

Article

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

María José Aliaño-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.alianogonzalez@alum.uca.es (M.J.A.-G.); marta.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

Received: 28 December 2019; Accepted: 24 January 2020; Published: 28 January 2020



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], juçara [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 açai [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 açai [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 açai, 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, $\geq 95\%$ 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 açai 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 açai samples in solid state, three açai juice in liquid state, an açai tablet in solid state, and an açai 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 $-20\text{ }^{\circ}\text{C}$ 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; β_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; β_i are the linear coefficients; β_{ij} are the cross-product coefficients; and β_{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 μ L and the chromatographic separation was performed on a reverse-phase C18 analytical column (Acquity UHPLC BEH C18, Waters) (2.1 mm \times 100 mm and 1.7 μ m 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 \cdot 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, 95% B; 7.50 min, 95% B. The total run time was 12 min, including 4 min for re-equilibration.

Analytes were determined, using an electrospray source operating in positive ionization mode, under the following conditions: desolvation gas flow = 700 L \cdot hr $^{-1}$, desolvation temperature = 500 °C, cone gas flow = 10 L \cdot hr $^{-1}$, source temperature = 150 °C, capillary voltage = 700 V, cone voltage = 20 V and trap collision energy = 4 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%).

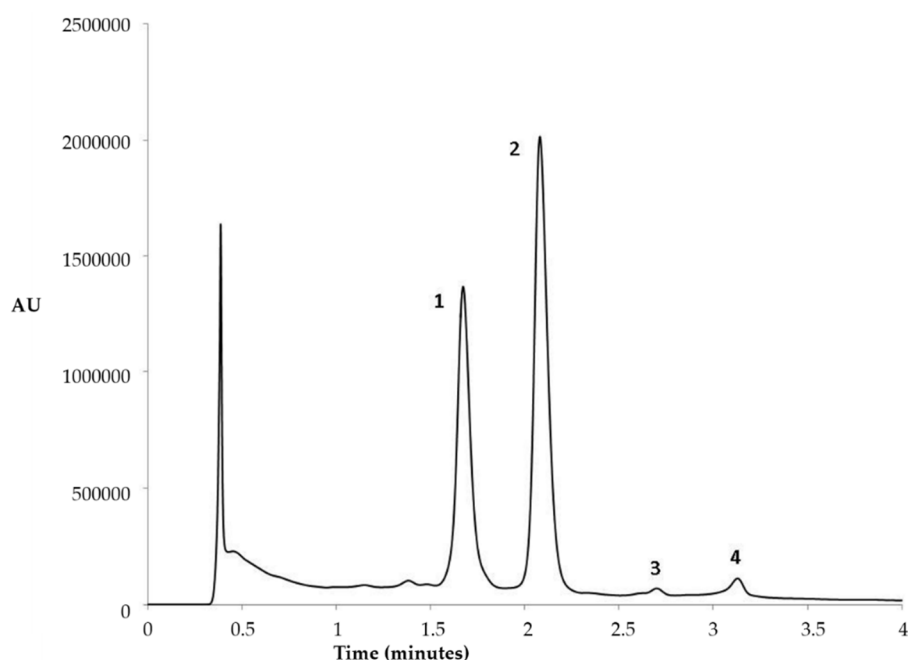


Figure 1. Chromatogram of the anthocyanins in the açai extract. 1. Cyanidin 3-*O*-glucoside; 2. Cyanidin 3-*O*-rutinoside; 3. Peonidin 3-*O*-glucoside; 4. Peonidin 3-*O*-rutinoside.

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 ($1.0 \text{ mg}\cdot\text{L}^{-1}$) was diluted in methanol at various concentrations ($0.1\text{--}500 \text{ mg}\cdot\text{L}^{-1}$) 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) = $1.649 \text{ mg}\cdot\text{L}^{-1}$; Limit of quantitation (LOQ) = $5.498 \text{ mg}\cdot\text{L}^{-1}$. Results are expressed as milligrams of gallic acid equivalent per gram of lyophilized açai.

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 açai. 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 açai 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 açai.

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 <i>p</i> -Value
β_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

