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Alternative Extraction Method of Bioactive Compounds from Mulberry (*Morus nigra* L.) Pulp Using Pressurized-Liquid Extraction

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Abstract

Black mulberry *(Morus nigra* L.*)* is one of the most important species of the genus *Morus* as its fruit contains substantial levels of anthocyanins and phenolic compounds which show a potentially positive effect on the human health. Nowadays, PLE is becoming a promising extraction technology. Therefore, the development of fast extraction methods of anthocyanins and phenolic compounds from mulberry pulp using pressurized-liquid extraction (PLE) has been studied in this paper. The operating conditions (solvent, temperature, pressure, purge time, pH, and flushing) were investigated by a Box–Behnken design. Analysis of the model clearly showed that the most influential factors were temperature and solvent composition. The optimum extraction conditions for anthocyanins were 47.2% methanol in water, a temperature of 75.5 °C, pressure of 200 atm, a purge time of 90 s, pH 3.01, and 50.2% for flushing. The best conditions for the extraction of phenolics were 74.6% methanol, 99.4 °C, 100 atm, 90 s purge, pH 7, and 100% flushing. The optimum extraction time was 10 min. The precision values of the methods were also evaluated and excellent results (RSD < 5%) were obtained. The developed methods were successfully applied to several mulberry marmalade samples. The results using PLE were compared to those achieved by UAE methods. Similar extraction yields were obtained for anthocyanins by PLE and UAE under optimized conditions; however, PLE required less methanol consumption. Besides, PLE showed higher extraction efficiency for total phenolic compounds. From the results, it can be concluded that pressurized-liquid extraction can be considered as an efficient alternative and powerful tool for the extraction of bioactive compounds from mulberries.

Keywords Anthocyanins · Box–Behnken design · Mulberry · Phenolic compounds · Pressurized-liquid extraction

Introduction

The mulberry tree belongs to the family *Moraceae* and this is native to Asia, where various parts of the plant have been used in traditional Chinese medicine (well known as medicinal mulberries in the Uygur region) for centuries (Zelova et al. 2014; Jiang and Nie 2015). Mulberry plants are widely cultivated in southern Europe as they grow in areas with mild temperatures and a Mediterranean climate (Elmaci and Altug

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2002). There are three main species of mulberry for fruit production: black (*Morus nigra* L.), red (*Morus rubra* L.), and white (*Morus alba* L.) (Hojjatpanah et al. 2011). *M. nigra* is one of the most important species of the genus *Morus* as its fruit contains substantial levels of anthocyanins and phenolic compounds, which show a potentially positive effect on the human health (Kutlu et al. 2011; Tomas et al. 2015; Sang et al. 2017). The anthocyanins that have been identified in mulberry are cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside as major components, and cyanidin-3-*O*-(6″-malonyl-glucoside) and cyanidin-3-*O*-(6″-dioxalyl-glucoside) as minor components (Machado et al. 2015). Apart from the health benefits, these compounds also contribute to the quality of *M. nigra* in terms of the characteristic color, its juicy fruit, and its slightly acidic flavor (Lee et al. 2002; Özgen et al. 2009). In order to establish criteria to determine the quality, origin and production control of these beneficial compounds, the development of fast and reliable methods for their extraction and analysis are required. Different conventional extraction methods that

