processes

MDPI

Article

Extraction of Anthocyanins from Red Raspberry for Natural Food Colorants Development: Processes Optimization and In Vitro Bioactivity

Rosiane Rocha, José Pinela *, Rui M.V. Abreu, Mikel Añibarro-Ortega, Tânia C.S.P. Pires, Ana Luísa Saldanha, Maria José Alves, António Nogueira, Isabel C.F.R. Ferreira and Lillian Barros *

Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal; roseyarine@hotmail.com (R.R.); ruiabreu@ipb.pt (R.M.V.A.); mikel@ipb.pt (M.A.-O.); tania.pires@ipb.pt (T.C.S.P.P.); ana.saldanha@ipb.pt (A.L.S.); maria.alves@ipb.pt (M.J.A.); ajmnogueira@ipb.pt (A.N.); iferreira@ipb.pt (I.C.F.R.F.)

* Correspondence: jpinela@ipb.pt (J.P.); lillian@ipb.pt (L.B.)

Received: 16 October 2020; Accepted: 9 November 2020; Published: 12 November 2020

Abstract: Heat (HAE)- and ultrasound (UAE)-assisted extraction methods were implemented to recover anthocyanins from red raspberry. Processing time, ethanol concentration, and temperature or ultrasonic power were the independent variables combined in five-level rotatable central composite designs coupled with response surface methodology (RSM) for processes optimization. The extraction yield and levels of cyanidin-3-*O*-sophoroside (C3S) and cyanidin-3-*O*-glucoside (C3G) were monitored by gravimetric and HPLC-DAD-ESI/MS^n methods, respectively, and used as response criteria. The constructed theoretical models were successfully fitted to the experimental data and used to determine the optimal extraction conditions. When maximizing all responses simultaneously, HAE originated slightly higher response values (61% extract weight and 8.7 mg anthocyanins/g extract) but needed 76 min processing at 38 °C, with 21% ethanol (*v*/*v*), while the UAE process required 16 min sonication at 466 W, using 38% ethanol (*v*/*v*). The predictive models were experimentally validated, and the purple-red extracts obtained under optimal condition showed antioxidant activity through lipid peroxidation and oxidative hemolysis inhibition, and antibacterial effects against food-related microorganisms, such as *Escherichia coli* and *Enterococcus faecalis*. These results highlight the potential of red raspberry extracts as natural food colorants with bioactive effects and could be exploited by industries interested in the production of anthocyanin-based products.

Keywords: *Rubus idaeus* L.; cyanidin glycosides; heat-/ultrasound-assisted extraction; process optimization; antioxidant/antimicrobial activity; natural colorants

1. Introduction

Food colorants are increasingly used in the food industry to enhance or sustain the sensory characteristics of food and to retain the desired color appearance [1]. In recent years, the intake of food products formulated with artificial colorants has become quite controversial due to the potential negative effects these molecules can have on consumer health. In this sense, many of the world's largest food companies have undertaken to replace these additives (chemically synthesized or chemically modified after extraction) with natural counterparts in order to meet current consumer expectations for natural-like, safer and healthy foods [1,2]. Despite this, the use of natural colorants is still limited in the industrial sector, partly due to the limited availability of options [2], although there are still many untapped natural sources.

Anthocyanins, carotenoids, betalains, and phenolic compounds are naturally occurring food colorants. Annatto, carminic acid, and some curcuminoids have also been studied, as well as other colorants that still remain to be assessed and evaluated to be authorized with an "E" code [3]. Among these molecules, anthocyanins show attractive colors ranging from red to purple and constitute an important group of water soluble plant pigments allowed to be used as food colorants [4]. In addition to their coloring capacity, anthocyanins impart positive effects on human health, being involved in cardiovascular disease prevention and obesity control, and can also be used as functional additives [5–7]. These pigments are particularly found in berries, among which red raspberry arises as an excellent source of anthocyanins (namely including cyanidin-3-*O*-glucoside and cyanidin-3-*O*-sophoroside) [6,8–11] and it seems to be promising to use their anthocyanin-rich extracts as natural bioactive colorants.

As with other bioactives, the anthocyanins recovery from plant materials generally follows the so-called "5-stages universal recovery process", a holistic approach that goes from the sample pre-treatment to the final bio-based product formulation, in which the extraction is a crucial step [12]. Among the existing extraction techniques, the conventional solid-liquid methods such as maceration, Soxhlet, or heat-assisted extraction (HAE) usually involve high solvent volumes or long processing times that can affect the compounds integrity and/or bioactivity or makes them expensive methodologies [13]. On the other hand, non-conventional methods such as ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE), and others intensified by high pressure and electrotechnologies, have been tested in an attempt to find the most efficient device to increase the extraction yield of intracellular compounds [14–16]. The UAE is a consolidated time-saving technique in solid-liquid extraction from bench-scale to industrial applications, which cavitation effects not only enables the disruption of the plant cell walls but also promotes the reduction of the particle size that benefits solvent-solute interactions [17]. However, the energy used to promote the disruption of plant cells can also affect the target compounds if applied excessively, as well as the other factors used in the extraction process. Therefore, relevant factors or independent variables should be combined in experimental designs coupled with an appropriate optimization method, such as the response surface methodology (RSM). Unlike one-factor-at-a-time approaches, RSM describes the relationship between independent variables and one or more response and allows to access interactions and optimize processes with a low number of experimental runs.

Regardless of the increasing number of studies on anthocyanins recovery from plant matrices, such as red raspberry [18,19], strawberry-tree (*Arbutus unedo* L.) fruit [20], passion fruit (*Passiflora edulis* Sims) epicarp [21], jabuticaba (*Myrciaria jaboticaba* (Vell.) Berg.) epicarp [7], and fig (*Ficus carica* L.) peel [14], industrial production and commercialization of such products remain insufficient [22,23]. In this context, optimization and comparison of conventional (HAE) and innovative (UAE) techniques seems to be a promising trend and good options for converting red raspberry into food colorants. Therefore, this study was carried out with the general objective of developing anthocyanin-rich extracts from red raspberry with potential to be used as natural bioactive food colorants. In the first instance, two extraction processes intensified by heat and ultrasound were optimized by combing three relevant independent variables on a five-level rotatable central composite design (RCCD) coupled with RSM. Then, after determining the optimal processing conditions that maximize the response values, two extracts were produced under such conditions to experimentally validate the predictive models and to assess their coloring potential and antioxidant and antimicrobial activities in vitro, in order to ensure their potential as food ingredient.

2. Material and Methods

2.1. Chemicals and Standards

Trifluoroacetic acid and high-performance liquid chromatography (HPLC)-grade acetonitrile were purchased from Fisher Scientific, Lisbon, Portugal. The standard compounds used for chromatographic quantifications (cyanidin-3-*O*-glucoside, from Extrasynthèse, Genay, France) and bioactivity assays

(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), from Sigma-Aldrich, St. Louis, MO, USA) had a purity level of at least 95%. 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) was also acquired from Sigma-Aldrich. *p*-Iodonitrotetrazolium chloride (INT) was acquired from Panreac Applichem, Barcelona, Spain. Tryptic soy broth (TSB) and Mueller-Hinton (MH) were purchased from Biolab, Budapest, Hungary. The antibiotics imipenem and vancomycin were purchased from Hikma Farmacêutica, S.A., Sintra, Portugal, while ampicillin was acquired from Fisher Scientific, Janssen Pharmaceutica NV, Belgium. All other reagents were of analytical grade and purchased from common sources.

2.2. Plant Material

Red raspberries (*Rubus idaeus* L., Fam. Rosaceae) of the cultivar "Kweli" were cultivated and supplied by the company "Ponto Agrícola Unipessoal, Lda" in Tabuado, Marco de Canaveses, Portugal. A batch of ripe fruits representative of the September 2018 harvest was lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA), powdered in a domestic grinder and sieved to ~20 mesh, and then homogenized to obtain a representative sample that was kept at −20 °C until analysis.

2.3. Color Measurements

The color of the dried red raspberry powder was measured with a colorimeter (model CR-400; Konica Minolta Sensing Inc., Japan) previously calibrated with a standard white tile. The parameters L^* (lightness), a^* (chromaticity from $^{(-)}$ green to $^{(+)}$ red), and b^* (chromaticity from $^{(-)}$ blue to $^{(+)}$ yellow) were registered using illuminant C and 8 mm diaphragm opening [24]. The CIELAB color values were then converted to RGB values using an online color converter.

2.4. Experimental Design

Two three-factor rotatable central compound designs (RCCD) with five levels each were implemented to optimize the anthocyanin extraction processes from red raspberry. The coded and natural values of the independent variables X_1 (time: t, min), X_2 (temperature: T, °C, or ultrasonic power: P, W) and X_3 (solvent ratio: S, % ethanol/water, v/v) are shown in Table 1. These variables and the respective range of values were selected based on previous optimization studies [7,15,20,21]. The Design-Expert software, Version 11 (Stat-Ease, Inc., Minneapolis, MN, USA) was used to generate the 20 experimental points of the RCCD design by entering the factor ranges in terms of alphas ($\alpha = 1.68$). These rotatable designs included 8 factorial points, 6 replicated center points, and 6 axial or star points chosen to allow rotatability, ensuring that the variance of the model prediction is constant at all points equidistant from the center point. The 20 experimental runs were randomized to minimize the effects of unexpected variability in the observed responses.

Table 1. Natural and codded values of the independent variables used in the five-level RCCD design implemented to optimize the heat- and ultrasound-assisted extraction processes using RSM.