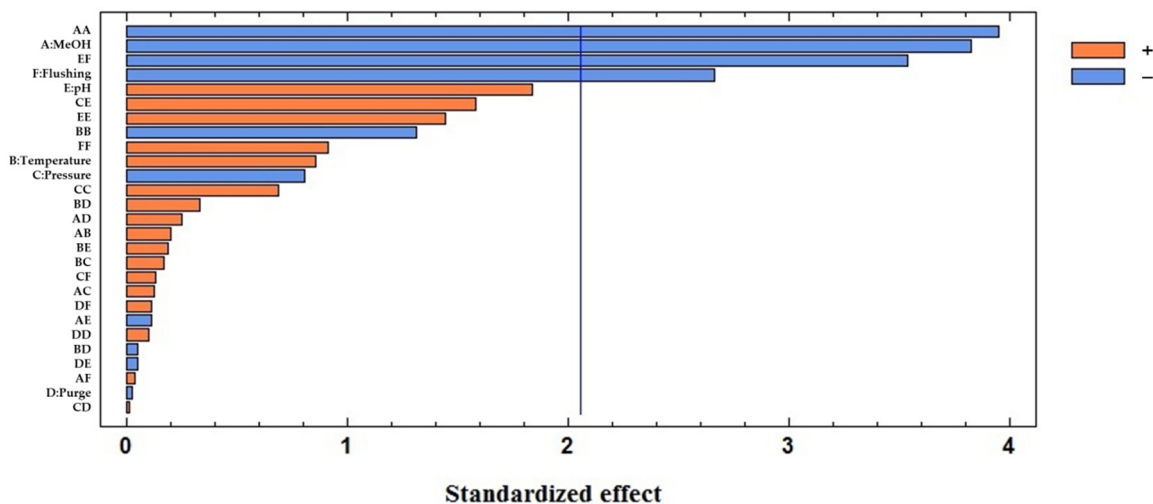


Figure 2. Standardized Pareto chart for total anthocyanins.

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.

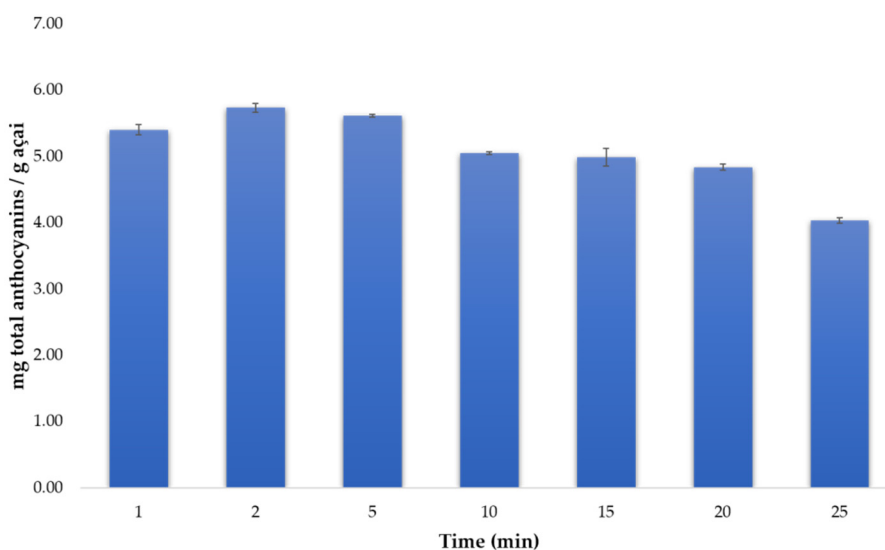


Figure 3. Average anthocyanin recoveries for extracts obtained using different extraction times ($n = 3$).

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 açai; RSD 5.85%) and two extraction cycles (5.912 milligrams anthocyanins per gram lyophilized açai; 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).

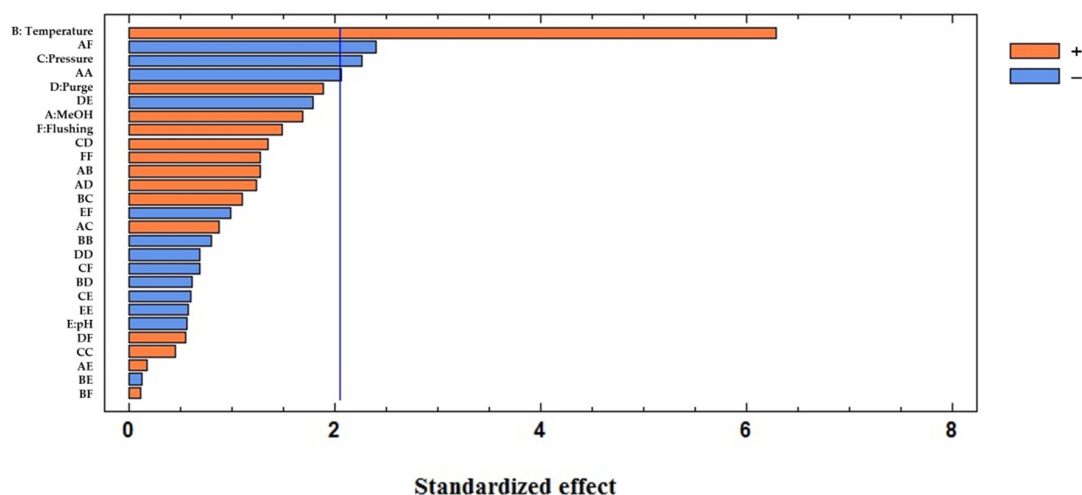


Figure 4. Standardized Pareto chart for total phenolic compounds.

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	<i>p</i> -Value
β_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:pH	−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.

