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Extraction of Antioxidants from Blackberry (*Rubus ulmifolius* L.): Comparison between Ultrasound- and Microwave-Assisted Extraction Techniques

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Abstract: Berries are considered functional food because of their potential health benefits to consumers due to their high concentrations in bioactive compounds. The extraction process of their antioxidant compounds is a crucial step. In this work, ultrasound (UAE) and microwave (MAE) assisted extraction have been evaluated and compared for the recovery of total phenolic compounds (TPC) and total anthocyanins (TA) from blackberry. Since several variables have an influence on the extraction processes efficiency, a response surface method using a Box–Behnken design (BBD) was chosen for the optimization of UAE and MAE variables. Six variables (solvent, temperature, amplitude, cycle, pH, and sample:solvent ratio) were optimized for UAE while the optimization for MAE was performed on four variables (solvent, temperature, pH, and sample:solvent ratio). It has been proven that solvent and temperature have a significant influence on the extraction of both TA and TPC. Only 10 and 5 min were necessary to complete the UAE and MAE procedures, respectively. A precision study was also carried out, and coefficient of variation lower than 5% was determined. Non-significant differences were obtained when using UAE and MAE at their respective optimum conditions. Thus, the results demonstrated a successful potential use of both techniques for the extraction of TA and TPC from blackberry. In conclusion, this work shows interesting perspectives for quality control analytical laboratories for the development of rapid extraction techniques to quantify these antioxidant compounds in blackberries.

Keywords: anthocyanins; antioxidants; blackberry; microwave-assisted extraction; phenolic compounds; *Rubus ulmifolius* L.; ultrasound-assisted extraction

1. Introduction

Rubus ulmifolius L. is well known for its high content in natural antioxidants. *Rubus ulmifolius* L. is a perennial bush that belongs to the Rosaceae family [1]. It is largely distributed throughout the world, since it has colonized from Europe to North Africa and spreads from sea level to heights up to 1100 m [2]. Its edible fruit, commonly known as blackberry, is a functional food that may play an important role in human health. It is characterized for its small size, an appealing dark black colour with a slightly acidic pleasant flavour [3,4]. Ripe blackberry has been widely used for hundreds of years as a traditional medicine because of its beneficial properties, which includes antioxidant, anti-inflammatory, antimicrobial, gastroprotective, and cardioprotective properties, among others. This fruit is characterized by its high contents in antioxidants, polyphenols, fibre, essential minerals, and vitamins [5,6]. The therapeutic properties of this fruit are mainly attributed to its high level of biologically active polyphenol components such as phenolic acids, flavonoids, condensed tannins, and anthocyanins [4]. Regarding the principal anthocyanins found in blackberries they are the following: cyanidin 3-*O*-glucoside; cyanidin 3-*O*-rutinoside; cyanidin 3-*O*-(6″-malonyl-glucoside); cyanidin 3-*O*-(6″-dioxalyl-glucoside) [7]. Therefore, the composition and properties of blackberries are currently a subject of great interest that has led quality control analytical laboratories to the development of new and rapid extraction and identification techniques to quantify the content of these bioactive and antioxidant compounds. The extraction method is a crucial step for a successful qualitative and quantitative analysis of the bioactive compounds in solid samples prior to the analytical determination [8]. Selecting the right extraction method not only ensures its efficiency but also reduces the decomposition of the extracted compounds during the process [9]. The extraction processes may include either conventional extraction methods such as distillation, solvent extraction, cold compression, or advanced extraction techniques such as ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), microwave-assisted extraction (MAE) or pressurized-liquid extraction (PLE) [10]. Nonetheless, the former are limited by some drawbacks, for instance, long extraction times, large amounts of organic solvents and high energy consumption. On the contrary, the latter largely reduce solvent volume demand, energy consumption, and processing length of time [11,12]. Over the last decade two advanced techniques such as UAE and MAE have been recognized as promising green extraction technologies [13]. UAE is an emerging powerful alternative to traditional methods for the extraction of antioxidant compounds from fruit [13–16]. Simple, economic, fast, low solvent consumption, improved method, and better extraction quality are the advantages that make it an effective and greener method for quality control [17]. UAE has been used to extract bioactive compounds from different fruits, including blueberry [18], mulberry [19], *Lycium ruthenicum* [17], or mandarin [15], among many others. As far as MAE is concerned, the reduction in the extraction time and the amount of solvents, the degree of automation, and its high performance are some of the advantages just to mention a few compared to conventional methods [20]. This technique has been recently used to extract antioxidant substances from a wide variety of matrices, such as myrtle [21], mango seed [22], or *Vernonia amygdalina* leaf [23].

In the bibliography, several studies can be found related to different extraction technologies specifically applied on blackberries. PLE of bioactive compounds from blackberry residues was studied by Da Fonseca-Machado et al. [5]. Total phenolics, monomeric anthocyanins, and antioxidant activity were evaluated. Two major (cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside) and two minor (cyanidin 3-*O*-(6″-malonyl-glucoside), and cyanidin 3-*O*-(6″-dioxalyl-glucoside)) compounds were identified. PLE was found to be effective in the recovery of anthocyanins and phenolics when compared to traditional methods such as maceration, and Soxhlet extraction. These authors also studied PLE in combination with UAE for the determination of the same bioactive compounds. The performance of UAE + PLE improved the extraction yields when compared to conventional Soxhlet extraction [24]. However, both works evaluated the influence of only two variables, the former studied the solvent type and temperature, and the latter evaluated the type of extraction and solvent. Supercritical fluid extraction was also studied for the extraction of the same bioactive compounds in blackberry wastes [25]. In this case, pressure, temperature, ultrasound power, and cosolvent were evaluated. The results presented in this work indicated that coupling ultrasound with SFE improve the extractions, reducing time and operational costs. Regarding the UAE methods found in literature, Aybastier et al. optimized this extraction technique for antioxidant compounds from blackberry leaves [26]. Total phenolics and antioxidant capacity were evaluated in this work. Ellagic acid, chlorogenic acid, caffeic acid, quercetin, myricetin, kaempferol, and kaempferol 3-*β*-*D*-glucopyranoside were identified as individual phenolics in the composition. However, only four variables were studied such as acid concentration, solvent percentage, extraction temperature, and extraction time. Other research work carried out by Ivanovic et al. employed UAE for a blackberry cultivar grown in Serbia [27]. The authors evaluated total phenolic compounds, tannins, and anthocyanin content. Nevertheless, a careful optimization of the UAE parameters would be needed. As far as MAE is concerned, there are no data available in literature of this type of extraction for TPC and TA from blackberry.

Since UAE is influenced by many factors including mainly solvent, temperature, amplitude, cycle, pH, and sample:solvent ratio, and MAE is mainly affected by solvent, temperature, pH, and sample:solvent ratio, they cannot be applied to every type of matrices, so it is necessary to determine their respective optimum extraction conditions [13]. Response surface methodology (RSM) is a suitable statistical tool that allows the optimization of multiple variables and their interactions while minimizing the number of experiments [28,29]. This tool is used to predict optimum experimental conditions to maximize or minimize, individually or simultaneously, several independent variables [30]. In this work, RSM was used to determine UAE and MAE optimum operating parameters. The optimization process was based on experiments completed according to a Box–Behnken design (BBD) which offers some advantages compared to other statistical designs, such as low reagent consumption, a shorter number of experiments and avoiding extreme treatment combinations [31]. In this way, a careful determination of the optimum extraction parameters for total anthocyanins (TA) and total phenolic compounds (TPC) was applied.