Coded Values	Natural Values					
	Heat-Assisted Extraction			Ultrasound-Assisted Extraction		
	***t* (min)**	***T* (°C)**	***S* (%)**	***t* (min)**	***P* (W)**	***S* (%)**
−1.68	2	20	0	2	5	0
−1	20	34	20	11	106	20
0	46	55	50	24	253	50
+1	72	76	80	36	400	80
+1.68	90	90	100	45	500	100

t: time; *T*: temperature; *P*: ultrasonic power; *S*: solvent (ethanol concentration).

2.5. Extraction Methods

The HAE was performed in a thermostated water bath using sealed vessels to avoid solvent evaporation. Sample weights (600 mg) were mixed with 20 mL of solvent (ethanol/water mixtures acidified with citric acid until pH 3) and processed by stirring at different levels of time (2–90 min), temperature (20–90 °C) and ethanol concentration (0–100%) (Table 1). An ultrasonic system (Ultrasonic homogenizer, model CY-500, Optic Ivymen Systen, Barcelona, Spain) equipped with a titanium probe was used in the UAE process, in which the samples were processed at different levels of time (2–45 min), ultrasonic power (5–500 W; at 20 kHz frequency) and ethanol concentration (0–100 %) (Table 1), while the temperature was kept constant. A solid/liquid ratio of 30 g/L was used in both methods. After extraction, the mixtures were centrifuged at 4000× *g* for 10 min and the supernatants were carefully collected. An aliquot of each supernatant was used to determine the extraction yield (extract weight, %, *w*/*w*) and the remainder was frozen and lyophilized for anthocyanins quantification.

2.6. HPLC Analysis of Anthocyanins

The dried extracts (~10 mg) were redissolved in 2 mL of methanol/water (20:80, *v*/*v*) and filtered through 0.22 µm syringe filter discs. The analysis was performed by high-performance liquid chromatography (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) coupled with a diode-array detector (DAD) and a Linear Ion Trap (LTQ XL) mass spectrometer (MS, Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. Compounds separation was achieved on an AQUA® (Phenomenex) reverse phase C18 column (150 mm × 4.6 mm, 5 µm). Elution was made with 0.1% trifluoroacetic acid and acetonitrile as previously described [25]. Anthocyanins identification was performed in double online detection using DAD (at 520 nm) coupled to the MS working in positive mode. The compounds identification was achieved by comparing their retention times, fragmentation pattern, and UV–Vis spectra with those of standards or data available in literature. Quantification was done with the calibration curve of the standard cyanidin-3-*O*-glucoside ($y = 134578x - 3 \times 10^6$; $r^2 = 0.9986$; LOD = 0.25 µg/mL; LOQ = 0.83 µg/mL). Data were recorded and processed using Xcalibur data system (Thermo Finnigan, San Jose, CA, USA) and the results were expressed in mg per g of extract.

2.7. Extraction Process Modelling and Statistical Analysis

The dependent variables Y_1 (extraction yield), Y_2 (cyanidin-3-*O*-sophoroside content, C3S), Y_3 (cyanidin-3-*O*-glucoside content, C3G), and Y_4 (total anthocyanin content, C3S + C3G) were used to optimize the recovery of anthocyanins from red raspberry. Fitting procedures, coefficient estimates, and statistical verification were performed using Design-Expert software. The analyses of variance (ANOVA) was used to assess the significance of the models generated and of all the terms that make up these models (polynomial equations), as well as the lack-of-fit. The method for testing statistical significance was performed by calculating the *p*-value from the F-value, considering the existence of significance for $p < 0.05$. Only the statistically significant terms were used in the construction of the mathematical models.

The coefficient of determination (R^2), the adjusted coefficient of determination (R^2_{adj}) and the adequate precision, interpreted as the proportion of variability of the dependent variable explained by the model [26], were used to estimate the adequacy of the polynomial equations to the responses. The R^2 and R^2_{adj} values are the main indicators of the model's significance; R^2 represents the variation around the average explained by the model, while R^2_{adj} results from an adjustment that considers the number of variables and significant terms in the model. Therefore, values close to 1 illustrate an agreement between theoretical and experimental data. For adequate precision, which is a measure of signal-to-noise ratio, the value must be greater than 4.

The lack-of-fit measures the quality of the model's fit to the experimental data. This test indicates whether the model adequately describes the functional relationship between the independent variables

and the obtained response. Thus, the lack-of-fit must be non-significant ($p > 0.05$) [26]. Design-Expert software was also used to generate the response surface graphics.

2.8. Models Validation and Evaluation of the Coloring and Bioactive Potential of the Extracts Obtained under Optimized Processing Conditions

The optimized global HAE and UAE conditions that maximize the recovery of anthocyanins from red raspberry were applied to obtain two extracts rich in these pigments, following the procedure described in Section 2.5. These new extracts were used in the process of experimental validation of the theoretical models, carried out through the analysis of the experimental responses (extraction yield and anthocyanins content) of these extracts and comparison with the model-predicted values. The color of these extracts was also evaluated, as well as their antioxidant and antimicrobial activity.

Coloring potential. The CIELAB color and the anthocyanin content of the extracts were determined as explained in Section 2.3. and Section 2.6., respectively.

Lipid peroxidation inhibition activity by thiobarbituric acid reactive substances (TBARS) formation inhibition. A porcine brain cell solution (1:2, *w*/*v*; 0.1 mL) was incubated with the extract solutions (0.02–10 mg/mL in water; 0.2 mL) or trolox (3.125–100 μg/mL in water) plus $FeSO_4$ (10 μM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 °C for 1 h. Then, trichloroacetic (28% *w*/*v*, 0.5 mL) and thiobarbituric (TBA, 2%, *w*/*v*, 0.38 mL) acids were added and the mixture was heated at 80 °C for 20 min. After centrifugation at 3000× *g* for 10 min, the malondialdehyde (MDA)-TBA complexes formed in vitro were monitored at 532 nm (Specord 200 spectrophotometer, Analytik Jena, Jena, Germany). The results were given as EC_{50} values (μg/mL).

Antihemolytic activity evaluation by the oxidative hemolysis inhibition assay (OxHLIA). An erythrocyte solution (2.8%, *v*/*v*; 200 μL) prepared in phosphate-buffered saline (PBS, pH 7.4) was mixed with 400 μL of either extract solution (0.03–1 mg/mL in PBS), trolox (7.81–250 μg/mL in PBS), PBS (control), or water (for complete hemolysis). After pre-incubation at 37 °C for 10 min with shaking, AAPH (200 μL, 160 mM in PBS) was added, and the optical density was measured at 690 nm every ~10 min in a microplate reader (Bio-Tek Instruments, ELX800) until complete hemolysis [27]. The results were given as IC_{50} values (μg/mL) at a Δt of 60 min.

Antibacterial activity. The extracts were redissolved at 0.156–20 mg/mL in a mixture of dimethyl sulfoxide + MH Broth (5:95, *v*/*v*) and tested against microorganisms isolated from patients hospitalized in local health units in Bragança and Vila Real, Portugal. The microdilution method and the INT colorimetric assay were used to assess the minimum inhibitory concentration (MIC). The lowest extract concentration that yielded no bacterial growth was defined as minimum bactericidal concentration (MBC) [28]. Three negative controls were prepared, one with MH Broth, another one with the extracts, and the third one with the medium, inoculum and antibiotic. Ampicillin (20 mg/mL), imipenem (1 mg/mL) and vancomycin (1 mg/mL) were the used positive controls.

Statistical analysis. For the results of color and antioxidant activity analyses, differences among samples were assessed using one-way analysis of variance (ANOVA). The fulfilment of the ANOVA requirements was tested by means of the Shapiro Wilk's and the Levene's tests to assess the normality and variance homogeneity of the data, respectively. Results were compared using Tukey's HSD or Tamhane's T2 multiple comparison tests, when homoscedasticity was verified or not, respectively. In addition, statistically significant differences between experimental and model-predicted optimal response values were assessed by applying a two-tailed paired Student's *t*-test. Statistical tests were performed at a 5% significance level using SPSS Statistics software (IBM SPSS Statistics for Windows, Version 22.0. IBM Corp., Armonk, NY, USA).

3. Results and Discussion

The intrinsic nature of the natural matrices, the type of extraction solvent, and the anthocyanins susceptibility to degradation under certain conditions of temperature, processing time, and pH influence their recovery and their integrity [1]. The selection of suitable extraction intensification methods and

determination of processing conditions that maximize the recovery of high-value added compounds has gained particular interest in the scientific and industrial field. In this sense, efforts have been made to develop more efficient and sustainable extraction processes capable of improving the extraction yield and selectivity [15,18–20]. The comparison of data achieved with different extraction techniques can, however, be a very difficult task, since compositional and physicochemical variations between plant materials make direct comparison difficult. In this study, to provide a better understanding of the potential of different intensification techniques and solvents, two solid-liquid extraction methods for anthocyanin recovery from red raspberry were investigated.

3.1. Anthocyanin Profile and Experimental Data for Process Optimization

Berries in general have been described as interesting food sources of antioxidant polyphenols, particularly anthocyanins [11,29]. The HPLC-DAD-ESI/MS^n analysis allowed identifying two cyanidin glycosides, namely cyanidin-3-*O*-sophoroside (tentatively identified based on the pseudomolecular ion $[M\text{-}H]^+$ at *m*/*z* 611 and the fragment at *m*/*z* 287) and cyanidin-3-*O*-glucoside (based on the pseudomolecular ion $[M\text{-}H]^+$ at *m*/*z* 449 and the fragment at *m*/*z* 287). These compounds were previously reported as the major anthocyanin constituents of red raspberry [8,9,30], which have been related to the deep purple-red color of this fruit.