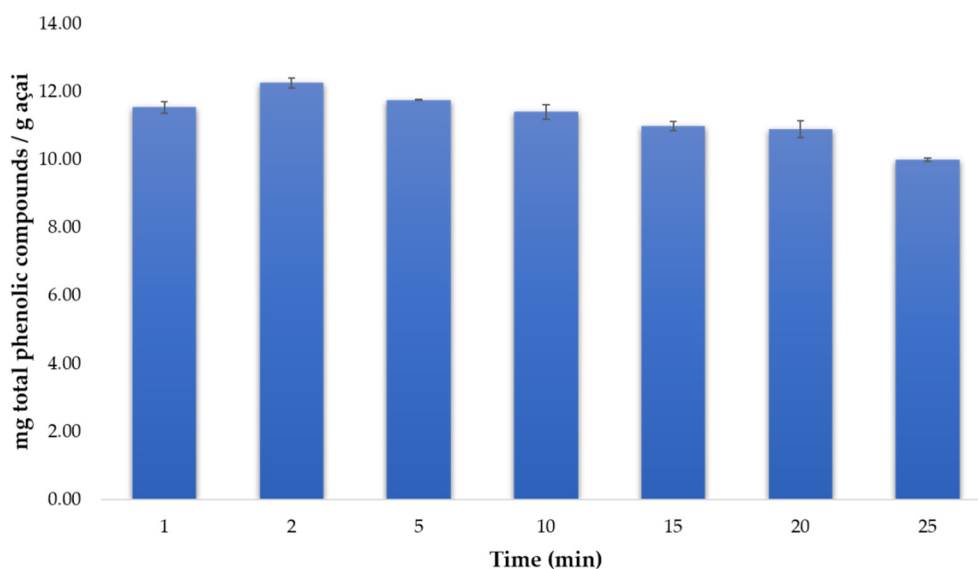


Figure 5. Average total phenolic compounds recovery rates for extracts obtained using different extraction times ($n = 3$).

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 ¹	Intermediate Precision ²
Average (mg total phenolic compounds/g açai)	12.265	12.274
Standard deviation	0.212	0.521
Relative standard deviation (%)	1.73	4.24

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

3.4. Application to Real Samples

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

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

Samples	mg Total Anthocyanins/g açai	mg Total Phenolics/g açai
Freeze-dried açai (1)	5.76 ± 0.47	12.25 ± 0.18
Freeze-dried açai (2)	4.83 ± 0.43	10.42 ± 0.62
Freeze-dried açai (3)	4.16 ± 0.45	9.48 ± 0.79
Açai juice	0.04 ± 0.00	2.25 ± 0.09
Açai juice food supplement	0.05 ± 0.00	2.15 ± 0.09
Concentrated açai-banana juice	0.01 ± 0.00	4.88 ± 0.14
Açai tablet	0.04 ± 0.00	2.02 ± 0.11
Açai 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 açai sample). This could be due to the fact that the amount of açai used in the production of these foods was very small, and manufacturers may have added other cheaper purple fruits rather than açai 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 açai. The most influential variable in the extraction of total anthocyanins from açai 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 açai 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 ($n = 2$).

Author Contributions: Conceptualization, E.E.-B. and G.F.B.; methodology, E.E.-B. and G.F.B.; software, M.F.-G.; formal analysis, M.J.A.-G. and C.C.; investigation, E.E.-B., C.C. and G.F.B.; resources, J.A.Á., M.P. and J.A.; data curation, M.J.A.-G., M.F.-G. and G.F.B.; writing—original draft preparation, M.J.A.-G.; writing—review and editing, G.F.B., M.F.-G. and E.E.-B.; supervision, G.F.B., M.F.-G. and E.E.-B.; project administration, G.F.B., J.A.Á., M.P. and J.A. All authors have read and agreed to the published version of the manuscript.

Funding: The authors acknowledge V. la Andaluza and the University of Cadiz for the support provided through the project OT2016/046.

Acknowledgments: The authors are grateful to the Instituto de Investigación Vitivinícola y Agroalimentaria (IVAGRO) for providing the necessary facilities to carry out the research and Programa de Fomento e Impulso de la Actividad de Investigación y Transferencia de la Universidad de Cádiz for the payment support for this manuscript.

Conflicts of Interest: The authors declare that they have no conflict of interest.

