Extraction of Anthocyanins and Total Phenolic Compounds from Açai (Euterpe oleracea Mart.) Using an Experimental Design Methodology. Part 1: Pressurized Liquid Extraction

María José Aliano-González 1, Marta Ferreiro-González 1, Estrella Espada-Bellido 1,*, Ceferino Carrera 1, Miguel Palma 1, José A. Álvarez 2, Jesús Ayuso 2 and Gerardo F. Barbero 1

1 Department of Analytical Chemistry, Faculty of Sciences, IVAGRO, University of Cadiz, 11510 Puerto Real, Cadiz, Spain; mariajose.aliangonzalez@alum.uca.es (M.J.A.-G.); mart.a.ferreiro@uca.es (M.F.-G.); ceferino.carrera@uca.es (C.C.); miguel.palma@uca.es (M.P.); gerardo.fernandez@uca.es (G.F.B.)

2 Department of Physical Chemistry, Faculty of Sciences, Institute of Biomolecules (INBIO), University of Cadiz, 11510 Puerto Real, Cadiz, Spain; joseangel.alvarez@uca.es (J.A.A.); jesus.ayuso@uca.es (J.A.)

* Correspondence: estrella.espada@uca.es; Tel.: +34-956-016355

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Abstract: Currently, açai is one of the most important fruits present in the world. Several studies have demonstrated its high content in phenolic compounds and anthocyanins. Both of them are responsible of interesting properties of the fruit such as anti-inflammatory, antioxidant or anticancer. In the present study, two optimized pressurized liquid extraction (PLE) methods have been developed for the extraction of anthocyanins and total phenolic compounds from açai. A full factorial design (Box–Behnken design) with six variables (solvent composition (25–75% methanol-in-water), temperature (50–100 °C), pressure (100–200 atm), purge time (30–90 s), pH (2–7) and flushing (50–150%)) were employed. The percentage of methanol in the extraction solvent was proven to be the most significant variable for the extraction of anthocyanins. In the case of total phenolic compounds, the extraction temperature was the most influential variable. The developed methods showed high precision, with relative standard deviations (RSD) of less than 5%. The applicability of the methods was successfully evaluated in real samples. In conclusion, two rapid and reliable PLE extraction methods to be used for laboratories and industries to determine anthocyanins and total phenolic compounds in açai and its derived products were developed in this work.

Keywords: açai; anthocyanins; Box–Behnken design; phenolic compounds; pressurized liquid extraction; UHPLC

1. Introduction

Açai is the fruit of a tropical palm tree (Euterpe oleracea Mart.) that is native to northern South America, and which usually grows in humid lowland forests [1]. It is a spherical dark grape with a size of around 1.0–1.8 cm [2]. This fruit is one of the most famous fruits of the Amazonian estuary, due to its nutritional properties (high contents of carbohydrates, proteins, fats and fibers) [3,4]. Açai pulp can be commercialized in fresh or frozen form, and it is commonly consumed like a thick soup [5].

During the last few years, it has been discovered that red or purple colors are characteristic of fruits with a high content of bioactive compounds [6–9], and this is the reason for why açai has been deeply studied [10–13]. Some researchers have discovered that it is one of the richest fruits in terms of natural antioxidants (anthocyanins, phenolic compounds, vitamins, etc.), among all of the fruits and vegetables known to date [14–16].
The high concentration of bioactive compounds in açai is responsible for several of the interesting properties of this fruit. In fact, açai has been commonly evaluated in the literature, in terms of its antioxidant [17–19], anti-microbial [20,21], anti-inflammatory [22,23] and anticancer [24,25] health benefits. In addition, it has been used with success in investigations into problems with microcirculation or cardiovascular diseases [26,27], obesity [28,29] or neurodegenerative diseases. In general, the treatment of illnesses with açai is due to its capacity for vasodilation, which supposes a high level of pain relief [23].

It has been demonstrated that the aforementioned important properties of açai are kindly related to its high concentration of phenolic compounds [2,30]. Phenolic compounds are considered to be secondary metabolites, which can be synthesized by plants [31,32]. Phenolic constituents are generally associated with health-promoting properties and the prevention of several degenerative diseases. Some studies have demonstrated their relation to antioxidant and anticancer properties in general [33–35]. The main phenolic compounds that could be found in açai are protocatechuic acid, epicatechin, benzoic acid, caffeic acid, chlorogenic acid, ferulic acid, syringic acid, vanillic acid, p-coumaric acid, orientin, isoorientin, vitexin, and procyanidins, amongst others [10,24].