As presented in Table 2, cyanidin-3-*O*-sophoroside (C3S) predominated over cyanidin-3-*O*-glucoside (C3G). The levels of C3S ranged from 3.64–5.51 mg/g extract and 3.63–5.9 mg/g extract with the HAE and UAE methods, while the C3G content ranged from 2.27–3.49 mg/g extract and 2.61–3.9 mg/g extract, respectively. In the HAE method, the higher concentrations were achieved with the runs 2 and 7, which combined a medium-high temperature (72 min; +1 level) with a medium-low temperature and solvent concentration (34 °C a 20% ethanol/water, *v*/*v*; −1 level), or a medium-low processing time (20 min; −1 level) with a medium-high temperature and solvent concentration (76 °C and 80% ethanol/water, *v*/*v*; +1 level), respectively. In turn, the higher anthocyanin concentrations in the UAE process were achieved with the run 1, characterized by medium-low processing condition (11 min, 106 W, and 20% ethanol/water, *v*/*v*; −1 level). In this method, the increase in time and solvent to medium-high condition (36 min and 80% ethanol/water, *v*/*v*) led to the lowest values (run 6). Regarding the 6 replicated center points of the RCCD design (Table 2), these resulted in mean values of 7.7 ± 0.1 mg/g and 7.2 ± 0.1 mg/g for the HAE and UAE methods, respectively. The extraction yield (or extract weight, *w*/*w*) obtained with the 20 runs of the five-level RCCD designs implemented to optimize the HAE and UAE processes was also significantly affected. It ranged from 41.22 to 60.36% with HAE and from 18.55 to 66.23% wit UAE (Table 2). Therefore, the most accentuated variance was verified with the UAE process, with the processing conditions at medium levels (center points) leading to the higher yields, while a decrease in power and solvent levels and an increase in extraction time (run 2) resulted in the lowest value.

Table 2. Experimental responses obtained under the extraction conditions defined in the RCCD design matrix for the extraction yield (%, *w*/*w*) and contents of cyanidin-3-*O*-sophoroside (C3S, mg/g extract), cyanidin-3-*O*-glucoside (C3G, mg/g extract) and the sum of both anthocyanins (total, mg/g extract) as a function of the extraction method. The natural values of the independent variables are presented in Table 1.

Run	Experimental Design Matrix			Experimental Responses							
	t	*T*/*P*	*S*	Heat-Assisted Extraction				Ultrasound-Assisted Extraction			
	min	°C/W	% (*v*/*v*)	Yield	C3S	C3G	Total	Yield	C3S	C3G	Total
1	−1	−1	−1	54.74	4.13	2.45	6.58	25.68	5.90	3.90	10.00
2	+1	−1	−1	58.11	5.51	3.49	9.00	18.55	4.15	2.80	6.95
3	−1	+1	−1	60.36	3.70	2.43	6.13	51.12	5.02	3.28	8.30
4	+1	+1	−1	60.24	4.27	2.62	7.05	57.74	4.31	2.67	6.98
5	−1	−1	+1	57.02	4.74	2.58	7.18	46.60	4.89	3.24	8.13
6	+1	−1	+1	51.78	4.28	2.76	7.51	48.93	3.63	2.61	6.24
7	−1	+1	+1	50.00	5.31	3.19	8.50	61.11	4.23	2.76	6.98
8	+1	+1	+1	45.45	3.72	2.35	6.07	60.37	4.34	2.76	7.09
9	−1.68	0	0	60.00	4.86	2.91	7.58	56.39	4.02	2.85	6.90
10	+1.68	0	0	59.83	3.65	2.28	6.21	59.84	4.44	3.26	7.31
11	0	−1.68	0	41.22	5.08	3.15	8.22	24.22	3.89	2.73	6.62
12	0	+1.68	0	56.93	4.37	2.75	7.11	54.00	4.73	3.11	7.84
13	0	0	−1.68	53.50	4.01	2.57	6.58	46.23	5.27	2.81	8.49
14	0	0	+1.68	50.82	4.12	2.64	6.76	55.92	4.47	3.18	7.63
15	0	0	0	50.62	4.69	3.00	7.69	63.42	4.28	2.78	7.06
16	0	0	0	48.85	4.68	2.97	7.65	65.18	4.22	2.95	7.17
17	0	0	0	55.35	4.80	3.00	7.80	66.21	4.40	2.83	7.23
18	0	0	0	52.00	4.66	2.95	7.76	64.65	4.42	2.86	7.28
19	0	0	0	53.70	4.66	2.93	7.59	51.58	4.26	2.85	7.35
20	0	0	0	52.93	4.56	2.96	7.52	66.23	4.36	2.86	7.25

t: time; *T*: temperature; *P*: ultrasonic power; *S*: solvent (ethanol concentration).

3.2. Models Fitting and Statistical Verification

RMS is a statistical tool suitable for modelling and optimizing extraction processes involving one or more response variables and allows to determine the optimal processing conditions with a reduced number of experimental trials, when compared with conventional one-factor-at-a-time approaches which do not take interactions into account [31]. To develop theoretical models capable of predicting and understanding the effects of the process independent variables on a given response, it is necessary to assess the accuracy of their fitting to the experimental data. In this study, the response values in Table 2 were fitted to a polynomial regression model using the Design-Expert software, but not all parameters were used in the models construction since some coefficients were non-significant (Supplementary Material Table S1); the significant ones were assessed at a 95% confidence level. The results of ANOVA and regression analyses are presented in Supplementary Material Table S1. The developed polynomial models, expressed in coded values, are presented in Equations (1)–(8).

For the HAE process:

$$Y_{(Yield)} = 52.34 - 3.64T + 2.89t^2 - 1.63tS - 2.64TS - 2.85t^2S + 2.94T^3 \quad (1)$$

$$Y_{(C3S)} = 4.67 - 0.36t - 0.21T - 0.12t^2 - 0.19S^2 - 0.24tT - 0.50tS + 0.21TS + 0.35tT^2 \quad (2)$$

$$Y_{(C3G)} = 2.97 + 0.21t - 0.10T - 0.13t^2 - 0.12S^2 - 0.23tT - 0.24tS + 0.14TS - 0.14t^3 \quad (3)$$

$$Y_{(Total)} = 7.66 - 0.28t - 0.32T - 0.18t^2 - 0.32S^2 - 0.54tT - 0.67tS + 0.28TS - 0.18tTS + 0.45tT^3 \quad (4)$$

For the UAE process:

$$Y_{(Yield)} = 62.96 + 10.30P - 8.90P^2 - 4.67S^2 - 4.84PS \quad (5)$$

$$Y_{(C3S)} = 4.30 + 0.12t - 0.27P - 0.27S + 0.21S^2 + 0.30tP + 0.16tS + 0.10PS - 0.57tP^2 + 0.18P^3 \quad (6)$$

$$Y_{(C3G)} = 2.85 + \begin{array}{c} 0.12t + 0.11P + 0.14S + 0.07t^2 + 0.04S^2 + 0.14tP + 0.14tS + 0.05PS \\ -0.25t^2P - 0.30t^2S - 0.41tP^2 \end{array} \quad (7)$$

$$Y_{(Total)} = 7.22 + \begin{array}{c} 0.12t + 0.36P - 0.26S + 0.32S^2 + 0.47tP + 0.33tS + 0.18PS - 0.62t^2P \\ -0.22t^2S - 0.89tP^2 \end{array} \quad (8)$$

In the mathematical models presented above, the coefficients in front of each term illustrate the effect of the independent variables and the interaction among them. The parametric values represent the expected change in response per unit change in factor value when all remaining factors are held constant. The higher the parametric value, the more significant is the weight of the variable. Additionally, a synergistic effect is indicated by a positive sign, while a negative sign indicates an antagonism [15]. Therefore, the complexity of the extraction trends is illustrated by the developed models. In each equation, the intercept corresponds to the overall average response of all the runs of the RSM design (Table 2). In the HAE equations, these values are lower for the extraction yield and higher for the anthocyanin levels, which could somehow translate a greater selectivity of this extraction method for the target compounds.

All models presented a non-significant (*ns*) lack of fit ($p > 0.05$) and an adequate precision greater than 12, which indicates that the model equations adequately describe the effects of the independent variables on the evaluated responses [32]. The coefficients R^2 and R^2_{adj} were greater than 0.93 and 0.86 in all cases, respectively (Supplementary Material Table S1), thus indicating that the variability of each response can be explained by the variables involved in the extraction processes. All models proved to be statistically adequate and, therefore, were used to navigate the design space in the optimization steps. The model coefficients are empirical and do not reflect physical or chemical significance, but they are useful to predict the outcome of untested experimental extraction conditions [33].