References

1. de Oliveira, M.d.S.P.; Schwartz, G. Açai—Euterpe oleracea. In *Exotic Fruits Reference Guide*, 1st ed.; Rodrigues, S., Silva, E., de Brito, E., Eds.; Academic Press: Cambridge, UK, 2018; pp. 1–5, ISBN 9780128031384.
2. Kang, J.; Li, Z.; Wu, T.; Jensen, G.S.; Schauss, A.G.; Wu, X. Anti-oxidant capacities of flavonoid compounds isolated from acai pulp (*Euterpe oleracea* Mart.). *Food Chem.* **2010**, *122*, 610–617. [[CrossRef](#)]
3. Lobato, K.B.d.S.; Alamar, P.D.; Caramês, E.T.d.S.; Pallone, J.A.L. Authenticity of freeze-dried açai pulp by near-infrared spectroscopy. *J. Food Eng.* **2018**, *224*, 105–111. [[CrossRef](#)]
4. Gordon, A.; Cruz, A.P.G.; Cabral, L.M.C.; de Freitas, S.C.; Taxi, C.M.A.D.; Donangelo, C.M.; de Andrade Mattietto, R.; Friedrich, M.; da Matta, V.M.; Marx, F. Chemical characterization and evaluation of antioxidant properties of Açai fruits (*Euterpe oleraceae* Mart.) during ripening. *Food Chem.* **2012**, *133*, 256–263. [[CrossRef](#)]
5. Schauss, G.A.; Jensen, X.W. *Flavor and Health Benefits of Small Fruit*; Qian, M., Rimando, A., Eds.; Oxford University Press: Oxford, UK, 2010; Volume 336, ISBN 0841225494.
6. Vázquez-Espinosa, M.; Espada-Bellido, E.; González de Peredo, A.V.; Ferreiro-González, M.; Carrera, C.; Palma, M.; Barroso, C.G.; Barbero, G.F. Optimization of Microwave-Assisted Extraction for the Recovery of Bioactive Compounds from the Chilean Superfruit (*Aristotelia chilensis* (Mol.) Stuntz). *Agronomy* **2018**, *8*, 240. [[CrossRef](#)]
7. Martinez, R.; Guimarães, D.; Berniz, C.; Abreu, J.; Rocha, A.; Moura, R.; Resende, A.; Teodoro, A. Açai (*Euterpe oleracea* Mart.) Seed Extract Induces Cell Cycle Arrest and Apoptosis in Human Lung Carcinoma Cells. *Foods* **2018**, *7*, 178. [[CrossRef](#)]
8. Espada-Bellido, E.; Ferreiro-González, M.; Carrera, C.; Palma, M.; Barroso, C.G.; Barbero, G.F. Optimization of the ultrasound-assisted extraction of anthocyanins and total phenolic compounds in mulberry (*Morus nigra*) pulp. *Food Chem.* **2017**, *219*, 23–32. [[CrossRef](#)] [[PubMed](#)]
9. Nile, S.H.; Park, S.W. Edible berries: Bioactive components and their effect on human health. *Nutrition* **2014**, *30*, 134–144. [[CrossRef](#)] [[PubMed](#)]
10. Lucas, B.F.; Zambiasi, R.C.; Costa, J.A.V. Biocompounds and physical properties of açai pulp dried by different methods. *LWT-Food Sci. Technol.* **2018**, *98*, 335–340. [[CrossRef](#)]
11. Menezes, E.M.d.S.; Torres, A.T.; Sabaa Srur, A.U. Valor nutricional da polpa de açai (*Euterpe oleracea* Mart.) liofilizada. *Acta Amaz.* **2008**, *38*, 311–316. [[CrossRef](#)]
12. Sadowska-Krepa, E.; Kłapcińska, B.; Podgórski, T.; Szade, B.; Tyl, K.; Hadzik, A. Effects of supplementation with acai (*Euterpe oleracea* Mart.) berry-based juice blend on the blood antioxidant defence capacity and lipid profile in junior hurdlers. A pilot study. *Biol. Sport* **2014**, *32*, 161–168. [[CrossRef](#)] [[PubMed](#)]
13. Yamaguchi, K.K.d.L.; Pereira, L.F.R.; Lamarão, C.V.; Lima, E.S.; da Veiga-Junior, V.F. Amazon açai: Chemistry and biological activities: A review. *Food Chem.* **2015**, *179*, 137–151. [[CrossRef](#)] [[PubMed](#)]
14. Pacheco-Palencia, L.A.; Talcott, S.T. Chemical stability of açai fruit (*Euterpe oleracea* Mart.) anthocyanins as influenced by naturally occurring and externally added polyphenolic cofactors in model systems. *Food Chem.* **2010**, *118*, 17–25. [[CrossRef](#)]
15. Brunshwig, C.; Leba, L.-J.; Saout, M.; Martial, K.; Bereau, D.; Robinson, J.-C. Chemical Composition and Antioxidant Activity of Euterpe oleracea Roots and Leaflets. *Int. J. Mol. Sci.* **2016**, *18*, 61. [[CrossRef](#)] [[PubMed](#)]