are employed to obtain extracts from plant materials can be found in the literature and these include distillation, solvent extraction, and cold compression, among others (Vinatoru 2001; Dranca and Oroian 2016). However, traditional extraction techniques generally require long process times, large amounts of organic solvents, cooling water and electricity, and they also lead to the degradation of specific compounds (Ivanovic et al. 2014; Machado et al. 2015). Compared to the commonly used extraction methods, novel extraction techniques such as ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, and pressurized-liquid extraction greatly reduce the energetic costs and process times (Wang and Weller 2006). Ultrasound-assisted extraction was recently published by the authors for the determination of anthocyanins and total phenolic compounds present in mulberries (Espada-Bellido et al. 2017). It was concluded from the results that UAE can be considered as an easy and economic tool for the extraction of both anthocyanins and total phenolic compounds from mulberry. Nowadays, PLE is becoming a promising extraction technology (Mustafa and Turner 2011; Santos et al. 2012). Pressurized-liquid extraction (PLE) is a technique that involves extraction using the solvent in its liquid state at high temperature and high pressure to provide greater solubility and extraction efficiency as compared to those techniques carried out at room temperature and atmospheric pressure (Mustafa and Turner 2011; Sousa et al. 2016). On the one hand, greater contact between the extracting liquid and the solid matrix is achieved because the elevated pressure keeps the solvent in the liquid state, thus forcing it to enter into the solid matrix. On the other hand, the high temperature increases the solubility of the compounds in the extraction solvent (Wang and Weller 2006; Setyaningsih et al. 2016). In recent studies new pressurized-liquid extraction methods have been proposed for the determination of anthocyanins and phenolic compounds in different matrices such as plant extracts of the genus *Phyllanthus amarus* (Sousa et al. 2016), *Schinus terebinthifolius* fruits (Feuereisen et al. 2017), the fruit of a tropical palm tree (juçara residues) (Garcia-Mendoza et al. 2017), and rice (Setyaningsih et al. 2016). All of these studies have highlighted the quicker and more efficient extractions obtained on using PLE. However, pressurized-liquid extraction methods for the determination of anthocyanins and total phenolic compounds in mulberries have not been optimized yet. A multivariate optimization using a Box–Behnken design (BBD) in a response surface methodology was applied. The extraction methods for total anthocyanins and total phenolic compounds were optimized by carrying out an experimental design of six independent variables. The concentration of anthocyanins and the concentration of total phenolic compounds were chosen as response variables. A BBD is more economical and efficient in comparison with its corresponding 3^k design, mainly because fewer runs are required (Santos et al.

2014). This design consists of a spherical response surface methodology in which the experimental points are located on the edges of a hypersphere and equidistant from the central point (Bezerra et al. 2008; Ding et al. 2016). This design has been selected for the optimization of extraction conditions in several recent studies (Ahmad et al. 2015; Katsampa et al. 2015; Li et al. 2016). Therefore, the aim of the work reported here was the development of two alternative PLE methods for the extraction of total phenolics and total anthocyanins from mulberries. Extraction techniques have not been extensively studied for mulberries. To the best of our knowledge, this is the first time that PLE technique has been used for this purpose. A comparison between the performance of PLE and UAE using the same samples was also evaluated to study the impact of high pressure treatments in comparison with ultrasound-assisted extraction.

Materials and Methods

Chemicals and Reagents

Methanol (Fisher Scientific, Loughborough, UK) and formic acid (Sharlau, Barcelona, Spain) were HPLC grade. Ultrapure water was supplied by a Milli-Q water purification system from Millipore (Bedford, MA, USA). Hydrochloric acid (Panreac, Barcelona, Spain) and sodium hydroxide (Panreac, Barcelona, Spain) for the adjustment of pH were of analytical grade. Anhydrous sodium carbonate (Panreac, Barcelona, Spain) and Folin–Ciocalteu reagent (Merck Millipore, Darmstadt, Germany) were used for the determination of total phenolic compounds by the Folin–Ciocalteu method. The phenolic compound standard (gallic acid, 97%) and the anthocyanin standard (cyanidin chloride, ≥ 95%) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Mulberry Samples

Mulberry fruits were harvested in their full ripeness stage from local mulberry trees in the Puerto Real region (Spain) in 2014. The mulberries were triturated in a Thermomix (Vorwerk, Spain) until a homogeneous sample was obtained for analysis. The triturated sample was stored in a freezer at − 20 °C prior to analysis. The final extraction method was applied to seven mulberry marmalades purchased from different commercial markets in Spain. The real samples were stored at 4 °C prior to analysis.

Pressurized-Liquid Extraction Procedure

PLE was performed using an ASE 200 extractor ("Acelerated Solvent Extractor," Dionex, Sunnyvale, CA, USA) equipped with stainless steel extraction cells (11 mL volume) and

collection vials (60 mL capacity). A cellulose filter (Dionex) was inserted at the bottom of the extraction cell. Washed sea sand (Panreac, Barcelona, Spain) was added until the bottom of the cell was covered (approximately 1 cm). Triturated mulberry (1.5 g approximately) was weighed, placed in the extraction cell and mixed homogeneously with sea sand. Finally, the cell was filled with sea sand. Based on the experimental design, the extraction cell was then filled with the appropriate extraction solvent, pressurized, and heated under controlled PLE conditions. Prior to each extraction, a fixed period of 5 min was applied to the extraction cell to allow the temperature to equilibrate. A period of 10 min was employed to carry out all the extractions of the experimental design. The extracts were filtered through a 0.20-µm nylon syringe filter (Membrane Solution, Dallas, USA). All extracts were then made up to a defined volume of 25 mL with the same solvent and stored at − 20 °C prior to chromatographic analysis.