Certain features regarding the overall effects of the independent variables on the extraction of anthocyanins from red raspberry can be inferred from the complexity of the polynomial equations constructed for the HAE and UAE processes. In the first method, the variables T and t significantly affected the extraction yield (extract weight) through negative linear and positive quadratic affects, respectively, which was also affected by the negative interaction of S with the variables T and t. For anthocyanins, the extraction was strongly affected by negative interactions between the three variables, mainly $t \times S$ and $t \times T$. In the UAE process, the variable P induced significant linear and negative quadratic effects on the extraction yield; the variable S also induced a negative quadratic effect and interacted negatively with P. As depicted in the polynomial equations of both methods, the anthocyanin extraction trend was more complex when using ultrasounds as intensification factor; this process was affected mostly by the linear effects of P and S and the interactions $t \times P$ and $t \times S$, as well as $t \times P^2$. These results justify the use of the RSM optimization tool, since the one-factor-at-a-time approaches do not assess interactive effects, which makes the determination of optimum processing values difficult.

3.3. Effect of Extraction Parameters on the Anthocyanin Content

The response surface graphs generated to illustrate the effect of the independent variables on the extraction yield and anthocyanin levels achieved with HAE and UAE are presented in Figures 1 and 2, respectively. In each 3D graph, the excluded independent variable was positioned at its individual optimal value, which is presented in Table 3.

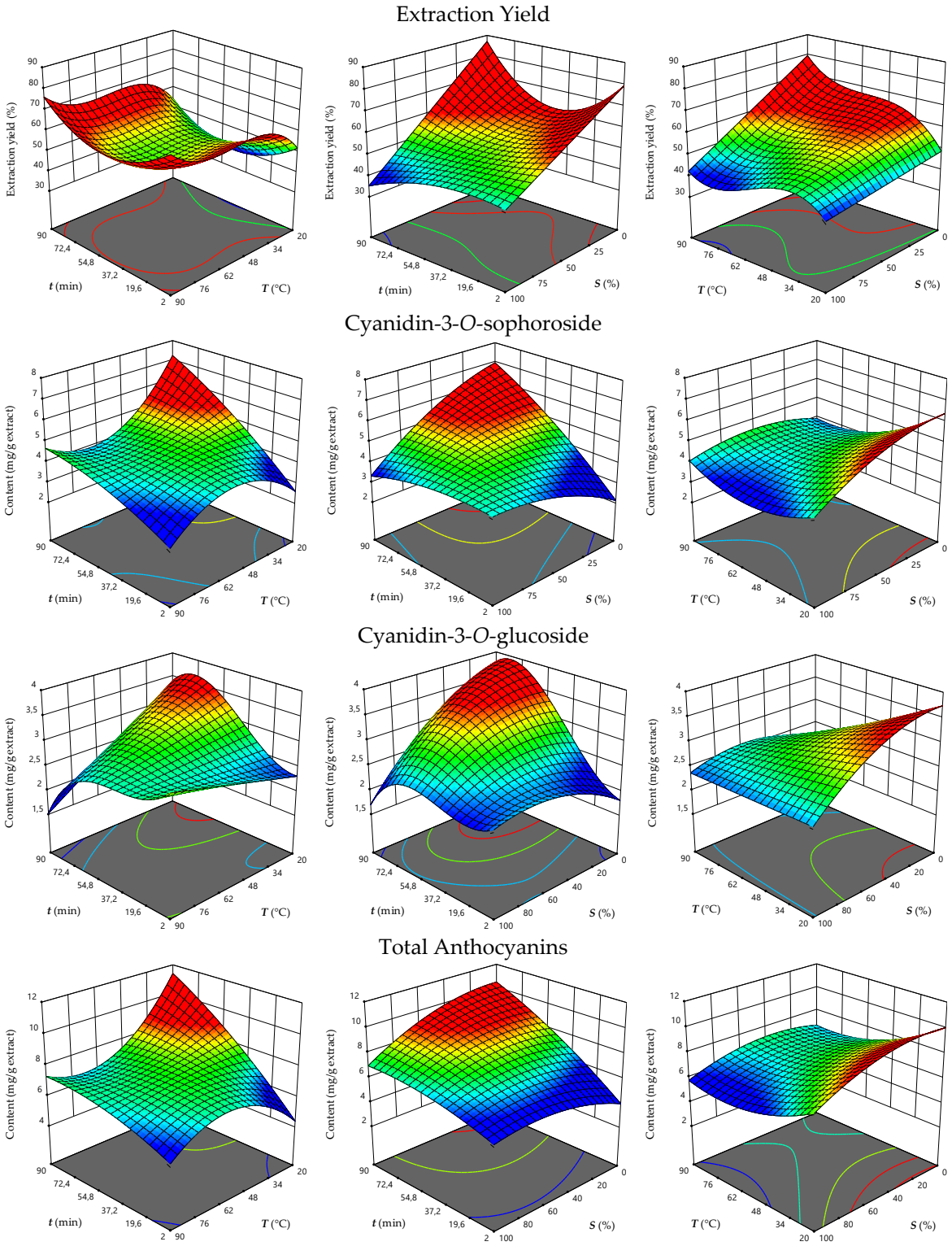

Figure 1. Response surface graphs for the effects of extraction time (*t*), temperature (*T*), and ethanol concentration (*S*) on the extraction yield (%) and levels of cyanidin-3-*O*-sophoroside (C3S), cyanidin-3-*O*-glucoside (C3G), and total anthocyanins (mg/g extract) obtained from red raspberry by the HAE process. In each graph, the excluded variable was positioned at its optimal value (Table 3).

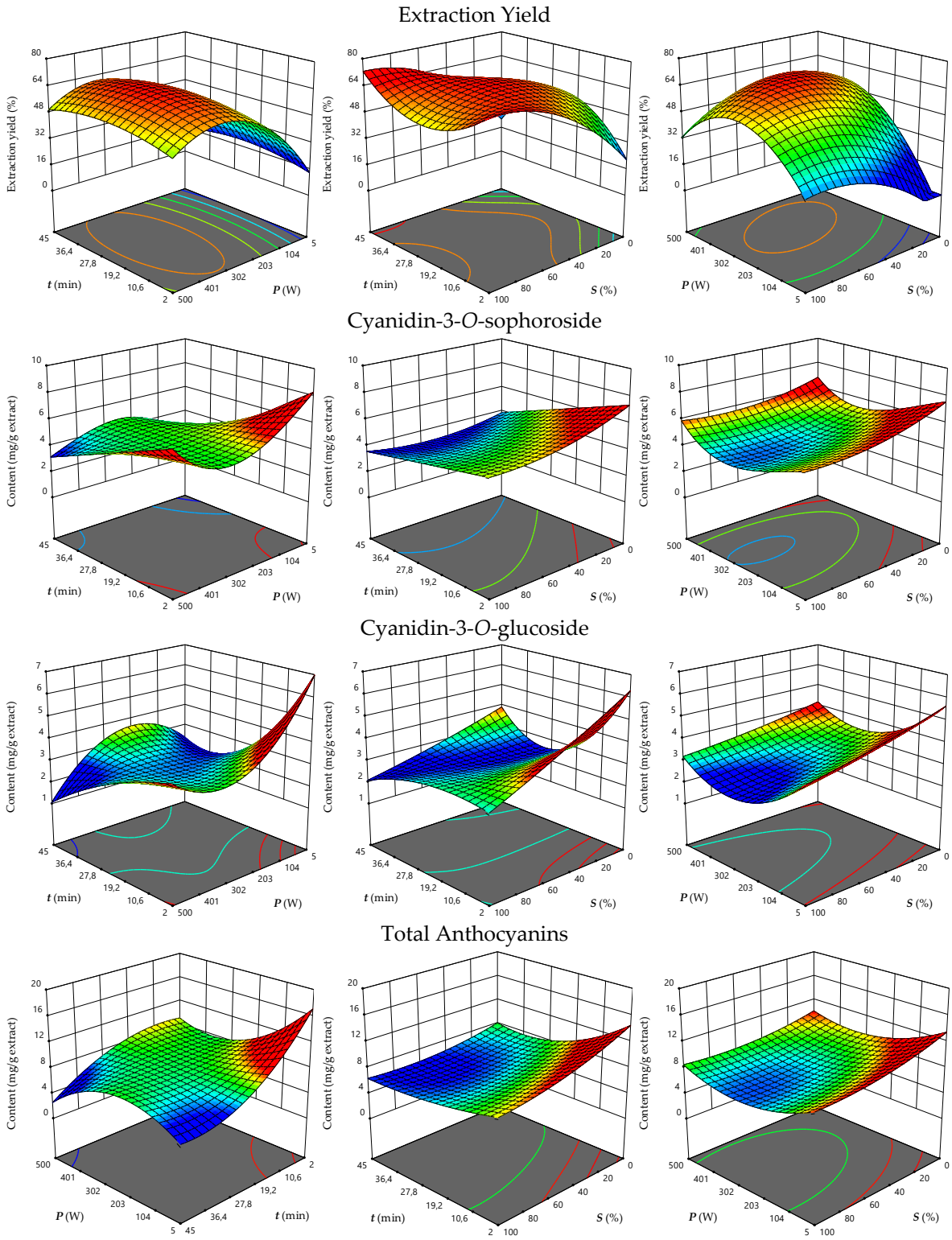

Figure 2. Response surface graphs for the effects of extraction time (*t*), ultrasonic power (*P*), and ethanol concentration (*S*) on the extraction yield (%) and levels of cyanidin-3-*O*-sophoroside (C3S), cyanidin-3-*O*-glucoside (C3G), and total anthocyanins (mg/g extract) obtained from red raspberry by the UAE process. In each graph, the excluded variable was positioned at its optimal value (Table 3).

Table 3. Optimal processing conditions that maximize the HAE and UAE extraction of anthocyanins from red raspberry and predicted and experimental responses.