16. Pacheco-palencia, L.A.; Hawken, P.; Talcott, S.T. Phytochemical, antioxidant and pigment stability of açai (*Euterpe oleracea* Mart.) as affected by clarification, ascorbic acid fortification and storage. *Food Res. Int.* **2007**, *40*, 620–628. [[CrossRef](#)]
17. Barbosa, P.O.; Pala, D.; Silva, C.T.; de Souza, M.O.; do Amaral, J.F.; Vieira, R.A.L.; Folly, G.A.d.F.; Volp, A.C.P.; de Freitas, R.N. Açai (*Euterpe oleracea* Mart.) pulp dietary intake improves cellular antioxidant enzymes and biomarkers of serum in healthy women. *Nutrition* **2016**, *32*, 674–680. [[CrossRef](#)]
18. Torma, P.d.C.M.R.; Brasil, A.V.S.; Carvalho, A.V.; Jablonski, A.; Rabelo, T.K.; Moreira, J.C.F.; Gelain, D.P.; Flôres, S.H.; Augusti, P.R.; Rios, A.d.O. Hydroethanolic extracts from different genotypes of açai (*Euterpe oleracea*) presented antioxidant potential and protected human neuron-like cells (SH-SY5Y). *Food Chem.* **2017**, *222*, 94–104. [[CrossRef](#)]
19. Censi, R.; Vargas Peregrina, D.; Lacava, G.; Agas, D.; Lupidi, G.; Sabbieti, M.; Di Martino, P. Cosmetic Formulation Based on an Açai Extract. *Cosmetics* **2018**, *5*, 48. [[CrossRef](#)]
20. Belda-Galbis, C.M.; Jiménez-Carretón, A.; Pina-Pérez, M.C.; Martínez, A.; Rodrigo, D. Antimicrobial activity of açai against *Listeria innocua*. *Food Control* **2015**, *53*, 212–216. [[CrossRef](#)]
21. Pina-Pérez, M.C.; Rivas, A.; Martínez, A.; Rodrigo, D. Effect of thermal treatment, microwave, and pulsed electric field processing on the antimicrobial potential of açai (*Euterpe oleracea*), stevia (*Stevia rebaudiana* Bertoni), and ginseng (*Panax quinquefolius* L.) extracts. *Food Control* **2018**, *90*, 98–104. [[CrossRef](#)]
22. Kang, J.; Thakali, K.M.; Xie, C.; Kondo, M.; Tong, Y.; Ou, B.; Jensen, G.; Medina, M.B.; Schauss, A.G.; Wu, X. Bioactivities of açai (*Euterpe precatoria* Mart.) fruit pulp, superior antioxidant and anti-inflammatory properties to *Euterpe oleracea* Mart. *Food Chem.* **2012**, *133*, 671–677. [[CrossRef](#)]
23. Kang, J.; Xie, C.; Li, Z.; Nagarajan, S.; Schauss, A.G.; Wu, T.; Wu, X. Flavonoids from açai (*Euterpe oleracea* Mart.) pulp and their antioxidant and anti-inflammatory activities. *Food Chem.* **2011**, *128*, 152–157. [[CrossRef](#)] [[PubMed](#)]
24. da Silveira, T.F.F.; de Souza, T.C.L.; Carvalho, A.V.; Ribeiro, A.B.; Kuhnle, G.G.C.; Godoy, H.T. White açai juice (*Euterpe oleracea*): Phenolic composition by LC-ESI-MS/MS, antioxidant capacity and inhibition effect on the formation of colorectal cancer related compounds. *J. Funct. Foods* **2017**, *36*, 215–223. [[CrossRef](#)]
25. Fragoso, M.F.; Romualdo, G.R.; Vanderveer, L.A.; Franco-Barraza, J.; Cukierman, E.; Clapper, M.L.; Carvalho, R.F.; Barbisan, L.F. Lyophilized açai pulp (*Euterpe oleracea* Mart.) attenuates colitis-associated colon carcinogenesis while its main anthocyanin has the potential to affect the motility of colon cancer cells. *Food Chem. Toxicol.* **2018**, *121*, 237–245. [[CrossRef](#)] [[PubMed](#)]
26. Coelho da Mota, D.S.; Sicuro, F.L.; Resende, A.C.; De Moura, R.S.; Bottino, D.A.; Bouskela, E. Effects of açai and cilostazol on skin microcirculation and viability of TRAM flaps in hamsters. *J. Surg. Res.* **2018**, *228*, 253–262. [[CrossRef](#)] [[PubMed](#)]
27. Poulouse, S.M.; Fisher, D.R.; Bielinski, D.F.; Gomes, S.M.; Rimando, A.M.; Schauss, A.G.; Shukitt-Hale, B. Restoration of stressor-induced calcium dysregulation and autophagy inhibition by polyphenol-rich açai (*Euterpe* spp.) fruit pulp extracts in rodent brain cells in vitro. *Nutrition* **2014**, *30*, 853–862. [[CrossRef](#)] [[PubMed](#)]
28. da Silva, R.C.; Batista, A.; Costa, D.C.F.d.; Moura-Nunes, N.; Koury, J.C.; da Costa, C.A.; Resende, Â.C.; Daleprane, J.B. Açai (*Euterpe oleracea* Mart.) seed flour prevents obesity-induced hepatic steatosis regulating lipid metabolism by increasing cholesterol excretion in high-fat diet-fed mice. *Food Res. Int.* **2018**, *111*, 408–415. [[CrossRef](#)] [[PubMed](#)]
29. Devalaraja, S.; Jain, S.; Yadav, H. Exotic fruits as therapeutic complements for diabetes, obesity and metabolic syndrome. *Food Res. Int.* **2011**, *44*, 1856–1865. [[CrossRef](#)] [[PubMed](#)]
30. Patil, V.M.; Masand, N. Anticancer Potential of Flavonoids: Chemistry, Biological Activities, and Future Perspectives. In *Studies in Natural Products Chemistry*, 1st ed.; Rahman, A., Ed.; Elsevier: Amsterdam, The Netherlands, 2019; Volume 59, pp. 401–430. ISBN 1572-5995.
31. Patrón-Vázquez, J.; Baas-Dzul, L.; Medina-Torres, N.; Ayora-Talavera, T.; Sánchez-Contreras, Á.; García-Cruz, U.; Pacheco, N. The Effect of Drying Temperature on the Phenolic Content and Functional Behavior of Flours Obtained from Lemon Wastes. *Agronomy* **2019**, *9*, 474. [[CrossRef](#)]
32. Duangpapeng, P.; Lertrat, K.; Lomthaisong, K.; Paul Scott, M.; Suriharn, B. Variability in Anthocyanins, Phenolic Compounds and Antioxidant Capacity in the Tassels of Collected Waxy Corn Germplasm. *Agronomy* **2019**, *9*, 158. [[CrossRef](#)]