Therefore, the objectives of this work were as follows: (i) to determine UAE optimum parameters for the extraction of TA and TPC; (ii) to determine MAE optimum parameters for the extraction of TA and TPC; (iii) to compare both extraction techniques. Moreover, as a supplementary study: (iv) to determine the optimum operating conditions for the simultaneous extraction of TA and TPC for each extraction technique by multi-response optimization based on the desirability function.

To the best of our knowledge, this is the first time that the efficacy of UAE and MAE has been optimized and compared for the extraction of TA and TPC from blackberry.

2. Materials and Methods

2.1. Blackberry and Jam Samples

In 2016 fresh blackberry fruit (*Rubus ulmifolius* L.) samples were harvested in their full ripeness stage from local blackberry bushes in Serradilla, a region within Caceres province, Spain. The blackberries were triturated by means of a Thermomix (Vorwerk, Spain) until a homogeneous sample was obtained for analysis. The sample once crushed was passed through a sieve 500 µm thick. The triturated samples were stored in a freezer at −20 °C prior to analysis. The suitability of the extraction methods was evaluated by the analysis of different blackberry pulps and blackberry jams, as the main ingredient in jams is blackberry pulp. Blackberry jam samples were purchased from different commercial markets in Spain. The same crushing and filtering procedure that was subjected to fresh blackberry samples was applied to jams. The real jam samples, once sieved, were stored at 4 °C prior to analysis.

2.2. Reagents and Solvents

Standards of gallic acid (commercially available standard for total phenolic compounds) and cyanidin chloride (commercially available standard for anthocyanins) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Methanol (Fischer Scientific, Loughborough, United Kingdom) and formic acid (Scharlau, Barcelona, Spain) used for the chromatographic separation were HPLC grade. Hydrochloric acid and sodium hydroxide (Panreac, Barcelona, Spain) used to regulate the pH of the extraction solvent were of analytical grade. For the determination of total phenolic compounds, Folin–Ciocalteu reagent (Merck KGaA, EMD Millipore Corporation, Darmstadt, Germany), and anhydrous sodium carbonate (Panreac, Barcelona, Spain) were used. Ultra-pure water was supplied by a Milli-Q water purification system from Millipore (Bedford, Massachusetts, USA).

2.3. Extraction Procedure

2.3.1. Ultrasound-Assisted Extraction

UAE was performed using a UP200S ultrasonic system (200 W, 24 kHz) (Hielscher Ultrasonics, Teltow, Germany). This compact ultrasonic system was immersed in a water bath coupled to a temperature controller (Frigiterm, J.P. Selecta, Barcelona, Spain) to maintain the desired extraction temperature. Each triturated sample (0.3 g approximately) was placed in a container. Based on the experimental design, the appropriate volume of solvent was added and each extraction was performed under controlled UAE conditions. Upon extraction, the extract was filtered through a 0.22 µm nylon syringe filter (Nylon Syringe Filter, FILTER-LAB, Barcelona, Spain) and stored at −20 °C prior to analysis.

2.3.2. Microwave-Assisted Extraction

MAE was conducted using a MARS 6 240/50 kit (One Touch Technology, CEM Corporation, Matthews, North Carolina, USA). Each triturated sample (0.3 g approximately) was placed in a container. Based on the experimental design, the adequate volume and type of solvent was added and each extraction was performed under controlled MAE conditions. The procedure consisted of a ramp of 3 min to reach the desired temperature, the temperature was then maintained for 5 min for the extraction of the compounds, and a period of 25 min was allowed to cool the sample down to room temperature. In all the experiments power output was set at 800 W, as this was enough to reach the desired temperatures. At the end of the extraction, the extract was centrifuged twice for 5 min at 7500 rpm (9.5 cm orbital radius) and transferred to a 25 mL volumetric flask. The extract was filtered through a 0.22 µm nylon syringe filter (Nylon Syringe Filter, FILTER-LAB, Barcelona, Spain) and stored at −20 °C prior to analysis.

2.4. Total Phenolic Compounds (TPC)

The TPC analyses were carried out by a modified Folin–Ciocalteu (FC) procedure [32] using a Helios Gamma (γ) Unicam UV–Vis spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). This is a suitable method for TPC determination when a high total polyphenol content is expected to be found in the solid sample [33]. The absorbance of the blue colour complex was measured at a maximum wavelength of 765 nm. Following the Lambert–Beer law, this absorbance is directly proportional to the concentration of total phenolic compounds in the measured sample. The extracts were filtered through a 0.45 µm syringe filter (Nylon Syringe Filter, FILTER-LAB, Barcelona, Spain) prior to their spectrophotometric analysis. The FC assay was performed by transferring 250 µL of extract, 12.5 mL of distilled water, 1.25 mL of FC reagent, and 5 mL of 20% anhydrous sodium carbonate into a volumetric flask (25 mL). The solution was made up to the mark with distilled water. Then, after 30 min based on the kinetics of the reaction, the absorbances were measured in the spectrophotometer at 765 nm. It was necessary to create a calibration curve under the same conditions and by the same procedure, using gallic acid as standard (1–1000 mg L^{-1}) and measuring the absorbance values ($y = 0.0012x + 0.0003$; $R^2 = 0.9998$). The results were expressed as micrograms of gallic acid equivalent per gram of fresh fruit.

2.5. Identification of Anthocyanins (TA)

The TA were identified by ultra-high performance liquid chromatography (UHPLC), on a chromatograph coupled to a quadrupole-time-of-flight mass spectrometer (QToF-MS) (Xevo G2 QToF, Waters Corp., Milford, MA, USA). The chromatographic separation was performed using a reverse phase C-18 analytical column (Acquity UHPLC BEH C18, Waters Corporation, Milford, Massachusetts, United States) with dimensions of 100 mm × 2.1 mm and a particle size 1.7 µm. The injection volume was 3 µL. Prior to the chromatographic analyses, all the extracts were filtered through a 0.22 µm syringe filter (Nylon Syringe Filter, FILTER-LAB, Barcelona, Spain). The mobile phase consisted on Milli-Q water acidified with 2% formic acid as solvent A and pure methanol as solvent B, both degassed and filtered. The elution 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% of B, at a flow rate of 0.4 mL min^{-1}. The total run time was 12 min, including 4 min for re-equilibration. The analytes were determined by means of an electrospray source operating in positive ionization mode under the following conditions: desolvation gas flow = 700 L h^{-1}, desolvation temperature = 500 °C, cone gas flow = 10 L h^{-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). The molecular ions $[M]^+$ of the anthocyanins identified in blackberry showed the following *m/z* ratios: cyanidin 3-*O*-glucoside, 449; cyanidin 3-*O*-rutinoside, 595; cyanidin 3-*O*-(6″-malonyl-glucoside), 535; and cyanidin 3-*O*-(6″-dioxalyl-glucoside), 593. Retention times (tr), anthocyanins names, molecular ions [M]+ and anthocyanidin fragments (*m/z*) of the identified anthocyanins are summarized in Table 1.

Table 1. Retention times (tr), anthocyanins names, molecular ions $[M]^+$ and anthocyanidin fragments (*m/z*) of the identified anthocyanins.