Anthocyanins are phenolic compounds that have been shown to be responsible for the red, purple, or blue color of some fruits such as açai. They have been found to have potential therapeutic effects such as anti-inflammatory [36,37], radio-protective [38,39] and chemoprotective properties [40]. Furthermore, they have been used with good results in the prevention and treatment of illness such as cancer [41–43]. Anthocyanidins are the sugar-free counterparts of anthocyanins, and a total of 20 have been identified to occur naturally. Six of these are more abundant in nature: pelargonidin, delphinidin, cyanidin, petunidin, peonidin and malvidin [44,45]. Four main anthocyanins have been identified in açai: cyanidin 3-O-glucoside, cyanidin 3-O-rutinoside, peonidin 3-O-glucoside, and peonidin 3-O-rutinoside [46].

Due to the increase in knowledge about açai, and due to the high demand for products rich in phenolic compounds, several studies related to the extraction of phenolic compounds have been carried out. Extraction techniques such as ultrasound-assisted extraction [47], Soxhlet [10], supercritical CO₂ [48] or pressurized liquid extraction [49] are some of the techniques that have been used for the study of the extraction of phenolic compounds and anthocyanins from açai. Nevertheless, to date, an exhaustive study of compound extraction with these techniques has not been reported for the quality control of the raw material, its storage, and the quality control process.

Pressurized liquid extraction (PLE) is a technique that involves extraction by using the solvent in its liquid state at elevated temperature and high pressure, to provide greater solubility and extraction efficiency, as compared to techniques that are carried out at room temperature and atmospheric pressure [49,50]. The sample is placed in an extraction chamber and pressurized with a solvent. The high temperatures facilitate the breaking of bonds between the analyte and the matrix, and this increases the ability of the solvent to dissolve the compounds of interest.

The main advantages of this technique are the short time (approximately 15 min) required to achieve an efficient extraction, and the inert extraction conditions (an N₂ atmosphere). In general, this technique is quite fast, uses minimal amounts of solvents, and does not require subsequent filtration [51,52]. The application of this technique to extract compounds from different matrices has increased significantly in recent years, especially in natural foods and plant metabolites. This technique has been applied for the extraction of phenolic compounds and anthocyanins from cocoa beans [53], blackberry [54], grape marc [55], jucara [56], pomegranate peel [57], mulberry [58], etc. However, there has been no evaluation of the potential for the extraction of biological compounds from açai by PLE, which can be used in quality control analysis of the raw material and its derived products.

Different techniques have been used to analyze extracts that are rich in phenolic compounds and anthocyanins. However, spectroscopic techniques and high-performance liquid chromatographic techniques have been the most commonly used for this purpose. High-performance liquid chromatography (HPLC) is an analytical technique that is used to separate different compounds...
based on their chemical interactions with chromatographic columns, allowing for their separation and analysis. This method could be applied, even in the case of non-volatile or thermolabile compounds. HPLC has been used to identify phenolic compounds and anthocyanins in grape skins [59], cornelian cherry fruit [60], Morus alba [61], olives [62], or açaí [63].

Ultra-high-performance liquid chromatography (UHPLC) is based on the HPLC technique, which involves a much higher pressure, and which supposes a better resolution. This technique has been used to identify phenolic compounds and anthocyanins in blueberry [64], fruit juices [65], spinach [66] or açaí [64].

The aim of the present work is the development and validation of new pressurized liquid extraction methods for the study of total anthocyanins and total phenolic compounds in açaí, with the purpose of providing a rapid and reliable extraction method that could be used in quality control laboratories.

2. Materials and Methods

2.1. Chemicals and Solvents

Different solvents were used for the chromatographic separation: methanol (MeOH) (Fisher Scientific, Loughborough, UK; HPLC grade), and formic acid (Panreac, Barcelona, Spain; HPLC grade), hydrochloric acid (Panreac, Barcelona, Spain; “For Analysis” grade) and ultrapure water from a Milli-Q water purification system from Millipore (Bedford, MA, USA).

An anthocyanin standard (cyanidin chloride, ≥95% purity) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), and total phenolic compounds were determined using the Folin–Ciocalteu reagent (EMD Millipore, Darmstadt, Germany), anhydrous sodium carbonate (Panreac, Barcelona, Spain) and gallic acid standard (Sigma-Aldrich Chemical Co., St. Louis, MO, USA).

