Furthermore, the quadratic interaction of temperature was also a very influential factor for the extraction of phenolics, since it usually improves the extraction kinetic of this type of bioactive compound [56].

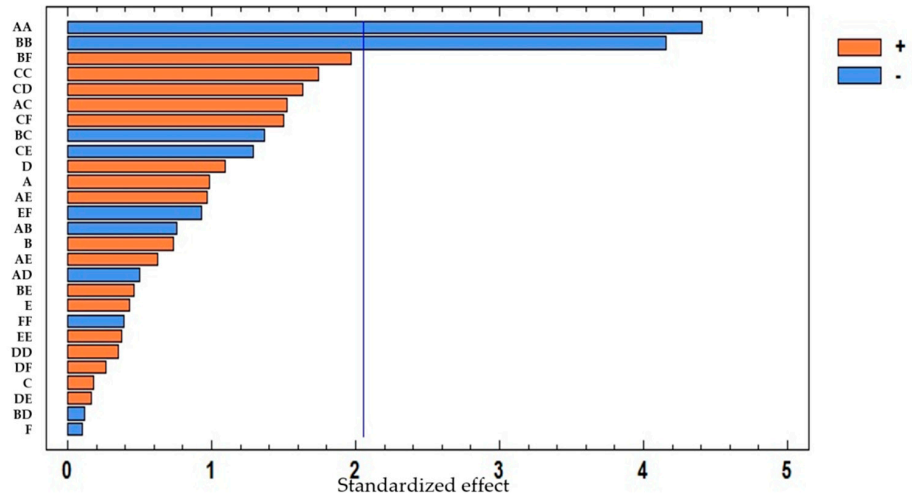

Figure 3. Standardized Pareto chart for TPC extraction. A: Solvent (% MeOH); B: Temperature (°C); C: Amplitude (%); D: Cycle (s); E: pH; F: Ratio (mL).

It could be observed from the *p*-values (Table 2) that the quadratic interaction of methanol in water had a *p*-value of 0.0002 and the quadratic interaction of temperature had a *p*-value of 0.0003. Both facts prove that a higher interaction enhances the extraction of TPC. Table 2 shows the optimal values for the extraction of TPC.

3.3.2. Optimum Extraction Time

The same as for total anthocyanins, the kinetics of the extraction of TPC was carried out. Several extractions for different periods ranging between 5 and 30 min were run at optimal extracting conditions (Table 2). All the analyses were performed in triplicate (Figure 4).

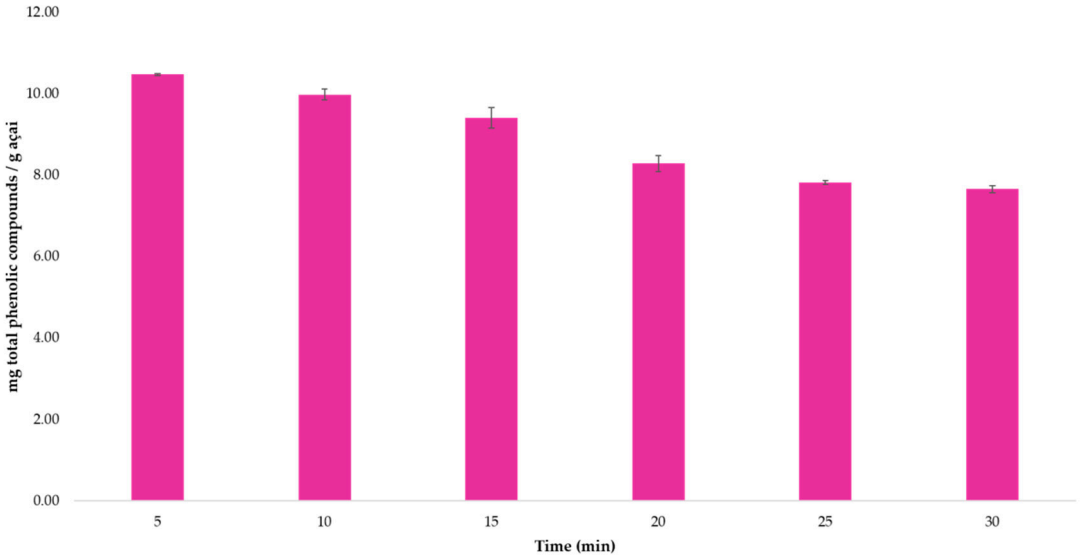

Figure 4. Mg of TPC/g açai recovered during the kinetic study (n = 3).