Peak	tr (min)	Anthocyanin	Molecular Ion $[M^+]$	Anthocyanidin Fragment (*m/z*)
1	1.55	cyanidin 3-*O*-glucoside	449	287
2	2.16	cyanidin 3-*O*-rutinoside	595	287
3	2.72	cyanidin 3-*O*-(6″-malonyl-glucoside)	535	287
4	3.24	cyanidin 3-*O*-(6″-dioxalyl-glucoside)	593	287

2.6. Separation and Quantification of Anthocyanins

The samples were analysed to separate the anthocyanins by UHPLC on an Elite LaChrom Ultra System (VWR Hitachi, Tokyo, Japan) equipped with an L-2200 U autosampler, a L-2300 column oven set at 50 °C, two L-2160 U pumps and a UV-Vis L-2420 U detector using 15 µL as the injection volume. The anthocyanins were separated by means of a 100 × 2.1 mm and a particle size of 2.6 µm Fused-Core C-18 column (Phenomenex Kinetex, CoreShell Technology, Torrance, California, USA). The detector was set at 520 nm, as this corresponds to anthocyanins maximum absorption. The extracts and the samples were filtered through a 0.22 µm syringe filter (Nylon Syringe Filter, FILTER-LAB, Barcelona, Spain). The mobile phase consisted on a gradient that used 5% acidified Milli-Q water with formic acid as solvent A and pure methanol as solvent B. It was previously filtered using a 0.22 µm filter (Nylon Membrane Filter, FILTER-LAB, Barcelona, Spain) and degassed by ultrasonic bath (Elmasonic S300, Elma Schmidbauer GmbH, Singen, Germany). The gradient was as follows: 0.0 min, 5% B; 1.0 min, 15% B; 2.0 min, 25% B; 2.3 min, 25% B; 3.6 min, 40% B; 5.9 min 70% B; 6.6 min, 100% B; 9.3 min, 100% B, at a flow rate of 0.7 mL min^{-1}. The quantification of the anthocyanins was based on a calibration curve, where cyanidin chloride was used as the reference standard. Known concentration standards were prepared between 0.05 and 30 mg L^{-1} ($y = 248738.73x - 2136.42$; $R^2 = 0.9997$). The limit of detection (0.186 mg L^{-1}) and quantitation (0.620 mg L^{-1}) was determined as the analyte concentration that corresponded to the standard deviation from the blank values signal ($n = 10$) plus 3 or 10 times, respectively, divided by the slope of the linear regression. Assuming that different anthocyanins have similar absorbances, and taking into account the molecular weight of each anthocyanin, a calibration curve was produced for each anthocyanin found in blackberry. This allowed the compounds of interest to be quantified. The results were expressed as micrograms of cyanidin chloride equivalents per gram of fresh fruit.

2.7. Response Surface Methodology (RSM)

A BBD was used to evaluate the response of the extraction process variables and to maximize the extraction of antioxidant compounds from blackberry. Two interdependent variables or responses were employed: (i) TPC (Y_{TP}, µg g^{-1}) and (ii) TA (Y_{TA}, µg g^{-1}) extraction yields. Regarding the independent variables or factors, six and four experimental variables were evaluated for UAE and MAE optimization, respectively. On the one hand, a careful parameter optimization for each particular target compound was carried out. On the other hand, optimal conditions to obtain the highest possible simultaneous concentration of TPC and TA were also studied for each extraction technique, UAE and MAE.

Therefore, a three-level BBD was employed to find the optimum UAE and MAE conditions for the extraction of TA and TPC from blackberry. In this study, the experimental designs consisted of 54 experiments for UAE and 27 experiments for MAE, which were randomly performed in duplicate, including six (UAE) and three (MAE) central point replicates, respectively. Each independent variable could take three different values, which were the minimum, medium or maximum values of the range studied. Table 2 and Table 5 show the experiments carried out for UAE and MAE, respectively. Once the response of the TPC and TA yield obtained in the optimization had been acquired, a second-order polynomial equation could be fitted for each yield to correlate the independent variables and the yield (Equation (1)):

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i \cdot x_i + \beta_{\mathbf{ii}} \cdot x_i^2 + \sum_i \sum_{i=1}^{k} \beta_{\mathbf{ij}} \cdot x_i x_j + r, \tag{1}$$

where x represents each variable that has an influence on the yield; y is the yield; β_i, β_{ij}, and β_{ii} are unknown coefficients for each main effect, the interactions i, j; and the quadratic effect i, i, respectively; and r is the residual value. Coefficients of determination (R^2) and lack of fit test were obtained for each fit.

Moreover, the multi-response optimization to determine the extraction conditions which maximize both yields (TPC and TA yields) was evaluated. This optimization was based on a desirability function (D) obtained through the following equation (Equation (2)) [11,31,34,35].

$$D = (d_1 \cdot d_2)^{\frac{1}{k}}, \tag{2}$$

where d_i are the individual normalized values, which vary from 0 to 1 for each of the two yields corresponding the lowest desirability to D = 0 and the highest desirability to D = 1 [11,36].

Analysis of variance (ANOVA) was used to estimate the effects of the variables on the final yield, the main, interactive and quadratic terms of the second order mathematical model, and the optimum levels of the significant variables. In addition, the relationships between variables and yields were represented in 3D-surface plots.

2.8. Statistical Analysis

All the experimental data from all the extractions carried out using either of the two extraction techniques were examined using Design Expert software 11 (Trial Version, Stat-Ease Inc., Minneapolis, MN, USA). This software was used to estimate the effects of the variables on the final response, the analysis of variance (ANOVA), the second order mathematical model, and the optimum levels of the significant variables. The software used for the figures has been Statgraphics Centurion XVIII (Trial version, Statpoint Technologies, Inc., USA).

3. Results and Discussion

3.1. Optimization of TA and TPC Separate Extraction by UAE

The effects of solvent composition, X_1 (25%, 50%, 75% methanol in water), temperature, X_2 (10, 40, 70 °C), ultrasound amplitude, X_3 (30%, 50%, 70%), cycle, X_4 (0.2, 0.45, 0.7 s), solvent pH, X_5 (2, 4.5, 7), and sample:solvent ratio, X_6 (0.3/10, 0.3/15, 0.3/20 g mL^{-1}) were optimized for the extraction of TA and TPC by UAE. Table 2 shows the matrix with the actual values at the three levels corresponding to each variable as well as the yields in the BBD for UAE. Since high temperatures might degrade individual anthocyanins, the maximum temperature was set at 70 °C for both TA and TPC extractions [37]. This maximum temperature was also adequate to avoid solvent evaporation. TA content varied between 931.61 and 1823.87 μg g^{-1} fresh weight and TPC ranged from 8661.9 to 14,429.3 μg g^{-1} fresh weight. Table 2 shows actual measured values for each trial of the experimental design as well as the predicted values according to the mathematical model. It can be seen that the experimental data and the model estimated values were very similar. The extraction of both types of compounds was correlated with the experimental conditions by a second-order mathematical equation that intended to evaluate the effect of the variables and their possible interactions. The suitability of the fitted model was determined by ANOVA.

Table 2. Matrix with real values for each factor and measured and predicted responses in the Box–Behnken design for ultrasound-assisted extraction (UAE).