2.2. Açai Samples

The extraction methods were developed using a lyophilized açaí sample (Mundo Arcoiris Sin Límites, S.L., Besalú, Girona, Spain) produced by ecological agriculture. Once the methods were developed, they were applied to commercial products: three freeze-dried açaí samples in solid state, three açaí juice in liquid state, an açaí tablet in solid state, and an açaí jam.

2.3. Extraction Procedure

Pressurized liquid extraction was performed using an ASE 200 (“Accelerated Solvent Extractor”, Dionex, Sunnyvale, CA, USA). The extracts were filtered through a 0.20 µm nylon syringe filter (Membrane Solution, Dallas, TX, USA) prior to chromatographic analysis. All extractions were performed in duplicate, using 0.5 g of sample in each experiment.

The variables studied during the optimization were the extraction solvent (25–75% MeOH in water), extraction temperature (50–100 °C), pressure (100–200 atm), purge time (30–90 s), pH of the extraction solvent (2–7), and flushing (50–150% of the total volume (11 mL) of the extraction cell). The extraction time employed for all the extractions of the experimental design was 10 min.

For the extraction procedure, a cellulose filter (Dionex) was fixed at the bottom of the extraction cell. Sea sand (thick grain) (Panreac, Barcelona, Spain) was added until the bottom of the cell was covered (approx. 1 cm). The sample (0.5 g) was added to the extraction cell and mixed homogeneously with the sea sand. Finally, the cell was filled with sea sand. A period of temperature equilibration of 5 min for the extraction cell was allowed, prior to each extraction. All extracts were then made up to a defined volume of 25 mL with the same solvent, and stored at −20 °C prior to chromatographic analysis.

2.4. Experimental Design and Optimization

The effects of the extraction variables on the response within the studied range were evaluated by the Box–Behnken statistical methodology, considering two different responses: the total amount of anthocyanins and total phenolic compounds. In this experimental design, six extraction variables
(each variable with three levels: low, medium and high) were studied. The six experimental variables and the three levels for each one were as follows: solvent composition (25–50–75% methanol in water), temperature (50–75–100 °C), pressure (100–150–200 atm), purge time (30–60–90 s), pH (2–4.5–7) and flushing (50–100–150% of the total volume of the extraction cell). These ranges of values were selected on the basis of prior knowledge about the extraction of phenolic compounds and anthocyanins. The experimental design for the 54 extractions in duplicate, including six experiments at the central point, along with the respective responses (total anthocyanins and total phenolic compounds) and the predicted values are shown in Table S1.

The results for the total amount of anthocyanins (mg of anthocyanins per gram of sample) determined by UHPLC were used as one of the response variables, and the results for the total phenolic compounds (milligrams of phenolic compounds per gram of sample), determined by the Folin–Ciocalteu method, were used as the other response variable. The predicted response value \( Y \), for the total anthocyanins and total phenolic compounds in each trial, can be fitted to a second-degree polynomial equation, as follows:

\[
Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{15} X_1 X_5 + \beta_{16} X_1 X_6 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{25} X_2 X_5 + \beta_{26} X_2 X_6 + \beta_{34} X_3 X_4 + \beta_{35} X_3 X_5 + \beta_{36} X_3 X_6 + \beta_{45} X_4 X_5 + \beta_{46} X_4 X_6 + \beta_{56} X_5 X_6 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{33} X_{32} + \beta_{44} X_{42} + \beta_{55} X_{52} + \beta_{66} X_{62}
\]

In this equation, \( Y \) is the aforementioned response; \( \beta_0 \) is the ordinate at the origin; \( X_1 \) (percentage of MeOH in the extraction solvent), \( X_2 \) (temperature of extraction), \( X_3 \) (pressure), \( X_4 \) (purge time), \( X_5 \) (pH of the extraction solvent) and \( X_6 \) (flushing) are the independent variables; \( \beta_i \) are the linear coefficients; \( \beta_{ij} \) are the cross-product coefficients; and \( \beta_{ii} \) are the quadratic coefficients.

The responses obtained from all the extractions were studied using Statgraphics Centurion XVII software (Statpoint Technologies, Inc., The Plains, VA, USA). This software was used to estimate the effects of the variables on the final response, the analysis of variance, the second-order mathematical model, the optimum levels of the significant variables, and the surface graphs.