	Optimal HAE Conditions			**Optimum**	
	***t* (min)**	***T* (°C)**	***S* (%, *v*/*v*)**	**Predicted**	**Experimental**
			For Each Response Variable		
Yield	76.64	84.73	28.93	65 ± 2%	-
C3S	64.66	27.01	29.17	5.71 ± 0.09 mg/g	-
C3G	63.11	20.70	34.65	3.59 ± 0.04 mg/g	-
Total	67.65	27.79	36.60	9.2 ± 0.1 mg/g	-
			Considering the Response Variables Together		
Yield	75.70	38.09	21.40	61 ± 2%	62 ± 2%
C3S				5.29 ± 0.08 mg/g	5.3 ± 0.2 mg/g
C3G				3.39 ± 0.04 mg/g	3.26 ± 0.06 mg/g
Total				8.7 ± 0.1 mg/g	8.5 ± 0.2 mg/g
	Optimal UAE Conditions			**Optimum**	
	***t*(min)**	***P*(W)**	***S*(%, *v*/*v*)**	**Predicted**	**Experimental**
			For Each Response Variable		
Yield	25.20	336.58	50.72	66 ± 2%	-
C3S	10.70	115.80	18.40	5.98 ± 0.09 mg/g	-
C3G	9.38	98.30	30.45	4.00 ± 0.05 mg/g	-
Total	11.11	99.90	19.08	10.1 ± 0.1 mg/g	-
			Considering the Response Variables Together		
Yield	15.61	465.90	37.58	58 ± 3%	58 ± 3%
C3S				5.03 ± 0.09 mg/g	5.04 ± 0.08 mg/g
C3G				3.30 ± 0.05 mg/g	3.31 ± 0.08 mg/g
Total				8.3 ± 0.1 mg/g	8.3 ± 0.2 mg/g

t: time; *T*: temperature; *P*: power; *S*: solvent (ethanol concentration); Yield: extraction yield; C3S: cyanidin-3-*O*-sophoroside; C3G: cyanidin-3-*O*-glucoside; Total: total anthocyanin content (C3S + C3G).

3.3.1. Heat-Assisted Extraction

The extraction yield was significantly influenced by the temperature applied in the HAE process (Equation (1) and Supplementary Material Table S1); the rise in temperature caused an increase in the extract weight obtained, manly when increased from approximately 20–35 °C and from 75 to 90 °C (Figure 1). This variable interacted negatively with the solvent, yielding higher extract weights when higher temperatures and lower ethanol concentrations were used. In turn, the negative interaction between solvent and time was responsible for the higher yields obtained with longer processing times and lower ethanol concentrations. Therefore, processing the red raspberry samples at 85 °C for 77 min, with 29% ethanol (*v*/*v*), allowed to maximize the extraction yield to 65 ± 2% (Table 3).

The three independent variables of the HAE process also affected the recovery of anthocyanins from red raspberry. The extraction trends were similar for C3S and C3G and, therefore, for the sum of both cyanidin glycosides. In general, the extraction was enhanced by longer processing times and lower temperatures and ethanol concentrations (Figure 1). The higher temperatures appeared to cause degradation of these thermosensitive pigments, while short extraction times were not enough to promote their recovery. In fact, it is interesting to note the negative interactions of processing time with temperature and solvent (Equations (2)–(4)), which show that, for longer extraction times, it is preferable to apply low temperatures and lower ethanol concentrations. For C3G, lower levels were obtained when processing the red raspberry samples for times above 70 min with high proportions of ethanol. In addition to the harmful effects of temperature, a low ethanol concentration was essential to promote the extraction of anthocyanins. It was also interesting to note that, when processing the samples for just over 2 min, the increase in temperature potentiated the extraction of total anthocyanins to a certain level, but later it was decreased with the higher temperature. Thus, to achieve the maximum

of 9.2 ± 0.1 mg/g extract, it was necessary to process the red raspberry at 28 °C for 68 min, using 37% ethanol (*v*/*v*) (Table 3). When comparing these conditions with those predicted for extraction yield, it is possible to conclude that compounds other than anthocyanins (possibly sugars) are being extracted when applying high temperatures. This result shows that there is a selectivity for anthocyanins as a function of the applied processing conditions.

As already mentioned, the extraction conditions are influenced by the nature of the raw material from which the anthocyanins are to be extracted, which justifies the different processing conditions that have been described in the literature. The optimized HAE conditions reported by Ghada et al. [21] for the extraction of C3G from passion fruit epicarp (78 min stirring at 20 °C using 29% ethanol, *v*/*v*) are close to those obtained in the present study and yielded 12 ± 2 mg/g extract. Backes et al. [14] described a temperature (~28 °C) similar to that required to process the red raspberry, a shorter extraction time of just 14 min, and pure ethanol as the best conditions to recover cyanidin-3-rutinoside from fig peel, with a yield of 6.0 ± 0.6 mg/g extract, a value lower than that obtained with red raspberry. In another study, Pinela et al. [15] optimized the HAE of delphinidin-3-*O*-sambubioside + cyanidin-3-*O*-sambubioside from roselle (*Hibiscus sabdariffa* L.) calyces and found 30 min processing at 30 °C, using just water as extraction solvent, as the best processing conditions to obtain 21 ± 1 mg/g extract. Using HAE, Albuquerque et al. [7] reached 81 ± 2 mg/g extract of delphinidin-3-*O*-glucoside + C3G when processing jabuticaba epicarp at 47 °C for 22 min with 9% ethanol (*v*/*v*). Low temperatures were required in all these studies as anthocyanins degrade readily during thermal processing, which has a direct impact on color shade. Despite this, López et al. [20] reported that the anthocyanins found in strawberry-tree fruit (where C3G has been identified as a major pigment) are better extracted when processing the plant material at 90 °C for 5 min with 80% ethanol (*v*/*v*), a difference that may be associated with the intrinsic characteristics of this fruit.

3.3.2. Ultrasound-Assisted Extraction

In the UAE process, the ultrasonic power was the variable that affected most the extraction yield, followed by the solvent (Equation (5) and Supplementary Material Table S1). The effect of the ultrasonic power is illustrated in Figure 2, where the curvature of the response surfaces and their projections perfectly show that medium-high power levels are the optimal processing ranges. It was also possible to verify that intermediate levels of time and solvent were favorable. In general, while low variable ranges were not sufficient to promote extraction, higher levels may have led to the compounds' degradation. The sonication of the samples at 337 W for 25 min, using 51% ethanol (*v*/*v*) as solvent, were the conditions that maximized the extraction yield to 66 ± 2% (Table 3).

The UAE extraction of both anthocyanins was promoted by medium-low conditions of the RCCD design variables (Figure 2). For longer extraction times, the degradation of anthocyanins was observed when processing the red raspberry samples with a high ultrasonic power, while the lower energy applied did not seem to be sufficient to promote mass transfer phenomena. As can be seen in the developed polynomial models in Equations (6)–(8), the interactions observed were all positive and translated an increase in the response value with the consequent decrease in the range of both variables represented in each of the 3D graphs. The Equation (8) polynomial model predicted that 10.1 ± 0.1 mg/g of total anthocyanins can be achieved by subjecting the plant material to 100 W for 11 min, using 19% ethanol (*v*/*v*), which were the optimal UAE conditions (Table 3).

The UAE of anthocyanins from red raspberry (cultivar 'Heritage') was previously optimized by Chen et al. [19]. The extraction time (6.2–302), ultrasonic power (232–568 W; at 22 kHz frequency) and liquid/solid ratio (0.6–7.4 mL/g) were the variables included in the experimental design, while the solvent consisted of 95% ethanol (*v*/*v*) acidified with 1.5 M HCl in all extractions. From 100 g of fresh fruits, it was possible to recover 34.5 mg of anthocyanins when sonicating the plant material at 400 W for 3.3 min at a liquid/solid ratio of 4:1 (mL/g). In comparison with a conventional method that yielded 35.1 mg anthocyanins when extracting at 71 °C for 53 min, UAE was a time- and solvent-saving methodology to due to its ability to disrupt plant tissues through acoustic cavitation

phenomena. The authors also concluded that UAE processing did not result in a noticeable degradation of anthocyanins due to the reduced processing time required, which was even lower than the optimum required in the present work. This difference may be justified by the different extraction solvent and liquid/solid ratios used. Regarding studies with other plant matrices, longer processing times (21 and 26 min) and ethanol concentrations (100 and 39%, *v*/*v*) were predicted for the anthocyanins recovery from fig peels [14] and roselle calyces [15], respectively, while the ultrasonic power was around 300 W in both cases, having yielded 9.6 ± 0.5 and 21 ± 1 mg/g, respectively.

The use of MAE has also been investigated for anthocyanins recovery from red raspberry. When irradiating the sample at 366 W for 12 min at a liquid/solid ratio of 4:1 (mL/g), Sun et al. [18] reached 43.42 mg/100 g of fresh fruits, a content higher than the 34.5 mg/100 g reported by Chen et al. [19] using an optimized UAE process. The developed MAE protocol was more efficient and faster than a conventional method due to the strong capacity of microwave irradiation to disrupt the plant tissue structure and enhance mass transfer. Backes and co-workers [14] compared three extraction methods and, based on the extraction yield achieved for fig peel anthocyanins, ranked the methods as follows: UAE (9.0 ± 0.8 mg/g extract), MAE (7.4 ± 0.8 mg/g extract) and HAE (5.8 ± 0.1 mg/g extract).