Run	**Factors**						**Responses (Fresh Weight)**			
	X_1 (%)	**X_2 (°C)**	**X_3 (%)**	**X_4 (s)**	**X_5**	**X_6 (g mL^{-1})**	**Y_{TA} (μg g^{-1})**		**Y_{TP} (μg g^{-1})**	
							Measured	**Predicted**	**Measured**	**Predicted**
1	50	40	30	0.45	2	10	1600.82	1639.82	11,294.9	11,058.5
2	50	40	70	0.45	2	10	1653.09	1671.87	12,811.9	12,208.3
3	50	40	30	0.45	7	10	1753.65	1727.65	11,921.7	12,081.3
4	50	40	70	0.45	7	10	1651.96	1716.24	11,921.7	12,221.9
5	50	40	30	0.45	2	20	1779.13	1734.33	11,294.9	11,228.2
6	50	40	70	0.45	2	20	1755.61	1762.13	12,799.4	12,406.3
7	50	40	30	0.45	7	20	1742.16	1742.85	11,545.6	12,382.8
8	50	40	70	0.45	7	20	1785.66	1727.19	12,548.6	12,551.5
9	50	10	50	0.2	2	15	1618.77	1648.17	10,041.1	10,407.3
10	50	70	50	0.2	2	15	1646.36	1728.32	11,796.4	12,193.9
11	50	10	50	0.7	2	15	1680.69	1672.9	12,423.3	12,068.5
12	50	70	50	0.7	2	15	1695.48	1669.94	12,297.9	12,099.9
13	50	10	50	0.2	7	15	1595.39	1637.79	10,542.6	10,677.9
14	50	70	50	0.2	7	15	1704.42	1695.36	12,548.6	12,966.0
15	50	10	50	0.7	7	15	1823.87	1758.75	12,924.8	12,464.5
16	50	70	50	0.7	7	15	1779.46	1733.21	13,300.9	12,997.4
17	25	40	30	0.2	4.5	15	1276.1	1233.94	9790.31	9954.34
18	75	40	30	0.2	4.5	15	956.935	947.121	12,297.9	12,070.1
19	25	40	70	0.2	4.5	15	1341.18	1276.12	10,291.8	10,362.9
20	75	40	70	0.2	4.5	15	931.61	984.435	12,799.4	12,604.0
21	25	40	30	0.7	4.5	15	1331.62	1287.95	10,668.0	10,690.9
22	75	40	30	0.7	4.5	15	962.905	1018.8	12,548.6	12,650.0
23	25	40	70	0.7	4.5	15	1248.06	1267.03	11,420.2	11,475.6
24	75	40	70	0.7	4.5	15	959.994	992.999	13,551.7	13,560.0
25	50	10	30	0.45	4.5	10	1642.19	1629.8	12,799.4	12,696.7
26	50	70	30	0.45	4.5	10	1567.99	1616.69	12,674.0	12,540.0
27	50	10	70	0.45	4.5	10	1730.47	1649.95	13,175.5	13,059.8
28	50	70	70	0.45	4.5	10	1610.16	1617.17	13,300.9	13,467.3
29	50	10	30	0.45	4.5	20	1660.89	1634.4	12,297.9	11,898.0
30	50	70	30	0.45	4.5	20	1660.74	1721.79	13,927.8	13,810.0
31	50	10	70	0.45	4.5	20	1679.53	1650.31	11,921.7	12,289.3
32	50	70	70	0.45	4.5	20	1686.17	1718.03	14,429.3	14,765.5
33	25	10	50	0.45	2	15	1095.24	1145.55	8661.9	9529.62
34	75	10	50	0.45	2	15	992.97	985.602	11,796.4	11,692.4
35	25	70	50	0.45	2	15	1311.5	1282.53	10,417.2	10,877.4
36	75	70	50	0.45	2	15	1037.31	925.805	12,297.9	12,162.6
37	25	10	50	0.45	7	15	1110.71	1205.37	9288.79	9486.78
38	75	10	50	0.45	7	15	989.131	1001.25	12,799.4	12,401.8
39	25	70	50	0.45	7	15	1295.56	1319.77	11,294.9	11,336.1
40	75	70	50	0.45	7	15	952.341	918.877	14,303.9	13,373.5
41	25	40	50	0.2	4.5	10	1304.64	1283.8	11,545.6	10,927.1
42	75	40	50	0.2	4.5	10	1024.03	975.192	12,172.5	12,510.0
43	25	40	50	0.7	4.5	10	1221.88	1209.13	10,417.2	10,378.5
44	75	40	50	0.7	4.5	10	894.625	918.182	10,918.7	11,804.7
45	25	40	50	0.2	4.5	20	1234.44	1220.04	10,166.4	9108.04
46	75	40	50	0.2	4.5	20	946.563	950.147	11,671.0	11,882.0
47	25	40	50	0.7	4.5	20	1299.96	1339.64	11,671.0	11,505.9
48	75	40	50	0.7	4.5	20	1057.39	1087.4	13,677.0	14,123.2
49	50	40	50	0.45	4.5	15	1640.56	1653.57	12,799.4	13,112.8
50	50	40	50	0.45	4.5	15	1670.51	1653.57	13,677.0	13,112.8
51	50	40	50	0.45	4.5	15	1625.19	1653.57	13,426.3	13,112.8
52	50	40	50	0.45	4.5	15	1699.1	1653.57	13,050.2	13,112.8
53	50	40	50	0.45	4.5	15	1676.91	1653.57	12,924.8	13,112.8
54	50	40	50	0.45	4.5	15	1609.12	1653.57	12,799.4	13,112.8

Y_{TA}: Yield of total anthocyanins; Y_{TP}: Yield of total phenolics.

In order to evaluate the adequate fitting of the model, the complete second order polynomial equation was evaluated using the coefficients of determination (R^2), which were 98.01% and 90.15% for TA and TPC, respectively. The R^2 values indicate how closely the model explains the variability of the response to be studied. These values were consistent with a statistically significant agreement between the measured and the estimated responses. However, for improved and extended information on the quality of the regression results, sums of squares, degrees of freedom, mean squares, F values and *p*-values for the regression, residuals, lack of fit, and pure error are also shown in Table 3. It was observed in both models that lack of fit tests obtained *p*-values higher than 0.05 (non-significant) which confirm the suitability of the model.

Table 3. Analysis of variance (ANOVA) of the quadratic model adjusted to the extraction of total anthocyanins and total phenolic compounds for UAE.