2.5. Analysis of Individual Anthocyanins

Ultra-performance liquid chromatography (UHPLC) coupled to quadrupole-time-of-flight mass spectrometry (Q-ToF-MS) (Xevo G2, Waters Corp., Milford, MA, USA) was used to identify anthocyanins. The injection volume was 3 µL and the chromatographic separation was performed on a reverse-phase C18 analytical column (Acquity UHPLC BEH C18, Waters) (2.1 mm × 100 mm and 1.7 µm particle size). The solvents for the identification of anthocyanins were water (2% formic acid) as solvent A and methanol as solvent B, at a flow rate of 0.4 mL·min⁻¹. The gradient employed was as follows: 0 min, 15% B; 3.30 min, 20% B; 3.86 min, 30% B; 5.05 min, 40% B; 5.35 min, 55% B; 5.64 min, 60% B, 5.94 min, 60% B, capillary voltage 20 V and trap collision energy = 5 eV. Full-scan mode was used (m/z = 100–1200). Masslynx software (version 4.1) was used to control the equipment, and for the acquisition and treatment of data. The anthocyanins identified in the extracts of açai and their molecular ions [M⁺] were: 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. Separation and Quantification of Anthocyanins by UHPLC-UV-vis

Once identified, the separation and quantification of anthocyanins were performed on an Elite UHPLC LaChrom Ultra System (VWR Hitachi, Tokyo, Japan), consisting of an L-2200U autosampler, an L2300 column oven, an L-2160U pump and an L-2420U UV-vis detector. The column oven was...
adjusted to 50 °C for the chromatographic separation. The UV-vis detector was set at 520 nm for the analysis. Anthocyanins were analyzed on a HaloTM C18 Hitachi LaChrom column (100 × 3 mm I.D., particle size 2.7 µm). A gradient method, using acidified water (5% formic acid, solvent A) and methanol (solvent B), working at a flow rate of 1.0 mL·min⁻¹, was employed for the chromatographic separation. The gradient employed was as follows: 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. The four anthocyanins were separated over less than 3.5 min. A characteristic chromatogram for the separation of anthocyanins in açai is shown in Figure 1. It was observed that the parameter that most modified the composition of individual anthocyanins was the percentage of methanol in the extraction solvent. In this sense, high percentages of water (25% methanol) favor the extraction of the most polar anthocyanins (mainly cyanidin 3-O-glucoside), while lower percentages in water (75% methanol) favor the extraction of less polar anthocyanins (peonidin 3-O-glucoside and peonidin 3-O-rutinoside). The average amounts of anthocyanins in the lyophilized açai studied (%) were as follows: cyanidin 3-O-glucoside (30.00%), cyanidin 3-O-rutinoside (63.81%), peonidin 3-O-glucoside (1.72%) and peonidin 3-O-rutinoside (4.47%).

![Figure 1. Chromatogram of the anthocyanins in the açai extract. 1. Cyanidin 3-O-glucoside; 2. Cyanidin 3-O-rutinoside; 3. Peonidin 3-O-glucoside; 4. Peonidin 3-O-rutinoside.](image)

The UHPLC method was used to obtain a calibration curve for cyanidin chloride ($y = 300568.88x - 28462.43$), which is the commercially available standard for anthocyanins. The regression equation and correlation coefficient ($R^2 = 0.9999$) were calculated using Microsoft Office Excel 2010. The limit of detection (0.198 mg·L⁻¹) and quantification (0.662 mg·L⁻¹) were obtained as three and 10 times, respectively, the standard deviation of the blank signal values divided by the slope of the calibration line. 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 using the calibration curve for cyanidin chloride, assuming that the different anthocyanins have similar absorbances, and taking into account their individual molecular weights. All analyses were carried out in triplicate. Results are expressed in milligrams of anthocyanins per gram of lyophilized açai.

2.7. Total Phenolic Content

The total phenolic contents of the extracts obtained from açai, both in the experimental design and in subsequent assays, were determined by the spectrophotometric Folin–Ciocalteu method described by...
Singleton and Rossi in 1965 [67], with modifications by Singleton et al. in 1998 [68] for vegetable extracts. Gallic acid was used as a standard. A methanolic solution of gallic acid (1.0 mg·L⁻¹) was diluted in methanol at various concentrations (0.1–500 mg·L⁻¹) to create the standard curve. The reaction was performed by transferring 0.25 mL of standard solution or extract sample into a 25 mL volumetric flask. Next, 12.5 mL of distilled water, 1.25 mL of Folin–Ciocalteu reagent and 5 mL of aqueous sodium carbonate solution (20% w/v) were added. The volumetric flask was flushed with distilled water and manually stirred for 30 s, then left to stand with protection from light at room temperature for 30 min. The absorbance of the solution was measured at 765 nm in a spectrophotometer (JASCO V-530, JASCO Corporation, Tokyo, Japan) with a quartz cuvette (10 mm path length). The calibration curve obtained for the standard of gallic acid was \( y = 0.0024x - 0.0031 \). The analytical parameters of the calibration curve were: \( R^2 = 0.9998 \); limit of detection (LOD) = 1.649 mg·L⁻¹; Limit of quantitation (LOQ) = 5.498 mg·L⁻¹. Results are expressed as milligrams of gallic acid equivalent per gram of lyophilized açaí.