3.3.3. HAE vs. UAE Processing

The two extraction methods tested in this study were compared in order to conclude which one could be the most suitable to produce red raspberry extracts with higher levels of anthocyanins. Table 3 presents the optimal processing conditions that maximize both the extraction yield and the total anthocyanin content as much as possible. This optimization step was done because it is important for the industry to obtain a high extract weight and also a high concentration of the target compounds from a given plant material. Supplementary Material Figure S1 shows the individual 2D responses of each independent variable involved in the extraction when both response variables are maximized simultaneously. Although the UAE process led to higher responses when maximized individually, HAE extraction appeared to be more suitable for maximizing response variables together (Table 3). As can be deduced from Figures 1 and 2 and Supplementary Material Figure S1, the optimal global extraction conditions are closer to the individual ones in the HAE method, since the trends observed for extraction yield and total anthocyanins are closer, which justifies the better performance of this method for both responses. In the HAE process, longer extraction times (76 min) and medium-low ethanol concentrations (21%, *v*/*v*) favored both responses, while low temperatures (28 °C) were suitable for anthocyanins and higher (85 °C) for the extract weight (Supplementary Material Figure S1). On the other hand, the extraction trends were more divergent for UAE, mainly for the variables ultrasonic power and solvent proportion. While the total anthocyanins and the extract weight were increased by 100 and 337 W and by 19 and 51% ethanol (*v*/*v*), respectively, the optimal global conditions were promoted by sonicating the samples at 466 W for 16 min, using 38% ethanol as the extraction solvent (Table 3).

The UAE emerged as a time-saving method when sonicating at 466 W, probably due to the ability of the ultrasound waves to snatch the plant cell walls, facilitating the solvent penetration and the consequent recovery of the solutes. Although the HAE method required a much longer processing time (76 min), the temperature needed was not very high (just 38 °C) and the percentage of solvent is slightly lower than that required for UAE. Therefore, to conclude which method is the most eco-sustainable, it would be necessary and interesting to calculate the energy costs associated with each one. Even so, industries interested in the developed processes will be able to choose the methodology that best adapts to their facilities and depending on the equipment they already have. Still, since this is a bench-scale study, subsequent scale-up steps will have to be established.

The adequacy of the HAE and UAE methods for the extraction of anthocyanins from different plant material has been the subject of previous work. López et al. [20] reported that the anthocyanins found in strawberry-tree fruit are also better extracted by HAE than when using UAE, in a process that yields 51% of extract weight with a total anthocyanin content of 745 μg/g extract when processing

at 90 °C for 5 min with 80% ethanol (*v*/*v*). However, these conditions are quite different from those achieved in the present study, where the higher temperatures affected the integrity of the red raspberry anthocyanins. The HAE (22 min processing at 47 °C with 9% ethanol, *v*/*v*) was also reported to be most efficient than UAE (24 min sonication at 500 W with 34% ethanol, *v*/*v*) to recover anthocyanins from jabuticaba epicarp in processes that yielded 81 ± 2 and 31 ± 2 mg/g extract, respectively [7]. There are also studies reporting that UAE processes are more appropriate for anthocyanins extraction. Pinela et al. [15] found that UAE is most suitable than HAE to recover delphinidin-3-*O*-sambubioside + cyanidin-3-*O*-sambubioside from roselle calyces, in a process that yield 61 ± 6% of extract weight and 48 ± 4 mg/g extract under optimal conditions (43 min sonication at 386 W, using 46% ethanol, *v*/*v*). UAE has also been identified as being more effective in recovering cyanidin-3-rutinoside from fig peel than HAE or MAE [14]. Therefore, it is possible be concluded that the extraction conditions are influenced by the compositional and structural complexity of the plant material, but the anthocyanidin and sugar molecule of the anthocyanin may also be a relevant structural feature.

3.4. Experimental Validation of the Models

The global processing conditions that maximize both the extraction yield and the recovery of anthocyanins from red raspberry by the HAE (76 min, 38 °C and 21% ethanol, *v*/*v*) and UAE (16 min, 466 W and 38% ethanol, *v*/*v*) methods (Table 3) were experimentally tested to evaluate the predictive accuracy of the models and to obtain anthocyanin-rich extracts for evaluation of the coloring and bioactive properties. As presented in Table 3, the HAE yielded 62 ± 2% and the UAE yielded 58 ± 3% of extract weight, values that did not differed from the predicted 61 ± 2% and 58 ± 3%, respectively. The HAE leaded to 5.3 ± 0.2 mg of C3S and 3.26 ± 0.06 mg of C3G per gram of crude extract, totalizing 8.5 ± 0.2 mg of anthocyanins. In turn, each gram of extract obtained with the UAE process contained 5.04 ± 0.08 mg of C3S and 3.31 ± 0.08 mg of C3G, totalizing 8.3 ± 0.2 mg of anthocyanins. The predictive capacity of the models was thus experimentally validated, since no statistically significant differences ($p > 0.05$) between experimental and model-predicted data were found when applying a Student's *t*-test.

3.5. Coloring and Bioactive Properties of the Extracts

Anthocyanins are among the most important and widely consumed naturally-occurring food colorants. Commercial anthocyanins, such as C3G, pelargonidin-3-glucoside and peonidin-3-glucoside have been used and widely study since their stability and color are affected by external factors, such as pH, temperature, and humidity, among others [1]. Therefore, the anthocyanins color varies depending on these factors. The color parameters recorded in the lyophilized red raspberry and in the two extracts obtained under optimized HAE and UAE extraction conditions are shown in Table 4. The extracts presented a slightly but significate ($p < 0.05$) darker shade (lower L^* values) and a less intense redness (lower a^* value) when compared to the raspberry sample. On the other hand, higher b^* values (yellowness) were measured in the extracts, especially in that obtained by the UAE, than in the lyophilized sample. However, as shown in Table 4, the color of the two extracts was difficult to distinguish with the naked eye. In addition, it was interesting to note that the purple-red color was somehow preserved with the applied extraction processes.

Table 4. Color parameters of the lyophilized red raspberry powder and extracts obtained under the optimized HAE and UAE conditions.

Sample	L^*: Lightness	a^*: Redness	b^*: Yellowness	RGB Color
Dry powder	37.2 ± 0.8 [a]	37.3 ± 0.4 [a]	10.1 ± 0.1 [c]	
HAE extract	35.8 ± 0.9 [b]	36 ± 1 [b]	15.1 ± 0.8 [b]	
UAE extract	35.8 ± 0.9 [b]	36 ± 1 [b]	16.1 ± 0.9 [a]	

In each column, different letters correspond to statistically significant differences ($p < 0.05$).

The preservation of food products from spoilage and deterioration during the processing and storage is a major issue in the food industry and can be achieved by adding bio-based ingredients with antioxidant and antimicrobial activity. The anthocyanin-rich extracts are increasingly used as food ingredients and known mainly for their coloring ability, but also display a wide range of bioactive properties and health-promoting effects [1]. The results of the antioxidant activity of the red raspberry extracts obtained under optimized extraction conditions are presented in Table 5. The HAE red raspberry extract showed a greater capacity to inhibit lipid peroxidation and antihemolytic activity than the UAE red raspberry extract, which can be attributed to the higher concentration of anthocyanins quantified in the HAE extract. Trolox, the commercial antioxidant used as a positive control gave a much lower value than those of the extracts. Despite this, trolox is a pure antioxidant compound, while plant extracts are complex mixtures of many compounds (e.g., polyphenols, vitamins, carbohydrates, etc.), some of which have no antioxidant effects, which reinforces the antioxidant potential of the extracts.

Table 5. Antioxidant and antibacterial activities of the red raspberry extracts obtained under the optimized HAE and UAE conditions and positive controls.

	HAE Red Raspberry Extract		**UAE Red Raspberry Extract**		**Positive Control**					
Antioxidant Activity							Trolox			
TBARS (EC_{50}, µg/mL)	28 ± 1 [b]		48 ± 1 [a]		5.4 ± 0.3 [c]					
OxHLIA (IC_{50}, µg/mL)	449 ± 14 [b]		520 ± 23 [a]		21.8 ± 0.6 [c]					
Antibacterial Activity					Ampicillin		Imipenem		Vancomycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Escherichia coli	5	>20	2.5	>20	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	>20	>20	>20	>20	10	20	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	10	>20	5	>20	20	>20	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	20	>20	20	>20	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	10	>20	10	>20	>20	>20	0.5	1	n.t.	n.t.
Enterococcus faecalis	2.5	>20	2.5	>20	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
Listeria monocytogenes	20	>20	20	>20	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	10	>20	5	>20	<0.15	<0.15	n.t.	n.t.	0.25	0.5

In each line, for antioxidant activity, different letters correspond to statistically significant differences ($p < 0.05$). MIC: minimum inhibitory concentration (mg/mL); MBC: minimum bactericidal concentration (mg/mL); MRSA: methicillin-resistant *Staphylococcus aureus*; n.t.: not tested.

In the TBARS formation inhibition assay, an EC_{50} of 28 µg/mL was obtained with the HAE extract Table 5, being more effective than the UAE extract in preventing the formation of reactive substances, such as malondialdehyde that results from the oxidation of polyunsaturated fatty acids present in the porcine brain cell membranes that were used as oxidizable substrate, thus being more effective in delaying the lipid peroxidation phenomena responsible for food deterioration. Among the range of extract concentrations tested, the lowest ones were unable to prevent the formation of TBARS that reacted with the thiobarbituric acid that was added to the mixture before incubation at 80 °C to form pink complexes that absorb a wavelength 532 nm.