UAE	Source	Sum of Squares	Degreesof Freedom	Mean Square	*F* Value	*p*-Value
Anthocyanins	Model	4.847×10^6	27	1.795×10^5	47.53	<0.0001
	Residual	98,193.97	26	3776.69		
	Lack of fit	92,338.78	21	4397.08	3.75	0.0736
	Pure error	5855.19	5	1171.04		
	Total	4.945×10^6	53			
Phenolics	Model	7.650×10^7	27	2.833×10^6	8.81	<0.0001
	Residual	8.362×10^6	26	3.216×10^5		
	Lack of fit	7.709×10^6	21	3.671×10^5	2.81	0.1270
	Pure error	6.523×10^5	5	1.305×10^5		
	Total	8.487×10^7	53			

Moreover, an acceptable agreement between the experimental data and the estimated values when only the significant terms (p-value < 0.05) of the polynomial equation were used was observed. Thus, the reduced equations that can reliably predict the experimental results were expressed as follows (Equations (3) and (4)):

$$Y_{TA}\ (\mu g\ g^{-1}) = 1653.57 - 140.21\ X_1 + 26.36\ X_6 - 568.28\ X_1{}^2 - 49.19\ X_{12} + 48.56\ X_{46} \tag{3}$$

$$\begin{gathered} Y_{TPC}\ (\mu g\ g^{-1}) = 13112.8 + 1050.05\ X_1 + 579.88\ X_2 + 329.64\ X_3 + 423.15\ X_4 + 292.03\ X_5 - \\ 1018.88\ X_1{}^2 - 438.83\ X_{24} + 517.19\ X_{26} - 391.98\ X_4{}^2 + 736.60\ X_{46} - 892.45\ X_5{}^2 \end{gathered} \tag{4}$$

The influencing factors were considered to be statistically significant at *p*-values less than 0.05, which would indicate that they were significantly different from zero at 95% confidence level (Table S1). In the case of TA, the *p*-values of the solvent composition and the sample:solvent ratio were less than 0.05, which indicated that both were significant factors that influenced the yield. The quadratic interaction of the solvent composition was also considered a significant factor with a *p*-value less than 0.01. The interaction between solvent composition and temperature, as well as the interaction between cycle and sample:solvent ratio indicated significant interactions between factors (p-value $<$ 0.05). The analysis of the model clearly shows that of all the linear terms, solvent composition was the most significant factor. It actually had a negative effect ($\beta_1 = -140.21$), which means that a decrease in this factor would favour the recovery of TA from the extract.

With regards to TPC, the *p*-values of solvent composition, temperature, amplitude, and cycle were less than 0.01, which indicates that these factors had significant effects. pH was also a significant factor for the extraction of TPC with a *p*-value less than 0.05. The quadratic interactions of solvent composition, pH, and cycle were significant on the yield, with *p*-values less than 0.01 for solvent and pH, and less than 0.05 for cycle. A significant interaction between cycle and sample:solvent ratio was obtained (p-value < 0.01). In this case, the analysis of the model showed that, of all the linear terms, solvent composition was the most significant factor, followed by temperature, cycle, amplitude, and pH.

In this case, solvent composition had a positive effect ($\beta_1 = 1050.05$), which means that the use of higher methanol contents in the solvent led to higher levels of TPC in the extract. The behaviour of the other factors was also the same, i.e., temperature ($\beta_2 = 579.88$), amplitude ($\beta_3 = 329.64$), cycle ($\beta_4 = 423.15$), and pH ($\beta_5 = 292.03$) had positive effects, which means that the use of higher temperature, cycle, and amplitude values as well as a higher pH would lead to a higher TPC content in the extract. In addition, solvent and pH showed significant quadratic effects with negative coefficients ($\beta_{11} = -1018.88$) and ($\beta_{55} = -892.45$), respectively.

Therefore, solvent composition was the most influential linear factor on UAE when both antioxidant compounds were considered, since it had a negative effect on TA yields and a positive effect on the extraction of TPC. It has been highlighted in the literature that hydroalcoholic mixtures (76% and 61% methanol in water for TA and TPC, respectively) are more efficient than pure solvents for the extraction of amphiphilic or moderately polar molecules, such as polyphenols [19,25]. Based on the principle of similarity, the similar polarity of the mixture of solvents and the compounds of interest easily dissolved the solute from plant cells [38]. Each solvent plays a specific role in the extraction process: on the one hand, the solubility of the phenolics is enhanced by the alcohol, and the desorption of the solute in the sample is favoured by the water [39].

Once the experiments had been carried out, the coefficients for the full quadratic polynomial equations that intend to predict the yields were established for both compound groups (Table S1). Two suitable second-order mathematical models based on a function of the independent variables were obtained to describe the yield values of Y_{TA} and Y_{TPC}.

For a better illustration of the interactive effects, the model data was complemented by plotting the trends in three-dimensional surface plots of the polynomial equations (Figure 1). The Figure 1a and 1b represented the 3D-surface plots of TA and TPC extractions, respectively, with respect to methanol percentage and temperature. The plots indicated that the optimum extraction temperature for both TA and TPC occurs with the upper values in the interval studied (between 60 and 68 °C). This can be attributed to the decrease in solvent viscosity as temperature was increased, which, in turn, increases the solubility of the antioxidant compounds [38]. However, higher temperatures were not studied since they might degrade individual anthocyanins and evaporate the solvent [37]. Regarding solvent composition, the optimum extraction conditions for TA were found to be in the middle values of the range studied and it was slightly higher for TPC. As above mentioned, hydroalcoholic mixtures are preferred for their greater efficiency compared to pure solvents when used for the extraction of moderately polar molecules.

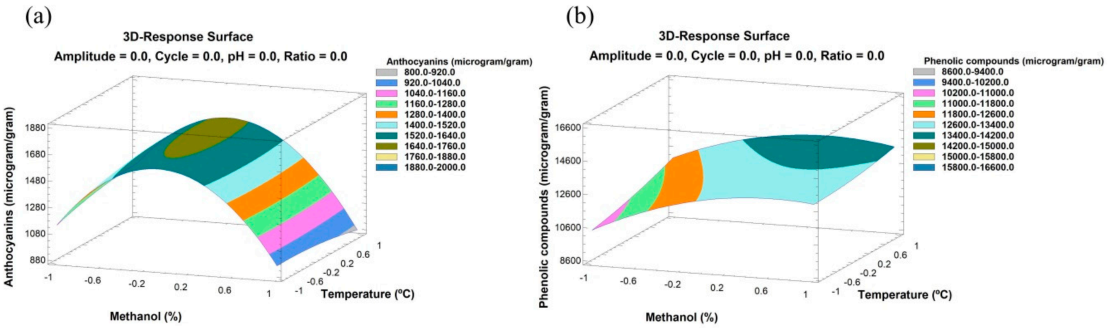

Figure 1. Response surface analysis of the extraction of total anthocyanins (TA) and total phenolic compounds (TPC) by means of UAE with respect to methanol percentage and temperature: (**a**) response surface plot for TA, and (**b**) response surface plot for TPC.

Table 4 summarizes the optimum conditions for the extraction of TA and TPC using UAE. It can be concluded that the optimum UAE conditions for TA were as follows: extraction solvent with 46% MeOH in water at pH 4.97, extraction temperature of 60.4 °C, ultrasound amplitude of 30%, cycle of 0.7 s, and 0.3:20 g mL^{-1} as the optimum solid:solvent ratio. The optimum UAE conditions for TPC were as follows: extraction solvent with 63.7% MeOH in water at pH 4.81, extraction temperature of 68.3 °C, ultrasound amplitude of 70%, cycle of 0.7 s, and 0.3:20 g mL^{-1} as the optimum solid:solvent ratio. The suitability of the model was confirmed by running a test under the optimum conditions previously determined. Experimental and predicted values were similar: TA (μg g^{-1}) (1792.73 ± 68.84 vs. 1830.06) and TPC (μg g^{-1}) (15,697.76 ± 603.21 vs. 15,466.0). Studies of re-extraction of the residue obtained after the extractions were carried out, obtaining quantities less than 5% of TPC and TA with respect to those extracted from the original pulp.

Table 4. Optimum conditions obtained for extraction of TA and TPC using UAE.