Folin–Ciocalteu Assay

The total phenolic content was determined using a modified Folin–Ciocalteu (FC) spectrophotometric method (Singleton and Rossi 1965; Singleton et al. 1999). This Folin–Ciocalteu method has been employed previously for the measurement of total phenolic compounds by many researchers (Patras et al. 2009; Santos et al. 2012; Machado et al. 2015; Sousa et al. 2016; Garcia-Mendoza et al. 2017). In this work, a series of tubes were prepared with 0.25 mL of extract or standard solution, 12.5 mL of water, 1.25 mL of Folin–Ciocalteu reagent and 5 mL of 20% aqueous sodium carbonate solution. After 30 min, the absorbance of each solution was measured at 750 nm (Waterhouse 2003) using a V-530 UV-vis spectrophotometer (Jasco, Madrid, Spain). The extracts were filtered through a 0.45-µm nylon filter (Membrane Solutions, Dallas, USA) prior to spectrophotometric analysis. The total phenolic content was calculated from a calibration curve with gallic acid as the standard (Patras et al. 2009; Garcia-Mendoza et al. 2017). The concentrations of gallic acid were prepared between 1 and 1000 mg L^{-1} and their absorbance values were measured. The regression equation ($y = 0.0012x + 0.0004$) and the correlation coefficient (0.9999) were calculated using the Microsoft Office Excel 2007 program (Table 1). Total phenolic compounds are expressed as micrograms of gallic acid equivalent per gram of fresh weight.

UHPLC/QToF-MS Analysis

Ultra-high performance liquid chromatography (UHPLC) coupled to quadrupole-time-of-flight mass spectrometry (QToF-MS) (Synapt G2, Waters Corp., Milford, MA, USA) was used to identify the anthocyanins. Separations were performed on a reverse-phase C18 analytical column (Acquity UPLC BEH C18, Waters) of 2.1 mm × 100 mm and 1.7 µm particle size. The mobile phase was a binary solvent system consisting of phase A (2% formic acid in water) and phase B (methanol) at a flow rate of 0.4 mL min^{-1}. The total programed gradient (12 min with 4 min for re-equilibration) was as follows (time, % solvent B): 0 min, 15%; 3.30 min, 20%; 3.86 min, 30%; 5.05 min, 40%; 5.35 min, 55%; 5.64 min, 60%; 5.95 min, 95%; and 7.50 min, 95%. An electrospray source was employed for the determination of the analytes. The electrospray operated in positive ionization mode under the following conditions: 700 L h^{-1} as desolvation gas flow, 500 °C as desolvation temperature, 10 L h^{-1} cone gas flow, 150 °C for source temperature, 700 V capillary voltage, 30 V cone voltage, and 20 eV collision energy. Full-scan mode was used ($m/z = 100–1200$). All PLE extracts were filtered through a 0.20-µm nylon syringe filter (Membrane Solutions, Dallas, USA) prior to chromatographic analysis. The injection volume was set to 3 µL. Under the above conditions, four anthocyanins were identified in the samples: cyanidin-3-O-glucoside ($m/z = 449$), cyanidin-3-O-rutinoside ($m/z = 595$), cyanidin-3-O-(6″-malonyl-glucoside) ($m/z = 535$) and cyanidin-3-O-(6″-dioxalyl-glucoside) ($m/z = 593$).

UHPLC Analysis

Separation and quantification of anthocyanins in PLE extracts were carried out on an Elite UHPLC LaChrom system (VWR Hitachi, Tokyo, Japan) with an L-2420 U UV-vis detector. The equipment consisted of an L-2200 U autosampler, an L2300 column oven, and two L-2160 U pumps. The column oven was set at 50 °C for the chromatographic analysis. Separations were performed on a Halo™ C18 Hitachi LaChrom column (100 × 3 mm I.D., particle size 2.7 µm). The mobile phase was a binary solvent consisting of phase A (5% formic acid in water) and phase B (methanol), working at a flow rate of 1.0 mL min^{-1}. The 10 min programed gradient was as follows (time, % solvent B): 0 min, 15%; 1.50 min, 20%; 3.30 min, 30%; 4.80 min, 40%; 5.50 min, 55%; 5.90 min, 60%; 6.60 min, 95%; 9.30 min, 95%; and 10 min, 100%. The UV-vis detector was set at 520 nm for the analysis. The extracts were filtered through a 0.20-µm nylon syringe filter (Membrane Solutions, Dallas, USA) prior to chromatographic analysis. The injection volume was set to 15 µL. All analyses were carried out in duplicate. Cyanidin chloride was used as the reference standard because it is the anthocyanidin standard that is commercially available for cyanidin anthocyanins. The standards of known concentration were prepared between 0.054 and 27 mg L^{-1}. The regression equation ($y = 300,568.88x − 28,462.43$) and the correlation coefficient (0.9999) were calculated using the Microsoft Office Excel 2007 program (Table 1). The four anthocyanins present in mulberry residues were quantified using the calibration curve for cyanidin chloride. Assuming that the four anthocyanins have similar absorbance and taking into account their