In the OxHLIA assay, 449 µg/mL was the HAE extract concentration required to protect half of the erythrocyte population from the oxidative hemolysis for a Δt of 60 min, while 520 µg/mL of UAE extract were required (Table 5). In this assay, the temperature-dependent free radical initiator AAPH is responsible for the formation of peroxyl radicals in the in vitro system that, in a first instance, attack the erythrocyte membranes and eventually cause hemolysis. Then, lipophilic radicals are generated as

a consequence of the initial lipid peroxidation reaction. This cell-based assay is suitable for measuring the antioxidant activity of natural extracts, since it uses physiologically relevant peroxyl radicals which mimics the lipid peroxyl radicals involved in lipid peroxidation chain reactions in vivo [34]. Additionally, erythrocytes have an intrinsically poor repair mechanism which makes them metabolically simplified model systems for assessing antioxidant activity [35].

The antioxidant activity of red raspberry extracts has been measured mostly by chemical based assays, namely for their DPPH and ABTS radicals scavenging activity and ferric (FRAP) and cupric (CUPRAC) reducing power, and biochemical methods such as β-carotene bleaching inhibition capacity [8,29,36,37]. Red raspberry extracts were already reported as more antioxidant than those obtained from blueberry, in which lower anthocyanin levels were detected [29]. The ability of an aqueous raspberry extract to protected human erythrocytes upon hydrogen peroxide-driven hemolysis was previously demonstrated by Gião et al. [35]. In an in vivo study, a decrease in oxidative stress biomarkers was observed in obese diabetic mice fed lyophilized red raspberry for 8 weeks in isocaloric diets (5.3% fruits (*w*/*w*), or control) [38]. The enhanced detoxifying cell defenses exerted by the red raspberry intake was linked to polyphenols and dietary fiber.

The red raspberry extracts produced under optimized processing conditions were also tested against food-related microorganisms, including Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis* and *Pseudomonas aeruginosa*) and Gram-positive (*Listeria monocytogenes*, *Enterococcus faecalis*, and MRSA—Methicillin-resistant *Staphylococcus aureus*) bacteria. As presented in Table 5, *E. coli* and *E. faecalis* were the most sensitive bacteria to the tested extracts and, in general, the UAE extract was more effective against *E. coli*, *M. morganii* and MRSA. Despite this, the minimum concentration required to inhibit most of the strains tested was the same for both extracts. On the other hand, an extract concentration above 20 mg/mL was required for a bactericidal effect. Therefore, the use of anthocyanin-rich red raspberry extracts as food ingredients can be useful to retard the microbial growth and thus help preventing food spoilage and food contamination incidents, in addition to the coloring effect. The antibacterial potential of hydroethanolic extracts of red and black raspberries was previously screened against common human pathogenic bacteria, among which *Corynebacterium diphtheriae* and *Moraxella catarrhalis* were the most sensitive to the extracts [10].

4. Conclusions

This study demonstrated the suitability of two extraction methods intensified by heat and ultrasounds for extracting anthocyanins from red raspberry. The three independent variables used in the optimization processes induced significant effects on the analyzed responses and the existence of interactions between them justified the use of RSM. Overall, HAE originated slightly higher response values but required a longer processing time than UAE (76 vs. 16 min, respectively). In terms of energy, 38 °C (in HAE) or 466 W (in UAE) were required to simultaneously maximize the extract weight and anthocyanins content. After statistical and experimental validation of the theoretical models, the color and biological activity of the extracts produced under the predicted optimal processing conditions were measured. Although the color of the HAE extract was slightly closer to that of the dry red raspberry powder, no sharp difference was noticed at naked eye. The HAE extract was also more antioxidant in vitro due to the lower extract concentrations required to inhibit the formation of TBARS and the oxidative hemolysis. Antibacterial effects were also observed especially against *E. coli* and *E. faecalis*. These results could be exploited by industries interested in the production of anthocyanin-based ingredients with coloring and bioactive capacity. In future studies, it will be interesting to investigate the stability of the developed anthocyanin-rich extracts when exposed to different factors and in real food matrices. The production of spray-dried red raspberry coloring powders will also be interesting to explore.

Supplementary Materials: The following are available online at http://www.mdpi.com/2227-9717/8/11/1447/s1, Figure S1: 2D response graphs for the effects of the independent variables on the extraction yield (%, *w*/*w*) and total anthocyanins content (mg/g extract) obtained from red raspberry by HAE and UAE when maximizing

all responses simultaneously. In each graph, the excluded variables were fixed at their global optimal value (Table 2), Table S1: Parametric values of the polynomial equations and statistical information of the model fitting procedure for both extraction processes. Parametric superscripted 1–3 stand for the variables time (t), temperature or ultrasonic power (T or P), and solvent (S), respectively.

Author Contributions: Conceptualization, J.P., A.N., I.C.F.R.F. and L.B.; methodology, J.P., R.M.V.A. and L.B.; validation, J.P., M.A.-O., T.C.S.P.P. and L.B.; formal analysis, R.R., J.P., M.A.-O., T.C.S.P.P. and A.L.S.; investigation, R.R., M.A.-O., J.P., R.M.V.A., T.C.S.P.P. and A.L.S.; resources, M.J.A., I.C.F.R.F. and L.B.; writing—original draft preparation, R.R. and J.P.; writing—review and editing, J.P., R.M.V.A., M.J.A., A.N., I.C.F.R.F. and L.B.; supervision, J.P., A.N. and L.B. All authors have read and agreed to the published version of the manuscript.

Funding: The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support through national funds FCT/MCTES to CIMO (UIDB/00690/2020). This research was funded by FEDER-Interreg España-Portugal programme through the projects 0377_Iberphenol_6_E and TRANSCoLAB 0612_TRANS_CO_LAB_2_P, and by the European Regional Development Fund (ERDF) through the Regional Operational Program North 2020, within the scope of Project *Mobilizador* Norte-01-0247-FEDER-024479: ValorNatural®. National funding by FCT, P.I., through the individual and institutional scientific employment program-contract for the J. Pinela and L. Barros research contracts, respectively.

Acknowledgments: The authors thank the company "Ponto Agrícola Unipessoal, Lda" for the plant material.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Martins, N.; Roriz, C.L.; Morales, P.; Barros, L.; Ferreira, I. Food Colorants: Challenges, Opportunities and Current Desires of Agro-Industries to Ensure Consumer Expectations and Regulatory Practices. *Trends Food Sci. Technol.* **2016**, *52*, 1–15. [CrossRef]
2. Chapman, S. *Guidelines on Approaches to the Replacement of Tartrazine, Allura Red, Ponceau 4R, Quinoline Yellow, Sunset Yellow and Carmoisine in Food and Beverages*; Food Standards Agency: London, UK, 2011.
3. Carocho, M.; Morales, P.; Ferreira, I. Natural Food Additives: Quo Vadis? *Trends Food Sci. Technol.* **2015**, *45*, 284–295. [CrossRef]
4. EFSA Panel on Food Additives and Nutrient Sources Added to Food (ANS). EFSA Scientific Opinion on the Re-Evaluation of Anthocyanins (E 163) as a Food Additive. *EFSA J.* **2013**, *11*, 3145. [CrossRef]
5. He, J.; Giusti, M.M. Anthocyanins: Natural Colorants with Health-Promoting Properties. *Annu. Rev. Food Sci. Technol.* **2010**, *1*, 163–187. [CrossRef]
6. Norberto, S.; Silva, S.; Meireles, M.; Faria, A.C.; Pintado, M.; Calhau, C. Blueberry Anthocyanins in Health Promotion: A Metabolic Overview. *J. Funct. Foods* **2013**, *5*, 1518–1528. [CrossRef]
7. Albuquerque, B.R.; Pinela, J.; Barros, L.; Oliveira, M.B.P.; Ferreira, I. Anthocyanin-Rich Extract of Jabuticaba Epicarp as a Natural Colorant: Optimization of Heat- and Ultrasound-Assisted Extractions and Application in a Bakery Product. *Food Chem.* **2020**, *316*, 126364. [CrossRef]
8. Sariburun, E.; Şahin, S.; Demir, C.; Türkben, C.; Uylaşer, V. Phenolic Content and Antioxidant Activity of Raspberry and Blackberry Cultivars. *J. Food Sci.* **2010**, *75*, C328–C335. [CrossRef]
9. Beekwilder, J.; Jonker, H.; Meesters, P.; Hall, R.D.; Van Der Meer, A.I.M.; De Vos, C.H.R. Antioxidants in Raspberry: On-Line Analysis Links Antioxidant Activity to a Diversity of Individual Metabolites. *J. Agric. Food Chem.* **2005**, *53*, 3313–3320. [CrossRef]
10. Krauze-Baranowska, M.; Majdan, M.; Hałasa, R.; Głód, D.; Kula, M.; Fecka, I.; Orzeł, A. The Antimicrobial Activity of Fruits from Some Cultivar Varieties of *Rubus idaeus* and *Rubus occidentalis*. *Food Funct.* **2014**, *5*, 2536–2541. [CrossRef]
11. Anjos, R.; Cosme, F.; Gonçalves, A.; Nunes, F.M.; Vilela, A.; Pinto, T. Effect of Agricultural Practices, Conventional vs Organic, on the Phytochemical Composition of 'Kweli' and 'Tulameen' Raspberries (*Rubus idaeus* L.). *Food Chem.* **2020**, *328*, 126833. [CrossRef]
12. Galanakis, C.M. Recovery of High Added-Value Components from Food Wastes: Conventional, Emerging Technologies and Commercialized Applications. *Trends Food Sci. Technol.* **2012**, *26*, 68–87. [CrossRef]
13. Chávez-González, M.; Sepúlveda, L.; Verma, D.K.; Luna-García, H.A.; Rodríguez-Durán, L.V.; Ilyina, A.; Aguilar, C.N. Conventional and Emerging Extraction Processes of Flavonoids. *Processes* **2020**, *8*, 434. [CrossRef]