UAE	TA	TPC
Methanol (%)	46	63.7
Temperature (°C)	60.4	68.3
pH	4.97	4.81
Ratio (g:mL)	0.3:20	0.3:20
Amplitude (%)	30	70
Cycle (s)	0.7	0.7
Experimental (μg g^{-1})	1792.73 ± 68.84	15,697.76 ± 603.21
Predicted (μg g^{-1})	1830.06	15,466.0

3.2. *Optimization of TA and TPC Separate Extraction by MAE*

The effects of solvent composition, X_1 (25%, 50%, 75% methanol in water), temperature, X_2 (50, 75, 100 °C), pH, X_3 (2, 4.5, 7), and sample:solvent ratio, X_4 (0.3:10, 0.3:15, 0.3:20 g mL^{-1}) were the variables to be controlled and optimized for MAE (Table 5).

Table 5. Matrix with real values for each factor and measured and predicted responses in the Box–Behnken design for microwave-assisted extraction (MAE).

Run	**Factors**				**Responses (Fresh Weight)**			
	X_1 (%)	**X_2 (°C)**	**X_3**	**X_4 (g mL^{-1})**	**Y_{TA} (μg g^{-1})**		**Y_{TP} (μg g^{-1})**	
					Measured	**Predicted**	**Measured**	**Predicted**
1	25	50	4.5	15	1621.25	1536.36	10,949.7	11,186.4
2	75	50	4.5	15	1563.46	1429.14	14,780.6	14,893.1
3	25	100	4.5	15	1987.62	2044.4	9982.97	10,422.5
4	75	100	4.5	15	1323.6	1330.94	14,847.6	15,163.1
5	50	75	2	10	2140.48	1969.04	15,080.7	14,812.8
6	50	75	7	10	1752.68	1792.01	8847.21	10,348.6
7	50	75	2	20	1972.25	1855.39	15,148.0	14,198.7
8	50	75	7	20	1895.13	1989.04	11,208.3	12,028.3
9	50	75	4.5	15	2121.59	1940.64	15,051.0	14,239.9
10	25	75	4.5	10	1662.33	1892.76	9111.25	9079.66
11	75	75	4.5	10	1676.08	1759.14	14,610.1	13,741.2
12	25	75	4.5	20	2091.89	2211.18	9725.54	10,050.4
13	75	75	4.5	20	1552.19	1524.12	14,348.5	13,836.1
14	50	50	2	15	826.374	1261.81	15,565.8	16,029.9
15	50	100	2	15	2003.25	2039.44	15,211.9	15,460.4
16	50	50	7	15	1646.65	1812.83	13,182.7	12,390.2
17	50	100	7	15	1678.12	1445.04	13,473.7	12,465.6
18	50	75	4.5	15	2037.76	1940.64	13,860.5	14,239.9
19	50	50	4.5	10	1945.26	1725.77	14,145.9	13,973.1
20	50	100	4.5	10	1762.86	1800.97	13,414.0	13,253.8
21	50	50	4.5	20	1800.67	1637.74	13,881.5	14,033.6
22	50	100	4.5	20	1877.72	1972.38	14,094.1	14,258.9
23	25	75	2	15	2091.72	1901.66	11,357.5	11,129.1
24	75	75	2	15	1518.01	1524.75	15,222.2	15,955.2
25	25	75	7	15	2044.97	1913.41	9155.48	8414.33
26	75	75	7	15	1404.39	1469.63	11,815.3	12,035.5
27	50	75	4.5	15	1662.58	1940.64	13,808.1	14,239.9

Y_{TA}: Yield of total anthocyanins; Y_{TP}: Yield of total phenolics.

To evaluate the appropriate fitting of the model, the complete second order polynomial model was evaluated using the coefficients of determination (R^2), which were 69.53% and 92.28% for TA and TPC, respectively. The value for TPC was consistent with a statistically significant agreement between the measured and estimated responses. Although a low agreement between experimental and predicted values for TA was observed with the coefficient of determination used, the lack of fit test was not significant (p-value = 0.61), which highlights the adequate fitting of the model. The complete ANOVA table for both antioxidant compounds including sums of squares, degrees of freedom, mean squares, F values and p-values for the regression, residuals, lack of fit, and pure error for MAE is shown in Table 6.

Table 6. Analysis of variance (ANOVA) of the quadratic model adjusted to the extraction of total anthocyanins and total phenolic compounds for MAE.

MAE	Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p*-Value
Anthocyanins	Model	1.579×10^6	14	1.128×10^5	1.96	0.1255
	Residual	6.922×10^5	12	57,681.13		
	Lack of fit	5.727×10^5	10	57,268.10	0.9585	0.6123
	Pure error	1.195×10^5	2	59,746.28		
	Total	2.271×10^6	26			
Phenolics	Model	1.145×10^8	14	8.181×10^6	10.24	0.0001
	Residual	9.588×10^6	12	7.99×10^5		
	Lack of fit	8.600×10^6	10	8.600×10^5	1.74	0.4195
	Pure error	9.883×10^5	2	4.941×10^5		
	Total	1.241×10^8	26			

In this case, the non-significant terms were also removed from the mathematical models to propose the reduced equations that can reliably predict the experimental results (Equations 5,6):

$$Y_{TA}\ (\mu g\ g^{-1}) = 1940.64 - 205.17\ X_1 - 286.35\ X_{23} \quad (5)$$

$$Y_{TPC}\ (\mu g\ g^{-1}) = 14{,}239.9 + 2111.82\ X_1 - 1658.63\ X_3 - 1763.29\ {X_1}^2 \quad (6)$$

In the case of TA, only the solvent composition had a linear significant factor ($p < 0.05$). Non-significant quadratic interactions were obtained. The interaction between temperature and pH proved to be significant with a p-value < 0.05. The analysis of the model showed the same behaviour as in UAE optimization, where of all the linear terms, solvent composition was also the most significant factor. The solvent composition had a negative effect ($\beta_1 = -205.17$), indicating that a decrease in this factor would favour the recovery of the TA in the extract (Table S2).

With regards to the TPC, both solvent composition and pH (p-value < 0.01) had significant effects. The quadratic effect of the solvent composition was also a significant factor for the extraction of TPC (p-value < 0.01). In this case, the analysis of the model showed that, of all the linear terms, solvent and pH were the most significant factors. The solvent composition had a positive effect ($\beta_1 = 2111.82$), which means that the use of a higher methanol content in the solvent led to a greater extraction of TPC. On the contrary, pH showed the opposite behaviour ($\beta_3 = -1658.63$), which indicates that the use of higher pH values would lead to lower levels of TPC in the extract. pH values less than two were not considered since such low levels might cause the acid hydrolysis of the compounds of interest [40]. In addition, the solvent showed a significant quadratic effect with a negative coefficient ($\beta_{11} = -1763.29$) (Table S2). Similar results have been found in the literature with regards to other vegetable matrices [41,42].

As aforementioned, the information for the model was completed by plotting the trends in three-dimensional surface plots of the polynomial equations for MAE (Figure 2).