The same optimal time was obtained for the extraction of TPC as for TA, i.e., 5 min. For periods longer than 5 min and mainly after 20 min of extraction, a slight decrease in the total content of TPC could be observed, probably due to the degradation of the compounds.

3.3.3. Precision Study

The intermediate precision and repeatability of the method were also obtained by running 30 extractions at the optimum conditions in three different days (12, 9, and 9 extractions). The results can be seen in Table 3, with an RSD of 4.2% for repeatability and an RSD of 1.7% for intermediate precision. These results demonstrated the high precision of the method developed for the extraction of the TPC from açai.

3.4. Re-Extraction Experiments

To evaluate the effectiveness of the method, a re-extraction study was carried out. To do so, after the extraction, the remaining açai residue was filtered and extracted again by applying the same extraction conditions. This study was carried out in triplicate for both TPC and TA. The amount of compounds obtained after the re-extraction was less than 5% of the total content of both types of compounds. These results demonstrate the effectiveness of the extraction methods developed in this study.

3.5. Application to Foods Made with Açai

The methods developed for TA and TPC by UAE were applied to eight real samples in triplicate: Three freeze-dried açai in solid state, three açai juices in liquid state, an açai tablet in solid state, and an

açai jam. High concentrations of phenolic compounds were obtained from the real samples (Table 4), with the lyophilized açai samples being the ones with the highest content. Besides, the content of TA in the real samples was very small (except in the freeze-dried açai samples); this may be due to the degradation processes of anthocyanins during storage or manufacturing [4]. It could also be due to the manufacturer's addition of other cheaper raw materials with anthocyanins to obtain the typical purple color of açai. This hypothesis was taken into account after obtaining some chromatograms of foods made with açai with chromatographic peaks of anthocyanins not present in this fruit [57].

Table 4. Average ± RSD values of TA and TPC in real açai samples (n = 3).

Samples	mg TA/g Sample	mg TPC/g Sample
Freeze-dried açai (1)	5.42 ± 0.06	12.04 ± 0.34
Freeze-dried açai (2)	4.55 ± 0.22	10.22 ± 0.41
Freeze-dried açai (3)	3.99 ± 0.15	9.17 ± 0.21
Açai juice	0.03 ± 0.00	2.09 ± 0.05
Açai juice food supplement	0.04 ± 0.00	2.05 ± 0.10
Concentrated juice açai-banana	0.01 ± 0.00	4.66 ± 0.19
Açai tablet	0.04 ± 0.00	1.91 ± 0.37
Açai jam	0.14 ± 0.00	1.16 ± 0.17

4. Conclusions

Two ultrasound-assisted methods were optimized for the extraction of the TA and TPC from açai.

The optimal conditions for each of the methods were determined followed by a kinetic study. The results from such kinetic study showed that an extraction time of five minutes was enough to obtain the maximum extractions of TA and TPC from açai. This would provide analysts with a rapid method of extraction.

The precision study (evaluated by repeatability and intermediate precision) for both extraction methods showed RSDs values below 5%.

The suitability of the extraction methods was tested by analyzing different presentations of real açai food samples. The results proved the applicability of the methods for the extraction of TA and TPC from açai-containing food. These results suggest that the methods developed can be applied in the industry for a reliable and quick extraction. Moreover, UAE presents several advantages over other extraction techniques since it is cheaper and easier to use.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/3/326/s1, Table S1. Box-Behnken design matrix with the values of the six variables for each experiment and measured and predicted responses (n = 2).

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