Table 1 Analytical characteristics for the determination of total phenolic compounds and anthocyanins

	Compound	Concentration range (mg L^{-1})	Linear equation	R^2	LOD (mg L^{-1})	LOQ (mg L^{-1})
Phenolic compounds	Gallic acid standard	1 − 1000	$y = 0.0012x + 0.0004$	0.9999	2.77	9.23
Anthocyanins	Cyanidin chloride standard	0.054–27	$y = 300{,}568.88x − 28{,}462.43$	0.9999	0.198	0.662
	Cyanidin-3-*O*-glucoside (C3G)		$y = 181{,}419.52x − 28{,}462.43$	0.9999	0.291	0.970
	Cyanidin-3-*O*-rutinoside (C3R)		$y = 136{,}897.98x − 28{,}462.43$	0.9999	0.386	1.285
	Cyanidin-3-*O*-(6-malonyl-glucoside) (C3MG)		$y = 152{,}385.61x − 28{,}462.43$	0.9999	0.347	1.155
	Cyanidin-3-*O*-(6″-dioxalyl-glucoside) (C3DG)		$y = 137{,}481.12x − 28{,}462.43$	0.9999	0.385	1.281

molecular weights, a calibration curve was calculated for each anthocyanin present in the mulberry, which allows the quantification of each of them. The results are expressed as micrograms of anthocyanins per g of fresh weight.

Performance of the Method

The chromatographic method used to determine anthocyanins and the colorimetric method used to determine total phenolic compounds were performed according to ISO 17025 and ICH guideline Q2 (R1) (ISO 2005; ICH 2006). Linearity range, precision, and detection and quantification limits of the method were evaluated. Linearity was estimated to express the ability of the method to obtain test results that are directly proportional to the concentration of anthocyanins and total phenolic compounds within the studied range. Appropriate dilution from a stock solution of gallic acid and cyanidin chloride were carried out to give concentrations ranging from 1 to 1000 mg L^{-1} and from 0.054 to 27 mg L^{-1}, respectively. The linearity of the calibration curves was evaluated by the coefficient of determination (R^2) after linear regression analysis. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the standard deviation obtained for the response (σ) and the slope (m) from the regression. The analytical properties of the colorimetric and chromatographic methods for the determination of total phenolic compounds and anthocyanins, respectively, are listed in Table 1.

Second-Order Experimental Design and Optimization

The effects of six independent variables (solvent composition, temperature, pressure, purge time, solvent pH, and flushing) on the dependent variables (extraction of anthocyanins and phenolics) were evaluated through a multivariate optimization using a response surface methodology (RSM). RSM is a collection of mathematical models based on the fit of the experimental data to a polynomial equation (Bezerra et al. 2008). RSM was found to be a useful tool for the identification of significant extraction

parameters as well as their interactions. Thus, a three-level, six-factor Box–Behnken design (BBD) was carried out for both types of compound. For statistical calculations, each independent variable or factor was coded at three different levels: − 1 (low), 0 (medium), and + 1 (high). The coded and uncoded levels of the six selected factors are provided in Table 2. The experimental results for total anthocyanins (Y_{TA}, µg g^{-1}) and total phenolic compounds (Y_{TP}, µg g^{-1}) were considered as the response variables. A total of 54 experiments were required, including six replicates at the center point.

The predicted response value Y for total anthocyanins and total phenolic compounds in each trial can be fitted to a second-degree polynomial equation, as follows:

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X^2_{ii} + \sum_{i=1}^{k-1} \sum_{i=1}^{k} b_{ij} X_i X_j$$

where Y is the aforementioned response (Y_{TA} and Y_{TP}) for the extraction of anthocyanins and total phenolic compounds, respectively; b_0 is the constant coefficient; X_i and X_j are the independent variables that affect the response Y; b_i ($i = 1,2,…, 6$) is the linear estimated coefficient; b_{ii} ($i = 1, 2, …, 6$) is the quadratic estimated coefficient, and b_{ij} ($i = 1, 2, …, 6; j = 1, 2, …, 6$) is the estimated cross-product coefficient. The design can predict how the independent variables will influence the extraction of anthocyanins and phenolics from mulberry samples.