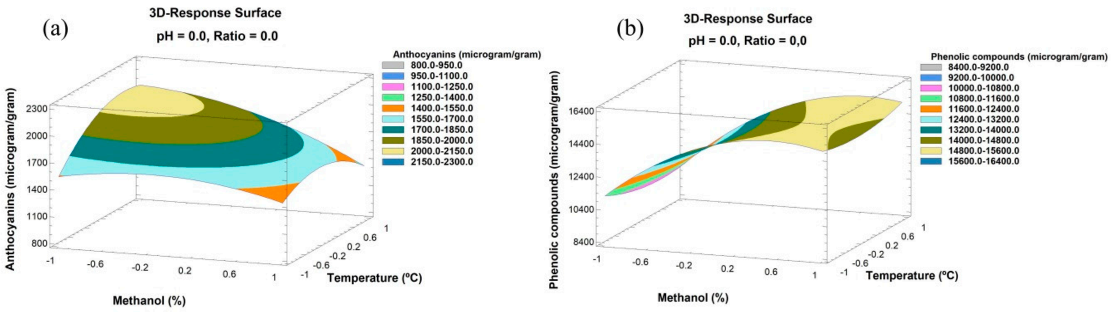

Figure 2. Response surface analysis of the extraction of TA and TPC by means of MAE with respect to methanol percentage and temperature: (**a**) response surface plot for TA, and (**b**) response surface plot for TPC.

The Figure 2a,b represent the 3D-surface plots of TA and TPC, respectively, with respect to methanol percentage and temperature. In this case, the plots indicate that the optimum extraction temperature for TA was in the upper limit of the interval studied, while for TPC it was around the intermediate values. Since these compounds are thermally sensitive and can, therefore, be easily degraded by the hydrolytic opening of the heterocyclic ring that would form chalcone in the case of anthocyanins, the use of higher temperatures was not recommended [5]. Moreover, working at temperatures higher than 70 °C may produce a change of phase and the evaporation of the methanol. Regarding solvent composition, the optimum extraction conditions for TA were found to be in the lower levels of the range studied. On the contrary, the optimum methanol percentage for TPC was close to its maximum value.

Table 7 summarizes the optimum conditions for the extraction of TA and TPC by means of MAE. It can be seen that the optimum MAE conditions for TA are as follows: extraction solvent with 26.3% MeOH in water at pH 2, extraction temperature of 100 °C, and 0.3:20 g:mL as the optimum solid:solvent ratio. The optimum MAE conditions for TPC were as follows: extraction solvent with 64% MeOH in water at pH 2, extraction temperature of 50.3 °C, and 0.3:12 g:mL as the optimum solid:solvent ratio. The suitability of the model was confirmed by running a test under the optimum conditions previously determined. Experimental and predicted values were similar: TA (µg g^{-1}) (2454.12 ± 65.90 vs. 2422.12) and TPC (µg g^{-1}) (16,277.29 ± 688.42 vs. 16,797.21). Studies of re-extraction of the residue obtained after the extraction have been carried out, obtaining quantities less than 5% of TPC and TA with respect to those extracted from the original pulp.

Table 7. Optimum conditions obtained for extraction of TA and TPC using MAE.

MAE	TA	TPC
Methanol (%)	26.3	64
Temperature (°C)	100	50.3
pH	2	2
Ratio (g:mL)	0.3:20	0.3:12
Experimental (µg g^{-1})	2454.12 ± 65.90	16,277.29 ± 688.42
Predicted (µg g^{-1})	2422.12	16,797.21

3.3. Extraction Time

In order to evaluate the kinetics of the extraction process, several extractions were run under the optimum UAE and MAE conditions for both TA and TPC. Different extraction times of 2, 5, 10, 15, 20, and 25 min were applied to the extraction of the sample in duplicate. It was observed that the recovery of both TA and TPC by UAE reached a maximum at 10 min and that this is sufficient for a substantial extraction. With regards to MAE, the maximum recovery was achieved in just 5 min. Longer extraction times for both extraction techniques led to lower recoveries, probably due to the degradation of the TA and TPC [43–45]. As a consequence, 10 and 5 min were established for UAE and MAE as their respective optimum extraction time for both antioxidants. These values are much lower than the extraction times found in the bibliography (around 30 min) for other similar extraction methods [5,24,27].

3.4. Repeatability and Intermediate Precision

The reliability of the developed method was studied in terms of repeatability and intermediate precision. The former is related to its precision under the same extraction conditions on the same day. The latter is related to its precision under the same extraction conditions on separate days. To determine the method repeatability, 10 extractions were performed on the first day of the study. In order to determine its intermediate precision, 10 more extractions were carried out on each of the next two consecutive days, which added up to 30 extractions altogether.

UAE repeatability obtained a coefficient of variation (CV) of 3.25% for TA and 3.98% for TPC, while for intermediate precision its coefficient of variation (CV) was 3.98% for TA and 4.16% for TPC. Regarding MAE, its repeatability coefficient of variation (CV) was 3.05% for TA and 3.88% for TPC, while its intermediate precision coefficient of variation (CV) was 3.29% for TA and 4.65% for TPC. All of the results were found to be less than 5% and were, therefore, within the acceptable limits (±10%) as determined by the Association of Official Agricultural Chemists International (AOAC International) [46]. Thus, an excellent precision level was observed in both of the two extraction methods for TA and TPC.

3.5. Comparison between UAE and MAE

In order to compare the suitability of each extraction method, a further study was carried out to quantify the concentrations of TA and TPC obtained from ten different blackberry jams and two blackberry fruit samples by means of UAE and MAE. The extractions were carried out in triplicate. The results are included in Table 8. It was observed that UAE and MAE were able to obtain similar extraction results. Non-significant differences were found between both UAE and MAE regarding extraction yields after the statistical analysis. However, higher TA concentrations were generally obtained when MAE was used. Regarding TPC, some jam samples (J-2, J-3, and J-7) differed significantly according to Tukey's test, and better recoveries in general were achieved when MAE was used.

Table 8. Concentrations of total anthocyanins and total phenolic compounds ($n = 3$) in different types of blackberry jams and blackberry fruits through UAE and MAE.

Sample	**Total Anthocyanins (μg g^{-1})**		**Total Phenolic Compounds (μg g^{-1})**	
	UAE	**MAE**	**UAE**	**MAE**
J-1	6.43 ± 0.40 ^{a}	6.62 ± 0.35 ^{a}	206.37 ± 12.34 ^{a}	227.33 ± 16.88 ^{a}
J-2	0.75 ± 0.07 ^{a}	0.81 ± 0.06 ^{a}	329.77 ± 14.97 ^{a}	360.85 ± 11.73 ^{b}
J-3	20.04 ± 0.81 ^{a}	20.95 ± 0.98 ^{a}	276.83 ± 12.71 ^{a}	312.61 ± 15.89 ^{b}
J-4	3.12 ± 0.28 ^{a}	3.29 ± 0.22 ^{a}	336.42 ± 20.25 ^{a}	358.07 ± 15.11 ^{a}
J-5	32.14 ± 1.02 ^{a}	33.02 ± 1.33 ^{a}	388.49 ± 22.44 ^{a}	412.89 ± 27.22 ^{a}
J-6	ND	ND	450.28 ± 16.98 ^{a}	462.73 ± 14.33 ^{a}
J-7	0.72 ± 0.06 ^{a}	0.71 ± 0.05 ^{a}	455.03 ± 20.30 ^{a}	507.44 ± 22.10 ^{b}
J-8	7.70 ± 0.37 ^{a}	7.91 ± 0.32 ^{a}	378.11 ± 25.67 ^{a}	391.93 ± 27.27 ^{a}
J-9	12.21 ± 0.22 ^{a}	12.49 ± 0.52 ^{a}	401.35 ± 16.20 ^{a}	420.77 ± 15.94 ^{a}
J-10	8.87 ± 0.38 ^{a}	8.83 ± 0.48 ^{a}	266.07 ± 12.03 ^{a}	281.09 ± 22.47 ^{a}
F-1	1792.73 ± 68.84 ^{a}	1844.55 ± 45.07 ^{a}	15,697.76 ± 603.21 ^{a}	16,525.08 ± 455.82 ^{a}
F-2	2298.54 ± 105.23 ^{a}	2454.12 ± 65.90 ^{a}	17,328.90 ± 509.36 ^{a}	16,277.29 ± 688.42 ^{a}

J: Jam samples; F: Fruit samples. ND, not detected as it was below the detection limit. Different letters in the same line for each kind of compounds indicate that the means differ significantly by Tukey's test ($p < 0.05$).