3. Results and Discussion

3.1. Optimization of the Extraction Method

A Box–Behnken design was used to optimize the extraction conditions for anthocyanins and total phenolic compounds from freeze-dried açaí. Six different extraction variables were studied in the following ranges: methanol in water (25–75%), temperature (50–100 °C), pressure (100–200 atm), purge time (30–90 s), solvent pH (2–7) and flushing (50–150%) of the total volume of the extraction cell. The extraction time was fixed initially at 10 min, and the amount of lyophilized açaí employed was 0.5 g. All experiments were performed in duplicate, and the results are presented in Table S1.

3.2. Anthocyanins Optimization

3.2.1. Extraction Method

Once the 54 extractions had been carried out in duplicate, extracts were measured by UHPLC-UV-vis to quantify the anthocyanins. Individual anthocyanins were added to determine the total anthocyanins. The Box–Behnken design was applied to determine which variables had the greatest influence on the total anthocyanin extraction, and to obtain the optimal conditions. Subsequently, the experimental values for total anthocyanins were correlated with the predicted values obtained by Equation (1). The data related to the fitting properties of the resulting model, expressed as percentage differences between the experimental values for the total anthocyanin recoveries and the calculated ones, are presented in Table S1. The resulting average difference was 4.63% and the range was 0.16% to 13.48%. These results indicate that the extraction variables control the final extract composition, in terms of recovery of anthocyanins, and that the experimental conditions can therefore be managed, to optimize the extraction of total anthocyanins from açaí.

Coefficients of the polynomial equation and their significance (p-values) are presented in Table 1. The p-values show that the most influential variables in the extraction of the total anthocyanins were the double effect of the extraction solvent (p-value: 0.0005), the percentage of methanol in water as extraction solvent (p-value: 0.0007), the pH-flushing ratio interaction (p-value: 0.0016), and the flushing (p-value: 0.0131). These results were graphically represented in standardized Pareto charts (Figure 2), where the significance of each of the variables analyzed in decreasing order can be easily seen. The sign of each effect is also observed from the two different colors of the bars. Positive signs indicate a direct relationship between the effect and the response variable, while negative signs refer to an inverse relationship.
Table 1. Coefficients for the second-order polynomial equation from the Box–Behnken design and the p-values for anthocyanins.

|                  | Anthocyanins Coefficient | Anthocyanins p-Value |
|------------------|--------------------------|----------------------|
| $\beta_0$        | 5.2101                   |                      |
| Solvent          | −0.9168                  | 0.0007               |
| Temperature      | 0.2052                   | 0.4000               |
| Pressure         | −0.1933                  | 0.4277               |
| Purge            | −0.0062                  | 0.9797               |
| pH               | 0.4409                   | 0.0775               |
| Flushing         | −0.6385                  | 0.0131               |
| Solvent:Solvent  | −1.4474                  | 0.0005               |
| Solvent:Temperature | 0.0827              | 0.8437               |
| Solvent:Pressure | 0.0525                   | 0.9005               |
| Solvent:Purge    | 0.0733                   | 0.8050               |
| Solvent:pH       | −0.0457                  | 0.9133               |
| Solvent:Flushing | 0.0154                   | 0.9708               |
| Temperature:Temperature | −0.4810            | 0.2007               |
| Temperature:Pressure | 0.0695               | 0.8684               |
| Temperature:Purge | −0.0213                  | 0.9596               |
| Temperature:pH    | 0.0551                   | 0.8527               |
| Temperature:Flushing | 0.1377                 | 0.7429               |
| Pressure:Pressure | 0.2527                   | 0.4965               |
| Pressure:Purge    | 0.0056                   | 0.9894               |
| Pressure:pH       | 0.6558                   | 0.1265               |
| Pressure:Flushing | 0.0388                   | 0.8959               |
| Purge:Purge       | 0.0377                   | 0.9189               |
| Purge:pH          | −0.0207                  | 0.9607               |
| Purge:Flushing    | 0.0460                   | 0.9126               |
| pH:pH             | 0.5286                   | 0.1610               |
| pH:Flushing       | −1.4685                  | 0.0016               |
| Flushing:Flushing | 0.3355                   | 0.3683               |

Figure 2. Standardized Pareto chart for total anthocyanins.