Table 2 Selected independent variables and their coded and uncoded levels used for the BBD

Variable, unit	Factors	Level		
	X	Low (− 1)	Medium (0)	High (+ 1)
Methanol in water (%)	X_1	25	50	75
Temperature (°C)	X_2	50	75	100
Pressure (atm)	X_3	100	150	200
Purge (s)	X_4	30	60	90
pH	X_5	3	5	7
Flushing (%)	X_6	50	75	100

Statistical Analysis

The results were statistically assessed by analysis of variance (ANOVA) to evaluate the quality of the model fitted to the experimental response. The software Statgraphics Centurion XVII (Statpoint Technologies, Inc., USA) was used to analyze the experimental design, to estimate the effects of the variables on the final response, the second-order mathematical model, the optimum levels of the significant variables, and the surface graphs.

Results and Discussion

Factors in the PLE Method

For the Box–Behnken design employed to study the optimal PLE conditions for anthocyanins and total phenolic compounds in mulberry, solvent composition, temperature, pressure, purge time, solvent pH, and flushing were optimized. As mentioned above, the six independent variables and their corresponding levels are shown in Table 2. The temperature was set to a maximum of 100 °C for the anthocyanins and phenolics extractions because the use of higher temperatures may lead to degradation of individual anthocyanins (Ju and Howard 2003; Arapitsas and Turner 2008). A total of 54 experimental runs, including six replicates at the center point, were carried out. All trials were performed in duplicate and in random order. The whole experimental design matrix employed to optimize PLE with the independent factors and their respective experimental data (Y_{TA} and Y_{TP}) is shown in Table 3. Once the experiments had been carried out, coefficients for the full quadratic polynomial equation for both compounds were established to predict the responses. Thus, two suitable second-order mathematical models were obtained to describe the response values of Y_{TA} and Y_{TP} as a function of the independent variables (Eqs. 1 and 2, respectively):

$$Y_{TA}\ (\mu g\ g^{-1}) = 98.91 - 9.71\ X_1 + 14.02\ X_2 + 8.36\ X_3 \qquad (1)$$
$$+ 2.00\ X_4 - 9.89\ X_5$$
$$+ 6.68\ X_6 - 52.88\ X_1{}^2 - 1.04\ X_1 X_2$$
$$+ 1.36\ X_1 X_3 - 0.74\ X_1 X_4 + 3.06\ X_1 X_5$$
$$+ 3.46\ X_1 X_6 - 16.49\ X_2{}^2 - 2.12\ X_2 X_3 - 0.15\ X_2 X_4$$
$$+ 4.43\ X_2 X_5 + 6.82\ X_2 X_6$$
$$+ 4.72\ X_3{}^2 - 0.13\ X_3 X_4$$
$$+ 3.80\ X_3 X_5 - 3.08\ X_3 X_6$$
$$+ 10.93\ X_4{}^2 - 4.81\ X_4 X_5 + 2.55\ X_4 X_6$$
$$+ 19.08\ X_5{}^2 + 10.05\ X_5 X_6 + 5.12\ X_6{}^2$$

$$Y_{TP}\ (\mu g\ g^{-1}) = 1419.34 + 121.37\ X_1 + 215.07\ X_2$$
$$+ 36.11\ X_3 - 6.29\ X_4 + 36.00\ X_5$$
$$+ 24.89\ X_6 - 42.85\ X_1{}^2 + 50.16\ X_1 X_2$$
$$+ 24.66\ X_1 X_3 - 2.50\ X_1 X_4$$
$$+ 62.67\ X_1 X_5 + 13.15\ X_1 X_6$$
$$+ 30.71\ X_2{}^2 - 86.76\ X_2 X_3 - 22.59\ X_2 X_4$$
$$+ 69.13\ X_2 X_5 - 17.82\ X_2 X_6$$
$$+ 33.10\ X_3{}^2 - 43.63\ X_3 X_4 - 67.53\ X_3 X_5$$
$$+ 7.66\ X_3 X_6 + 32.50\ X_4{}^2$$
$$+ 30.31\ X_4 X_5 + 3.91\ X_4 X_6 - 31.13\ X_5{}^2$$
$$+ 75.73\ X_5 X_6 - 24.42\ X_6{}^2 \qquad (2)$$