33. Vázquez-Espinosa, M.; González de Peredo, A.V.; Ferreiro-González, M.; Carrera, C.; Palma, M.; Barbero, G.F.; Espada-Bellido, E. Assessment of Ultrasound Assisted Extraction as an Alternative Method for the Extraction of Anthocyanins and Total Phenolic Compounds from Maqui Berries (*Aristotelia chilensis* (Mol.) Stuntz). *Agronomy* **2019**, *9*, 148. [[CrossRef](#)]
34. González de Peredo, A.V.; Vázquez-Espinosa, M.; Espada-Bellido, E.; Jiménez-Cantizano, A.; Ferreiro-González, M.; Amores-Arrocha, A.; Palma, M.; Barroso, C.G.; Barbero, G.F. Development of New Analytical Microwave-Assisted Extraction Methods for Bioactive Compounds from Myrtle (*Myrtus communis* L.). *Molecules* **2018**, *23*, 2992. [[CrossRef](#)]
35. González de Peredo, A.V.; Vázquez-Espinosa, M.; Espada-Bellido, E.; Ferreiro-González, M.; Amores-Arrocha, A.; Palma, M.; Barbero, G.F.; Jiménez-Cantizano, A. Alternative Ultrasound-Assisted Method for the Extraction of the Bioactive Compounds Present in Myrtle (*Myrtus communis* L.). *Molecules* **2019**, *24*, 882. [[CrossRef](#)]
36. Duarte, L.J.; Chaves, V.C.; Nascimento, M.V.P.d.S.; Calvete, E.; Li, M.; Ciralo, E.; Ghigo, A.; Hirsch, E.; Simões, C.M.O.; Reginatto, F.H.; et al. Molecular mechanism of action of Pelargonidin-3-O-glucoside, the main anthocyanin responsible for the anti-inflammatory effect of strawberry fruits. *Food Chem.* **2018**, *247*, 56–65. [[CrossRef](#)]
37. Phan, M.A.T.; Bucknall, M.; Arcot, J. Interactive effects of β -carotene and anthocyanins on cellular uptake, antioxidant activity and anti-inflammatory activity in vitro and ex vivo. *J. Funct. Foods* **2018**, *45*, 129–137. [[CrossRef](#)]
38. de Pascual-Teresa, S. Molecular mechanisms involved in the cardiovascular and neuroprotective effects of anthocyanins. *Arch. Biochem. Biophys.* **2014**, *559*, 68–74. [[CrossRef](#)]
39. Wang, L.-S.; Stoner, G.D. Anthocyanins and their role in cancer prevention. *Cancer Lett.* **2008**, *269*, 281–290. [[CrossRef](#)]
40. Su, C.-C.; Wang, C.-J.; Huang, K.-H.; Lee, Y.-J.; Chan, W.-M.; Chang, Y.-C. Anthocyanins from Hibiscus sabdariffa calyx attenuate in vitro and in vivo melanoma cancer metastasis. *J. Funct. Foods* **2018**, *48*, 614–631. [[CrossRef](#)]
41. Long, H.-L.; Zhang, F.-F.; Wang, H.-L.; Yang, W.-S.; Hou, H.-T.; Yu, J.-K.; Liu, B. Mulberry anthocyanins improves thyroid cancer progression mainly by inducing apoptosis and autophagy cell death. *Kaohsiung J. Med. Sci.* **2018**, *34*, 255–262. [[CrossRef](#)]
42. Mazewski, C.; Liang, K.; Gonzalez de Mejia, E. Inhibitory potential of anthocyanin-rich purple and red corn extracts on human colorectal cancer cell proliferation in vitro. *J. Funct. Foods* **2017**, *34*, 254–265. [[CrossRef](#)]
43. Zhou, L.; Wang, H.; Yi, J.; Yang, B.; Li, M.; He, D.; Yang, W.; Zhang, Y.; Ni, H. Anti-tumor properties of anthocyanins from *Lonicera caerulea* 'Beilei' fruit on human hepatocellular carcinoma: In vitro and in vivo study. *Biomed. Pharmacother.* **2018**, *104*, 520–529. [[CrossRef](#)]
44. Celli, G.B.; Tan, C.; Selig, M.J. Anthocyanidins and Anthocyanins. In *Encyclopedia of Food Chemistry*, 1st ed.; Melton, L., Shahidi, F., Varelis, P., Eds.; Elsevier: Amsterdam, The Netherlands, 2019; Volume 3, pp. 218–223. ISBN 978-0-12-814045-1.
45. Santos-Buelga, C.; González-Paramás, A.M. Anthocyanins. In *Encyclopedia of Food Chemistry*, 1st ed.; Melton, L., Shahidi, F., Varelis, P., Eds.; Elsevier: Amsterdam, The Netherlands, 2019; Volume 1, pp. 10–21, ISBN 978-0-12-814045-1.
46. Garzón, G.A.; Narváez-Cuenca, C.-E.; Vincken, J.-P.; Gruppen, H. Polyphenolic composition and antioxidant activity of açai (*Euterpe oleracea* Mart.) from Colombia. *Food Chem.* **2017**, *217*, 364–372. [[CrossRef](#)]
47. Oliveira, A.F.A.; Mar, J.M.; Santos, S.F.; da Silva Júnior, J.L.; Kluczkowski, A.M.; Bakry, A.M.; Bezerra, J.d.A.; Nunomura, R.d.C.S.; Sanches, E.A.; Campelo, P.H. Non-thermal combined treatments in the processing of açai (*Euterpe oleracea*) juice. *Food Chem.* **2018**, *265*, 57–63. [[CrossRef](#)]
48. de Cássia Rodrigues Batista, C.; de Oliveira, M.S.; Araújo, M.E.; Rodrigues, A.M.C.; Botelho, J.R.S.; da Silva Souza Filho, A.P.; Machado, N.T.; Carvalho, R.N. Supercritical CO₂ extraction of açai (*Euterpe oleracea*) berry oil: Global yield, fatty acids, allelopathic activities, and determination of phenolic and anthocyanins total compounds in the residual pulp. *J. Supercrit. Fluids* **2016**, *107*, 364–369. [[CrossRef](#)]
49. Alcázar-Alay, S.C.; Cardenas-Toro, F.P.; Osorio-Tobón, J.F.; Barbero, G.F.; Meireles, M.A. Obtaining anthocyanin-rich extracts from frozen açai (*Euterpe oleracea* Mart.) pulp using pressurized liquid extraction. *Food Sci. Technol.* **2017**, *37*, 48–54. [[CrossRef](#)]