It can be observed that the quadratic interaction of the percentage of methanol and the individual effect of the percentage of methanol are the most influential factors. This is due to the necessity of using a solvent or a mixture of solvents with similar polarity to extract the compounds of interest. A mixture with 43% methanol in water was the optimum percentage for the extraction of anthocyanins. This value is consistent with other solvent mixtures that are used to extract these kinds of compounds from other
vegetable matrixes, such as blackberry [69], and black bamboo [70], amongst others. The optimum values for the extraction of total anthocyanins are shown in Table 2.

Table 2. Optimum extraction values for total anthocyanins.

| Variable      | Optimum Value |
|---------------|---------------|
| % MeOH        | 43            |
| Temperature (°C) | 81.0         |
| Pressure (atm)  | 200           |
| Time of purge (s) | 60           |
| pH            | 7.00          |
| Flushing (%)  | 50            |

3.2.2. Kinetics of the Extraction Process

In order to assess the kinetics of the extraction process, several extractions at different times (between 1 and 25 min) were carried out, using the optimum extraction conditions (Table 2). The results for the recovery of the total anthocyanins are presented in Figure 3. All analyses were performed in triplicate. It can be seen from Figure 3 that the optimum extraction time was 2 min (excluding the 5 min for temperature equilibration). An extraction time of 2 min under these optimal conditions was sufficient to obtain the maximum recovery of total anthocyanins. Extraction times greater than 2 min led to a lower final amount of anthocyanins being extracted, due to their degradation during the process.

![Figure 3. Average anthocyanin recoveries for extracts obtained using different extraction times (n = 3).](image)

3.2.3. Extraction Cycles

Once the extraction method for total anthocyanins had been optimized, it was necessary to study the influence of the extraction cycles on the total anthocyanins extracted by pressurized liquid extraction. For this reason, a comparison between the total anthocyanins extracted under the optimized conditions with one and two extraction cycles was studied. These two experiments were carried out in triplicate. Non-significant differences between one extraction cycle (5.768 milligrams anthocyanins per gram lyophilized acai; RSD 5.85%) and two extraction cycles (5.912 milligrams anthocyanins per gram lyophilized acai; RSD 4.42%) were observed, which indicates that one extraction cycle is sufficient for the quantitative extraction of total anthocyanins.
3.2.4. Repeatability and Intermediate Precision of the Method

The repeatability and intermediate precision of the developed method were evaluated. In this study, a total of 30 extractions were performed, and these were distributed as follows: 12 extractions performed on the first day of the study, and nine more extractions on each of the two subsequent consecutive days. It can be observed from the results (Table 3) that the developed method showed good repeatability (RSD 1.20%) and intermediate precision (RSD 2.71%).

Table 3. Repeatability and intermediate precision of the method for extraction of total anthocyanins.

|                          | Repeatability ¹ | Intermediate Precision ² |
|--------------------------|-----------------|--------------------------|
| Average (mg total anthocyanins/g açai) | 5.732           | 5.713                    |
| Standard deviation       | 0.069           | 0.155                    |
| Relative standard deviation (%) | 1.20            | 2.71                     |

¹ Repeatability (n = 12); ² Intermediate precision (n = 27).

3.3. Phenolic Compounds Optimization

3.3.1. Extraction Method

The same extraction experiments that were carried out to obtain the best conditions for the extraction of total anthocyanins by the Box–Behnken design, were used for the optimization of total phenolic compounds extraction. In this case, total phenolic compounds were measured in the extracts by the Folin–Ciocalteu methodology previously described. With these values, the Box–Behnken design was applied to determine the variables that have the greatest influence on the extraction of the total phenolic compounds, and to obtain the optimal conditions for total phenolic extraction. In a similar way to the anthocyanins, the experimental values for total phenolic compounds were correlated with the predicted values. The experimental and predicted values for the total phenolic compounds extracted, and the differences between them (%) are presented in Table S1. The resulting average difference between the experimental and predicted values was 3.31%, and this ranged from 0.10% to 7.84%. These results showed that there was a good correlation between the experimental and predicted values. Thus, the model obtained can be used to predict the amount of total phenolic compounds obtained under specific experimental conditions.