The estimated values obtained with the model were very similar to those observed in all experiments. Anthocyanins and phenolic compounds Pareto charts were used to obtain the statistically significant factors (Fig. 1). The effects were display in decreasing order of significance. The length of each bar of the Pareto chart is proportional to the value of a t-statistic calculated by dividing the corresponding estimated effect by its standard error. The vertical line that crosses each chart shows the factors or combination of factors with a significant effect. Thus, any bar beyond the vertical line can be considered statistically significant at a 95% confidence level. In the case of anthocyanins (Fig. 1a), the p values for methanol (X_1), temperature (X_2), pressure (X_3), pH (X_5), and flushing (X_6) were less than 0.05, which indicates that these factors had a significant linear influence on the response. Temperature, pressure, and flushing had positive effects ($b_2 = 14.02$, $b_3 = 8.36$, and $b_6 = 6.68$), respectively, and this indicates that an increase in these factors favored the recovery of anthocyanins in the extract. By contrast, methanol ($b_1 = -9.71$) and pH ($b_5 = -9.89$) had a negative effect, which indicates that the extraction of anthocyanins was more favorable with low percentages of methanol and low pH values. Moreover, methanol, temperature, purge, and pH had a significant quadratic influence on the response variable, with p values of less than 0.05. In fact, the percentage of methanol had the most significant effect on the extraction of anthocyanins from mulberries. Methanol and temperature presented negative coefficients ($b_{11} = -52.88$ and $b_{22} = -16.49$, respectively) whereas purge and pH had positive coefficients ($b_{44} = 10.93$ and $b_{55} = 19.08$, respectively). In addition to the linear and quadratic influences, a slight interaction between pH and flushing (p value < 0.1) was observed ($b_{56} = 10.05$). As far as total phenolic compounds are concerned (Fig. 1b), X_1 and X_2 had significant positive linear influences on the response ($b_1 = 121.37$ and $b_2 = 215.07$), with p values lower than 0.05. Non-significant quadratic effects were obtained (p value > 0.1). However, a slight interaction between

Table 3 Box–Behnken design matrix with coded independent variables and experimental results

| Run	X_1	X_2	X_3	X_4	X_5	X_6	Y_{TA} (µg g⁻¹)	Y_{TP} (µg g⁻¹)
1	0	0	−1	0	−1	−1	109.44	1156.75
2	0	0	+1	0	−1	−1	134.38	1452.49
3	0	0	−1	0	+1	−1	69.17	1300.00
4	0	0	+1	0	+1	−1	138.12	1418.28
5	0	0	−1	0	−1	+1	120.39	1133.94
6	0	0	+1	0	−1	+1	143.42	1432.44
7	0	0	−1	0	+1	+1	149.12	1672.78
8	0	0	+1	0	+1	+1	158.58	1608.49
9	0	−1	0	−1	−1	0	122.80	1256.70
10	0	+1	0	−1	−1	0	143.01	1788.14
11	0	−1	0	+1	−1	0	142.74	1337.30
12	0	+1	0	+1	−1	0	143.04	1552.21
13	0	−1	0	−1	+1	0	76.31	1175.98
14	0	+1	0	−1	+1	0	106.86	1619.01
15	0	−1	0	+1	+1	0	57.71	1151.65
16	0	+1	0	+1	+1	0	106.98	1730.50
17	−1	0	−1	−1	0	0	67.47	1213.44
18	+1	0	−1	−1	0	0	49.12	1591.07
19	−1	0	+1	−1	0	0	66.48	1182.27
20	+1	0	+1	−1	0	0	57.70	1711.29
21	−1	0	−1	+1	0	0	73.45	1355.53
22	+1	0	−1	+1	0	0	49.60	1692.75
23	−1	0	+1	+1	0	0	76.07	1203.65
24	+1	0	+1	+1	0	0	53.54	1586.77
25	0	−1	−1	0	0	−1	76.63	1096.38
26	0	+1	−1	0	0	−1	99.20	1765.33
27	0	−1	+1	0	0	−1	94.17	1397.16
28	0	+1	+1	0	0	−1	105.63	1506.92
29	0	−1	−1	0	0	+1	57.97	1197.07
30	0	+1	−1	0	0	+1	105.20	1582.58
31	0	−1	+1	0	0	+1	78.94	1436.92
32	0	+1	+1	0	0	+1	120.30	1687.53
33	−1	−1	0	0	−1	0	56.68	1414.67
34	+1	−1	0	0	−1	0	25.30	976.16
35	−1	+1	0	0	−1	0	82.80	1468.78
36	+1	+1	0	0	−1	0	50.20	1496.08
37	−1	−1	0	0	+1	0	35.96	1023.41
38	+1	−1	0	0	+1	0	19.78	1100.74
39	−1	+1	0	0	+1	0	70.82	1757.99
40	+1	+1	0	0	+1	0	47.49	1770.79
41	−1	0	0	−1	0	−1	63.93	1306.68
42	+1	0	0	−1	0	−1	43.39	1541.43
43	−1	0	0	+1	0	−1	67.87	1102.25
44	+1	0	0	+1	0	−1	46.53	1618.44
45	−1	0	0	−1	0	+1	67.44	1153.16
46	+1	0	0	−1	0	+1	56.27	1648.79
47	−1	0	0	+1	0	+1	77.13	1172.66
48	+1	0	0	+1	0	+1	74.08	1533.18
49	0	0	0	0	0	0	98.87	1456.20
50	0	0	0	0	0	0	103.48	1380.68
51	0	0	0	0	0	0	95.44	1400.60
52	0	0	0	0	0	0	94.40	1414.32
53	0	0	0	0	0	0	101.40	1442.16
54	0	0	0	0	0	0	99.89	1422.10