50. Cea Pavez, I.; Lozano-Sánchez, J.; Borrás-Linares, I.; Nuñez, H.; Robert, P.; Segura-Carretero, A. Obtaining an Extract Rich in Phenolic Compounds from Olive Pomace by Pressurized Liquid Extraction. *Molecules* **2019**, *24*, 3108. [[CrossRef](#)]
51. Otero, P.; Quintana, S.; Reglero, G.; Fornari, T.; García-Risco, M. Pressurized Liquid Extraction (PLE) as an Innovative Green Technology for the Effective Enrichment of Galician Algae Extracts with High Quality Fatty Acids and Antimicrobial and Antioxidant Properties. *Mar. Drugs* **2018**, *16*, 156. [[CrossRef](#)]
52. Leyva-Jiménez, F.J.; Lozano-Sánchez, J.; Borrás-Linares, I.; Arráez-Román, D.; Segura-Carretero, A. Comparative study of conventional and pressurized liquid extraction for recovering bioactive compounds from *Lippia citriodora* leaves. *Food Res. Int.* **2018**, *109*, 213–222. [[CrossRef](#)]
53. Okiyama, D.C.G.; Soares, I.D.; Cuevas, M.S.; Crevelin, E.J.; Moraes, L.A.B.; Melo, M.P.; Oliveira, A.L.; Rodrigues, C.E.C. Pressurized liquid extraction of flavanols and alkaloids from cocoa bean shell using ethanol as solvent. *Food Res. Int.* **2018**, *114*, 20–29. [[CrossRef](#)]
54. Machado, A.P.D.F.; Pasquel-Reátegui, J.L.; Barbero, G.F.; Martínez, J. Pressurized liquid extraction of bioactive compounds from blackberry (*Rubus fruticosus* L.) residues: A comparison with conventional methods. *Food Res. Int.* **2015**, *77*, 675–683. [[CrossRef](#)]
55. Pereira, D.T.V.; Tarone, A.G.; Cazarin, C.B.B.; Barbero, G.F.; Martínez, J. Pressurized liquid extraction of bioactive compounds from grape marc. *J. Food Eng.* **2019**, *240*, 105–113. [[CrossRef](#)]
56. Garcia-Mendoza, M.d.P.; Espinosa-Pardo, F.A.; Baseggio, A.M.; Barbero, G.F.; Maróstica Junior, M.R.; Rostagno, M.A.; Martínez, J. Extraction of phenolic compounds and anthocyanins from juçara (*Euterpe edulis* Mart.) residues using pressurized liquids and supercritical fluids. *J. Supercrit. Fluids* **2017**, *119*, 9–16. [[CrossRef](#)]
57. Sumere, B.R.; de Souza, M.C.; dos Santos, M.P.; Bezerra, R.M.N.; da Cunha, D.T.; Martinez, J.; Rostagno, M.A. Combining pressurized liquids with ultrasound to improve the extraction of phenolic compounds from pomegranate peel (*Punica granatum* L.). *Ultrason. Sonochem.* **2018**, *48*, 151–162. [[CrossRef](#)] [[PubMed](#)]
58. Espada-Bellido, E.; Ferreira-González, M.; Barbero, G.F.; Carrera, C.; Palma, M.; Barroso, C.G. Alternative Extraction Method of Bioactive Compounds from Mulberry (*Morus nigra* L.) Pulp Using Pressurized-Liquid Extraction. *Food Anal. Methods* **2018**, *11*, 2384–2395. [[CrossRef](#)]
59. Guaita, M.; Bosso, A. Polyphenolic Characterization of Grape Skins and Seeds of Four Italian Red Cultivars at Harvest and after Fermentative Maceration. *Foods* **2019**, *8*, 395. [[CrossRef](#)]
60. David, L.; Danciu, V.; Moldovan, B.; Filip, A. Effects of In Vitro Gastrointestinal Digestion on the Antioxidant Capacity and Anthocyanin Content of Cornelian Cherry Fruit Extract. *Antioxidants* **2019**, *8*, 114. [[CrossRef](#)]