It can be observed from the Pareto chart (Figure 4) and the resulting p-values (Table 4) that the predominant factor in the extraction of total phenolic compounds from açai is temperature (p-value: 0.0000). Other significant factors are the solvent:flushing interaction (p-value: 0.0240), the extraction pressure (p-value: 0.0323), and the double effect of solvent extraction (p-value: 0.0497).

![Figure 4. Standardized Pareto chart for total phenolic compounds.](image-url)
Table 4. Coefficients for the second-order polynomial equation from the Box–Behnken design and $p$-values for total phenolic compounds.

| Total Phenolic Compounds | Coefficient | $p$-Value |
|--------------------------|-------------|-----------|
| $\beta_0$                | 11.6945     |           |
| Solvent                  | 0.2706      | 0.1036    |
| Temperature              | 1.0078      | 0.0000    |
| Pressure                 | −0.3627     | 0.0323    |
| Purge                    | 0.3031      | 0.0700    |
| pH                       | 0.0880      | 0.5881    |
| Flushing                 | 0.2393      | 0.1478    |
| Solvent:Solvent          | −0.5043     | 0.0497    |
| Solvent:Temperature      | 0.3544      | 0.2133    |
| Solvent:Pressure         | 0.2441      | 0.3876    |
| Solvent:Purge            | 0.2431      | 0.2270    |
| Solvent:pH               | −0.0335     | 0.9051    |
| Solvent:Flushing         | −0.6661     | 0.0240    |
| Temperature:Temperature  | −0.1950     | 0.4333    |
| Temperature:Pressure     | 0.3050      | 0.2824    |
| Temperature:Purge        | −0.1686     | 0.5493    |
| Temperature:pH           | −0.1110     | 0.5768    |
| Temperature:Flushing     | 0.0305      | 0.9133    |
| Pressure:Pressure        | 0.0437      | 0.8599    |
| Pressure:Purge           | 0.3765      | 0.1870    |
| Pressure:pH              | −0.1650     | 0.5578    |
| Pressure:Flushing        | −0.1343     | 0.5002    |
| Purge:Purge              | −0.1688     | 0.4969    |
| Purge:pH                 | −0.4982     | 0.0846    |
| Purge:Flushing           | 0.1262      | 0.6534    |
| pH:Flushing              | −0.1404     | 0.5715    |
| pH:Flushing              | −0.2732     | 0.3344    |
| Flushing:Flushing        | 0.3133      | 0.2123    |

In contrast to anthocyanins, where the percentage of methanol in the extraction solvent was the most influential variable, for the total phenolic compounds, the solvent had little influence on the final response. This is due to the fact that the variability in the polarity of phenolic compounds is very large, and as a consequence, there is no specific percentage of methanol that could satisfy the extraction of all phenolics. However, in this case temperature is a very influential factor as it improves the extraction kinetics for this kind of compounds. The optimum values for the total phenolic compounds obtained from the experimental design are presented in Table 5.

Table 5. Optimum extraction values for total phenolic compounds.

| Variable          | Optimum Value |
|-------------------|---------------|
| % MeOH            | 42            |
| Temperature (°C)  | 99.6          |
| Pressure (atm)    | 100           |
| Time of purge (s) | 65            |
| pH                | 3.02          |
| Flushing (%)      | 150           |

3.3.2. Kinetics of the Extraction Process

The kinetics for the extraction of total phenolic compounds were also evaluated. Several extractions were run under the optimum extraction conditions (Table 5) using extraction times of 1–25 min. The results for the recovery of total phenolic compounds are presented in Figure 5.
All analyses were performed in triplicate. An extraction time of 2 min (excluding the 5 min for temperature equilibration) under the optimal conditions was sufficient for the quantitative extraction of the total phenolic compounds from açai. Extraction times of greater than 2 min led to a lower amount of total phenolic compounds extracted.

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3.3.3. Extraction Cycles

Once the extraction method for total phenolic compounds had been optimized, it was necessary to study the influence of the number of extraction cycles by pressurized liquid extraction. For this reason, a comparison between the total phenolic compounds extracted under the optimized conditions, with one or two extraction cycles, was studied. These two experiments were carried out in triplicate. As for the total anthocyanins, significant differences were not observed between one extraction cycle (12.337 milligrams total phenolic compounds per gram lyophilized açai; RSD 5.24%) and two extraction cycles (12.483 milligrams total phenolic compounds per gram lyophilized açai; RSD 4.11%), thus indicating that only one cycle is sufficient for the quantitative extraction of total phenolic compounds.