temperature and pH (p value < 0.1) was also observed with a positive effect ($b_{25} = 69.13$). Analysis of the model clearly shows that the most influential factors were temperature and solvent composition, both of which had a marked effect on the extraction. Similar results were obtained in our previous studies (Espada-Bellido et al. 2017) and other authors also obtained similar results with other matrices (Santos et al. 2012; Truong et al. 2012; Cai et al. 2016). The use of a higher extraction temperature leads to a more efficient extraction because the target compounds have higher solubility in the solvent and the diffusion and mass transfer of the extracted molecules are also enhanced (Galvan d'Alessandro et al. 2012). The behavior observed can be attributed to the decreases in solvent viscosity, water polarity, and surface tension (Mustafa and Turner 2011; Azmir et al. 2013; Sousa et al. 2016). However, it is important to highlight that temperatures above 100 °C can produce degradation of individual anthocyanins and, as a consequence, this variable was not studied over a wider range (Kechinski et al. 2010; Fischer et al. 2013).

Evaluation of the Fitted Model

The suitability of the model was validated by analysis of variance (ANOVA). The regression coefficients (R^2) obtained from the analysis of variance (ANOVA) were 89.7 and 75.0% for anthocyanins and total phenolic compounds, respectively. The measured concentrations were compared with the predicted values obtained with the polynomial models and a statistically significant agreement was obtained between them.

Nevertheless, insignificant dependent terms ($p > 0.1$) were omitted from the models. An acceptable correlation between experimental data and estimated values was obtained. Thus, the models provided a satisfactory fit to the data with regard to total anthocyanins and phenolics extraction. Both reduced mathematical models that can reliably predict the experimental results describing the effect of the independent variables on the extraction of total anthocyanins and total phenolic compounds were expressed by Eqs. 3 and 4, respectively:

Fig. 1 Pareto charts for the standardized effects: **a** total anthocyanins and **b** total phenolic compounds

(a)

Anthocyanins Pareto Chart

(b)