14. Backes, E.; Pereira, C.; Barros, L.; Lage, M.P.; Genena, A.K.; Barreiro, M.F.; Ferreira, I. Recovery of Bioactive Anthocyanin Pigments from *Ficus carica* L. Peel by Heat, Microwave, and Ultrasound Based Extraction Techniques. *Food Res. Int.* **2018**, *113*, 197–209. [CrossRef] [PubMed]
15. Pinela, J.; Prieto, M.; Pereira, E.; Jabeur, I.; Barreiro, M.F.; Barros, L.; Ferreira, I. Optimization of Heat-and Ultrasound-Assisted Extraction of Anthocyanins from *Hibiscus sabdariffa* Calyces for Natural Food Colorants. *Food Chem.* **2019**, *275*, 309–321. [CrossRef]
16. Corrales, M.; Toepfl, S.; Butz, P.; Knorr, D.; Tauscher, B. Extraction of Anthocyanins from Grape By-Products Assisted by Ultrasonics, High Hydrostatic Pressure or Pulsed Electric Fields: A Comparison. *Innov. Food Sci. Emerg. Technol.* **2008**, *9*, 85–91. [CrossRef]
17. Wu, Z.; Ferreira, D.F.; Crudo, D.; Bosco, V.; Stevanato, L.; Costale, A.; Cravotto, G. Plant and Biomass Extraction and Valorisation under Hydrodynamic Cavitation. *Processes* **2019**, *7*, 965. [CrossRef]
18. Sun, Y.; Liao, X.; Wang, Z.; Hu, X.; Chen, F. Optimization of Microwave-Assisted Extraction of Anthocyanins in Red Raspberries and Identification of Anthocyanin of Extracts Using High-Performance Liquid Chromatography–Mass Spectrometry. *Eur. Food Res. Technol.* **2006**, *225*, 511–523. [CrossRef]
19. Chen, F.; Sun, Y.; Zhao, G.; Liao, X.; Hu, X.; Wu, J.; Wang, Z. Optimization of Ultrasound-Assisted Extraction of Anthocyanins in Red Raspberries and Identification of Anthocyanins in Extract Using High-Performance Liquid Chromatography–Mass Spectrometry. *Ultrason. Sonochem.* **2007**, *14*, 767–778. [CrossRef]
20. López, C.J.; Caleja, C.; Lage, M.P.; Barreiro, M.F.; Barros, L.; Ferreira, I. Optimization and Comparison of Heat and Ultrasound Assisted Extraction Techniques to Obtain Anthocyanin Compounds from *Arbutus unedo* L. Fruits. *Food Chem.* **2018**, *264*, 81–91. [CrossRef]
21. Ghada, B.; Pereira, E.; Pinela, J.; Prieto, M.; Pereira, C.; Calhelha, R.C.; Stojković, D.; Sokovic, M.; Zaghdoudi, K.; Barros, L.; et al. Recovery of Anthocyanins from Passion Fruit Epicarp for Food Colorants: Extraction Process Optimization and Evaluation of Bioactive Properties. *Molecules* **2020**, *25*, 3203. [CrossRef]
22. Corrado, S.; Sala, S. Food Waste Accounting Along Global and European Food Supply Chains: State of the Art and Outlook. *Waste Manag.* **2018**, *79*, 120–131. [CrossRef] [PubMed]
23. European Commission. *Frequently Asked Questions: Reducing Food Waste in the EU*; European Comission: Brussels, Belgium, 2019.
24. Pinela, J.; Barreira, J.C.; Barros, L.; Verde, S.C.; Antonio, A.L.; Carvalho, A.M.; Oliveira, M.B.P.; Ferreira, I. Suitability of Gamma Irradiation for Preserving Fresh-Cut Watercress Quality During Cold Storage. *Food Chem.* **2016**, *206*, 50–58. [CrossRef]
25. Gonçalves, G.A.; Soares, A.A.; Correa, R.C.; Barros, L.; Haminiuk, C.W.; Peralta, R.M.; Ferreira, I.; Bracht, A. Merlot Grape Pomace Hydroalcoholic Extract Improves the Oxidative and Inflammatory States of Rats with Adjuvant-Induced Arthritis. *J. Funct. Foods* **2017**, *33*, 408–418. [CrossRef]
26. Albuquerque, B.R.; Ferreira, I.; Vazquez, J.; Barreiro, M.F.; Barros, L.; Ferreira, I. Recovery of Bioactive Compounds from *Arbutus unedo* L. Fruits: Comparative Optimization Study of Maceration/Microwave/Ultrasound Extraction Techniques. *Food Res. Int.* **2018**, *109*, 455–471. [CrossRef] [PubMed]
27. Lockowandt, L.; Pinela, J.; Roriz, C.L.; Pereira, C.; Abreu, R.M.; Calhelha, R.C.; Alves, M.J.; Barros, L.; Bredol, M.; Ferreira, I. Chemical Features and Bioactivities of Cornflower (*Centaurea cyanus* L.) Capitula: The Blue Flowers and the Unexplored Non-Edible Part. *Ind. Crop. Prod.* **2019**, *128*, 496–503. [CrossRef]
28. Kuete, V.; Ango, P.Y.; Fotso, G.W.; Kapche, D.G.W.F.; Dzoyem, J.P.; Wouking, A.G.; Ngadjui, B.T.; Abegaz, B.M. Antimicrobial Activities of the Methanol Extract and Compounds from *Artocarpus communis* (Moraceae). *BMC Complement. Altern. Med.* **2011**, *11*, 42. [CrossRef]
29. De Souza, V.R.; Pereira, P.A.P.; Da Silva, T.L.T.; Lima, L.C.D.O.; Pio, R.; Queiroz, F. Determination of the Bioactive Compounds, Antioxidant Activity and Chemical Composition of Brazilian Blackberry, Red Raspberry, Strawberry, Blueberry and Sweet Cherry Fruits. *Food Chem.* **2014**, *156*, 362–368. [CrossRef]
30. Bowen-Forbes, C.S.; Zhang, Y.; Nair, M.G. Anthocyanin Content, Antioxidant, Anti-Inflammatory and Anticancer Properties of Blackberry and Raspberry Fruits. *J. Food Compos. Anal.* **2010**, *23*, 554–560. [CrossRef]
31. Leichtweis, M.G.; Pereira, C.; Prieto, M.; Barreiro, M.F.; Barros, L.; Ferreira, I. Ultrasound as a Rapid and Low-Cost Extraction Procedure to Obtain Anthocyanin-Based Colorants from *Prunus spinosa* L. Fruit Epicarp: Comparative Study with Conventional Heat-Based Extraction. *Molecules* **2019**, *24*, 573. [CrossRef]
32. Iberahim, N.; Sethupathi, S.; Goh, C.L.; Bashir, M.J.; Ahmad, W. Optimization of Activated Palm Oil Sludge Biochar Preparation for Sulphur Dioxide Adsorption. *J. Environ. Manag.* **2019**, *248*, 109302. [CrossRef]

33. Pinela, J.; Prieto, M.; Barros, L.; Carvalho, A.M.; Oliveira, M.B.P.; Saraiva, J.A.; Ferreira, I.C. Cold Extraction of Phenolic Compounds from Watercress by High Hydrostatic Pressure: Process Modelling and Optimization. *Sep. Purif. Technol.* **2018**, *192*, 501–512. [CrossRef]
34. Tai, A.; Sawano, T.; Ito, H. Antioxidative Properties of Vanillic Acid Esters in Multiple Antioxidant Assays. *Biosci. Biotechnol. Biochem.* **2012**, *76*, 314–318. [CrossRef] [PubMed]
35. Gião, M.S.; Leitão, I.; Pereira, A.; Borges, A.B.; Guedes, C.J.; Fernandes, J.C.; Belo, L.; Santos-Silva, A.; Hogg, T.; Pintado, M.E.; et al. Plant Aqueous Extracts: Antioxidant Capacity via Haemolysis and Bacteriophage P22 Protection. *Food Control* **2010**, *21*, 633–638. [CrossRef]
36. Milivojevic, J.; Maksimović, V.; Nikolić, M.; Bogdanović, J.; Maletić, R.; Milatović, D.P. Chemical and Antioxidant Properties of Cultivated and Wild *Fragaria* and *Rubus* Berries. *J. Food Qual.* **2011**, *34*, 1–9. [CrossRef]
37. Valdés, A.; Pérez, S.E.M.; Butsko, M.; Moya, M.S.P.; Beltrán, A. Authentication of "Adelita" Raspberry Cultivar Based on Physical Properties, Antioxidant Activity and Volatile Profile. *Antioxidants* **2020**, *9*, 593. [CrossRef]
38. Noratto, G.D.; Chew, B.P.; Atienza, L.M. Red Raspberry (*Rubus idaeus* L.) Intake Decreases Oxidative Stress in Obese Diabetic (db/db) Mice. *Food Chem.* **2017**, *227*, 305–314. [CrossRef]