3.3.4. Repeatability and Intermediate Precision of the Method

The repeatability and intermediate precision of the method were also evaluated with different extractions under the optimum extraction conditions, on three consecutive days. For repeatability, 12 extractions were carried out on the same day. For intermediate precision, nine more extractions per day were carried out on two consecutive days. It was observed (Table 6) that the developed method showed good repeatability (RSD 1.73%) and intermediate precision (RSD 4.24%).

| Table 6. Repeatability and intermediate precision of the method for total phenolic compounds. |
|-----------------------------------------------|-----------------|-----------------|
|                                               | **Repeatability**<sup>1</sup> | **Intermediate Precision**<sup>2</sup> |
| Average (mg total phenolic compounds/g açai)  | 12.265          | 12.274          |
| Standard deviation                            | 0.212           | 0.521           |
| Relative standard deviation (%)               | 1.73            | 4.24            |

<sup>1</sup> Repeatability (<i>n</i> = 12); <sup>2</sup> Intermediate precision (<i>n</i> = 27).
3.4. Application to Real Samples

In order to study the applicability of both extraction methods, eight real samples: three freeze-dried açaí samples, an açaí juice, syrups such as an açaí juice food supplement and a concentrated açaí-banana juice, an açaí tablet, and an açaí jam, were evaluated. The results are presented in Table 7. High concentrations of total phenolic compounds were determined in real samples of açaí products. Lyophilized açaí samples had the highest contents of total phenolic compounds. The other foods evaluated also contained considerable amounts of phenolic compounds (açaí juice, juices, tablets and jam).

Table 7. Total amount of anthocyanins and phenolic compounds in real samples (n = 3).

| Samples                        | mg Total Anthocyanins/g açaí | mg Total Phenolics/g açaí |
|--------------------------------|-------------------------------|---------------------------|
| Freeze-dried açaí (1)         | 5.76 ± 0.47                   | 12.25 ± 0.18              |
| Freeze-dried açaí (2)         | 4.83 ± 0.43                   | 10.42 ± 0.62              |
| Freeze-dried açaí (3)         | 4.16 ± 0.45                   | 9.48 ± 0.79               |
| Açaí juice                    | 0.04 ± 0.00                   | 2.25 ± 0.09               |
| Açaí juice food supplement    | 0.05 ± 0.00                   | 2.15 ± 0.09               |
| Concentrated açaí-banana juice| 0.01 ± 0.00                   | 4.88 ± 0.14               |
| Açaí tablet                   | 0.04 ± 0.00                   | 2.02 ± 0.11               |
| Açaí jam                      | 0.15 ± 0.03                   | 1.25 ± 0.08               |

It is noteworthy to highlight that the concentrations of total anthocyanins in the real samples were quite small (except for the freeze-dried açaí sample). This could be due to the fact that the amount of açaí used in the production of these foods was very small, and manufacturers may have added other cheaper purple fruits rather than açaí to obtain the desired color. This situation was demonstrated for several samples, as some of the chromatograms showed a series of peaks with retention times of over four minutes, which were probably indicative of other anthocyanins from different berries [71]. Other possible reasons for these discrepancies could be due to the differences in manufacturing processes or storage methods of the products, which can lead to severe anthocyanin degradation [72].

4. Conclusions

Two pressurized liquid extraction methods have been developed for the extraction of both total anthocyanins and total phenolic compounds from açaí. The most influential variable in the extraction of total anthocyanins from açaí was the double effect of the extraction solvent. Other influential variables were the percentage of methanol in water as the extraction solvent, the pH:flushing ratio and the flushing volume. The optimum extraction conditions for anthocyanins were: 43% MeOH in the extraction solvent at 81.0 °C and 200 atm, a 60 s purge, pH 7.00 and 50% flushing, based on the volume of the extraction cell (11 mL).

Concerning the total phenolic compounds, the most influential variable was temperature. Other significant factors were the ratio solvent:flushing, the extraction pressure, and the double effect of solvent extraction. The optimum extraction conditions for total phenolic compounds were: 42% MeOH in the extraction solvent at 99.6 °C and 100 atm, a 65 s purge, pH 3.92 and 150% flushing, based on the volume of the extraction cell (11 mL). An extraction time of 2 min (with 5 min for equilibration) was the optimum extraction time for the quantitative extraction of both total anthocyanins and total phenolic compounds. The two methods showed good repeatability (RSD < 2%) and intermediate precision (RSD < 5%), and were successfully applied to real açaí samples.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/2/183/s1, Table S1. Box–Behnken design matrix with the values of the six variables for each experiment, and the measured and predicted responses (n = 2).
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