While recoveries were slightly greater and they were completed in a shorter time when MAE was used, UAE completed the extractions at lower temperature. This suggests that depending on laboratory equipment requirements and availability either technique can be equally appropriate and pose particular advantages for the extraction of antioxidants from blackberry.

3.6. Optimization of TA and TPC Simultaneous Extraction

Although most of the studies found in the literature are focused on the individual optimization for the extraction of the antioxidant compounds, the simultaneous optimization has several advantages for quality control applications in analytical laboratories in industry, for instance as monetary and time saving. Thus, multi-response optimization was also performed for both techniques according to the results obtained from the BBD to determine the best extraction conditions as a compromise between TA and TPC optimum conditions. Therefore, two responses TA and TPC were simultaneously optimized by the desirability function. For UAE, the maximum desirability was obtained when extraction solvent with 46.5% MeOH in water at pH 4.16, extraction temperature of 67.5 °C, ultrasound amplitude of 66.8%, cycle of 0.7 s, and 0.3:20 g mL^{-1} as the optimum sample:solvent ratio (Table 9) were applied. Figure 3a represents the contour plot of the D values as a function of both methanol percentage and temperature, while the rest of variables remained constant. It can be seen that the highest D values were obtained when the percentage of methanol was around the mid values within the interval evaluated. These results highlight the importance of determining optimum simultaneous conditions, since a similar yield of antioxidant compounds is obtained when the percentage of methanol used is lower in comparison to the specific optimum conditions for TPC.

Table 9. Optimum conditions obtained for simultaneous extraction of TA and TPC using UAE and MAE.

Variables	UAE	MAE
Methanol (%)	46.5	51
Temperature (°C)	67.5	100
pH	4.16	2
Ratio (g:mL)	0.3:20	0.3:15
Amplitude (%)	66.8	-
Cycle (s)	0.7	-

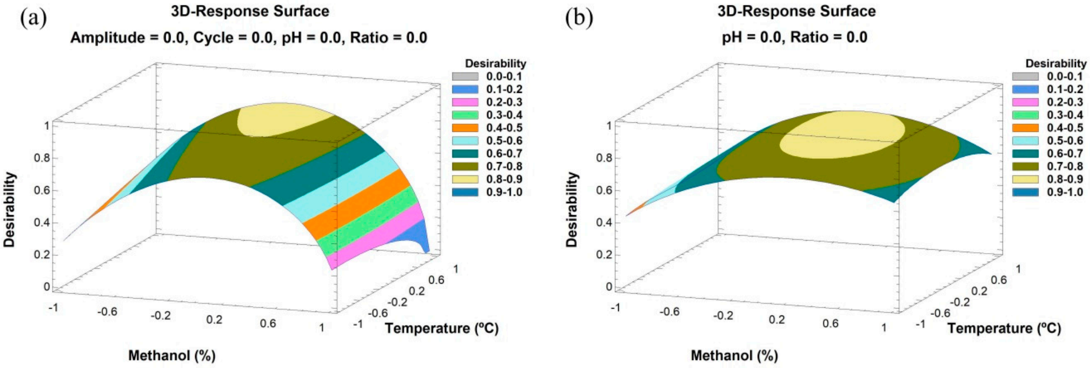

Figure 3. Response surface analysis of the extraction of TA and TPC simultaneously by means of UAE and MAE with respect to methanol percentage and temperature: (**a**) response surface plot for the desirability function when UAE was used, and (**b**) response surface plot for the desirability function when MAE was used.

Multi-response optimization was also performed for MAE and in this case, a maximum desirability was obtained using an extraction solvent at 51% MeOH in water at pH 2, at 100 °C, and 0.3:15 g mL^{-1} as the optimum sample:solvent ratio (Table 9). Figure 3b represents the contour plot of the D values as a function of both the methanol percentage and the temperature, while the rest of the variables remain constant. It can be observed that the highest D values were obtained when the percentage of methanol was around the mid values within the interval evaluated, as a compromise between the optimum conditions for TA and for TPC. Regarding temperature, a high value was necessary for the simultaneous extraction of both antioxidant groups.

After completing this optimization process and its corresponding multi-response value, a confirmation study was carried out based on the optimized parameters. The simultaneous extraction of TA and TPC when optimized extraction parameters were applied, were very similar to those obtained when separate extraction had been performed. Simultaneous and separate values were: UAE TA (µg g^{-1}) (1687.77 ± 50.08 vs. 1792.73 ± 68.84) and TPC (µg g^{-1}) (14,809.73 ± 455.78 vs. 15,697.76 ± 603.21) and MAE TA (µg g^{-1}) (2409.11 ± 69.16 vs. 2454.12 ± 65.90) and TPC (µg g^{-1}) (15,677.97 ± 729.73 vs. 16,277.29 ± 688.42).

4. Conclusions

As an alternative to conventional and traditional extraction techniques, the suitability of two advanced and green extraction techniques has been evaluated for the extraction of the antioxidant compounds found in blackberry. To the best of our knowledge, this is the first study to optimize and compare UAE and MAE efficacy for the extraction of TA and TPC from blackberry. Solvent composition was the most influential factor on UAE for both antioxidant compounds, with a negative effect on TA yields and a positive effect on the extraction of TPC. As far as MAE is concerned, solvent composition was also the most significant factor with a negative effect of the TA recovery. With regards to the TPC, both solvent composition and pH had significant effects. Rapid extraction runs of 10 min and 5 min are proposed for UAE and MAE respectively. The developed method showed high precision for both antioxidants (CV < 5%). Additionally, the comparative studies showed non-significant differences between both methods. Although slightly greater recoveries and in a shorter time were obtained when MAE was used, UAE obtained optimum results at lower temperatures. These results suggest the suitability of both as extraction techniques to obtain TA and TPC from such an important functional food as blackberry for our alimentary industry.

An additional study was completed on the simultaneous extraction of TA and TPC by UAE and MAE. A reduction of solvent consumption was observed for TPC, maintaining the extraction efficiency. UAE and MAE proved to be similarly suitable for the extraction of TA and TPC from blackberry samples and at similar optimum conditions. This work showed interesting results for quality control analytical laboratories regarding the development of new and rapid extraction techniques to quantify the content of these antioxidant compounds.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/11/745/s1. Table S1. Coefficients for the second-order polynomial equation from the Box–Behnken design for UAE; Table S2. Coefficients for the second-order polynomial equation from the Box–Behnken design for MAE.

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