61. Polumackanycz, M.; Sledzinski, T.; Goyke, E.; Wesolowski, M.; Viapiana, A. A Comparative Study on the Phenolic Composition and Biological Activities of *Morus alba* L. Commercial Samples. *Molecules* **2019**, *24*, 3082. [[CrossRef](#)]
62. Aprile, A.; Negro, C.; Sabella, E.; Luvisi, A.; Nicoli, F.; Nutricati, E.; Vergine, M.; Miceli, A.; Blando, F.; De Bellis, L. Antioxidant Activity and Anthocyanin Contents in Olives (cv Cellina di Nardò) during Ripening and after Fermentation. *Antioxidants* **2019**, *8*, 138. [[CrossRef](#)]
63. de Moura, R.S.; Ferreira, T.S.; Lopes, A.A.; Pires, K.M.P.; Nesi, R.T.; Resende, A.C.; Souza, P.J.C.; da Silva, A.J.R.; Borges, R.M.; Porto, L.C.; et al. Effects of *Euterpe oleracea* Mart. (AÇAI) extract in acute lung inflammation induced by cigarette smoke in the mouse. *Phytomedicine* **2012**, *19*, 262–269. [[CrossRef](#)]
64. Fibigr, J.; Šatinský, D.; Solich, P. A UHPLC method for the rapid separation and quantification of anthocyanins in acai berry and dry blueberry extracts. *J. Pharm. Biomed. Anal.* **2017**, *143*, 204–213. [[CrossRef](#)]
65. Albuquerque, T.G.; Silva, M.A.; Oliveira, M.B.P.P.; Costa, M.B.P.P.; Oliveira, H.S. Analysis, Identification, and Quantification of Anthocyanins. In *Fruit Juices*; Rajauria, G., Tiwari, B.K., Eds.; Academic Press: San Diego, CA, USA, 2018; Volume 8, pp. 693–737. ISBN 978-0-12-802230-6.
66. Singh, J.; Jayaprakasha, G.K.; Patil, B.S. An optimized solvent extraction and characterization of unidentified flavonoid glucuronide derivatives from spinach by UHPLC-HR-QTOF-MS. *Talanta* **2018**, *188*, 763–771. [[CrossRef](#)]
67. Singleton, V.L.; Rossi, J.A. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.
68. Singleton, V.L.; Orthofer, R.; Lamuela-Raventós, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In *Methods in Enzymology*, 1st ed.; Packer, L., Ed.; Elsevier: Amsterdam, The Netherlands, 1998; Volume 299, pp. 152–178, ISBN 9780121822002.

69. Wang, L.; Weller, C.L. Recent advances in extraction of nutraceuticals from plants. *Trends Food Sci. Technol.* **2006**, *17*, 300–312. [[CrossRef](#)]
70. Shang, Y.F.; Kim, S.M.; Um, B.H. Optimisation of pressurised liquid extraction of antioxidants from black bamboo leaves. *Food Chem.* **2014**, *154*, 164–170. [[CrossRef](#)]
71. Gardana, C.; Ciappellano, S.; Marinoni, L.; Fachechi, C.; Simonetti, P. Bilberry adulteration: Identification and chemical profiling of anthocyanins by different analytical methods. *J. Agric. Food Chem.* **2014**, *62*, 10998–11004. [[CrossRef](#)]
72. Sui, X.; Bary, S.; Zhou, W. Changes in the color, chemical stability and antioxidant capacity of thermally treated anthocyanin aqueous solution over storage. *Food Chem.* **2016**, *192*, 516–524. [[CrossRef](#)]



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).