Phenolic compounds Pareto Chart
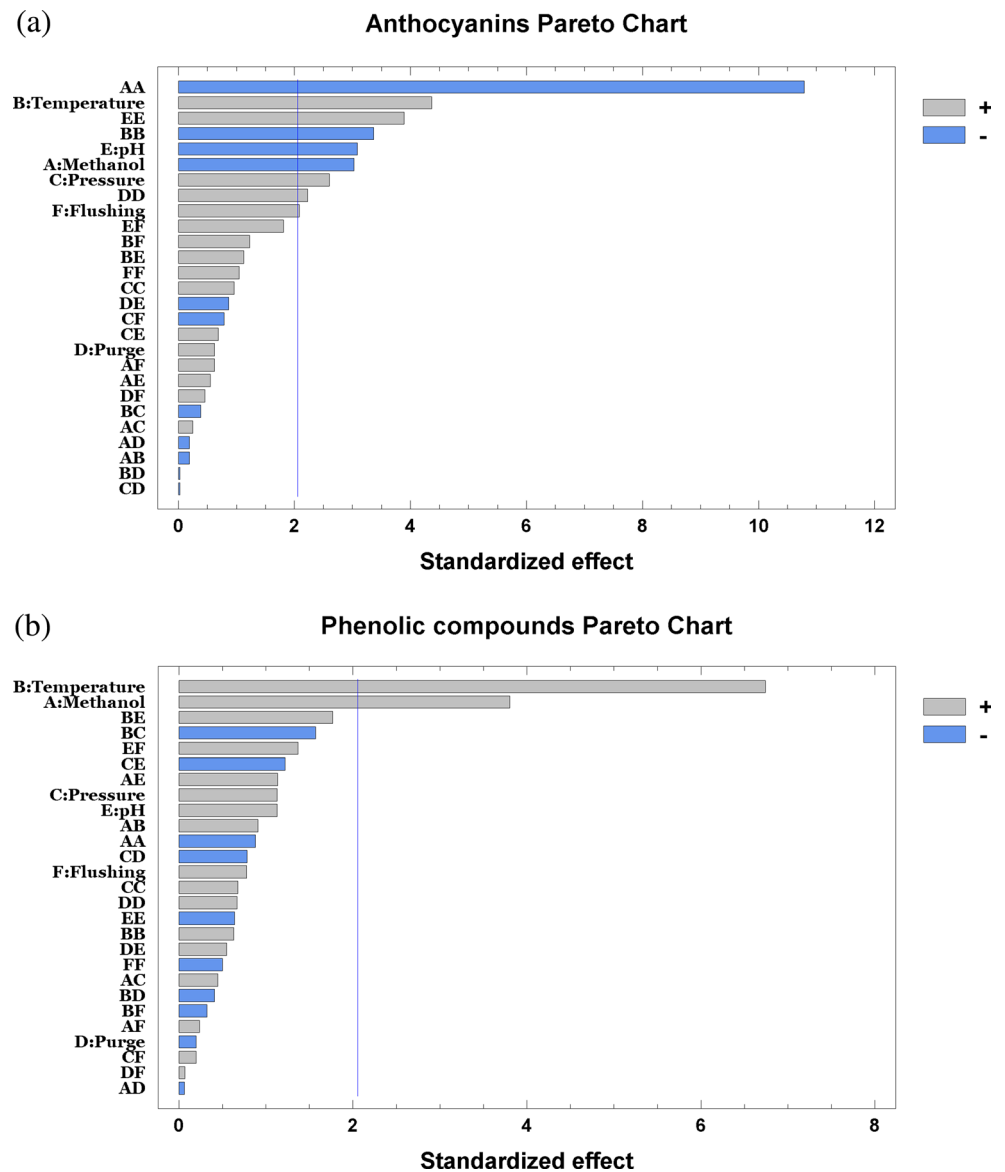
$$Y_{TA} \left(\mu g\ g^{-1} \right) = 98.91 - 9.71\ X_1 + 14.02\ X_2$$
$$+ 8.36\ X_3 - 9.89\ X_5$$
$$+ 6.68\ X_6 - 52.88\ X_1{}^2 - 16.49\ X_2{}^2$$
$$+ 10.93\ X_4{}^2 + 19.08\ X_5{}^2$$
$$+ 10.05\ X_5 X_6 \tag{3}$$

$$Y_{TP} \left(\mu g\ g^{-1} \right) = 1419.34 + 121.37\ X_1 + 215.07\ X_2$$
$$+ 69.13\ X_2 X_5 \tag{4}$$

3D-Response Surface Plots

Three-dimensional (3D) response surface plots were obtained with the software using the fitted model in order to improve our understanding of both the main and interaction effects of the most influential parameters (Liu and Chiou 2005; Abul Kalam et al. 2016; Ding et al. 2016). The influences of two factors on a particular response, with the rest of the variables kept constant, are represented graphically in the 3D plots shown in Fig. 2. Temperature and percentage of methanol in the extraction solvent were selected as the most significant factors. On the one hand, the combined effect of methanol and temperature on the

total anthocyanins recovery is represented in Fig. 2a. The response increased with both factors to a point close to the average value of each experimental range studied and thereafter the extraction efficiency decreased. On the other hand, the interactive effect of temperature and methanol on the phenolics extraction is represented in Fig. 2b. An increase in both temperature and percentage of methanol led to an increase in the extraction of phenolics. It can be seen that temperature did not have a maximum value in the middle of the range as for anthocyanins, but an increasing linear trend was observed and the optimum temperature was at the very end of the range evaluated.

Optimal Conditions

Optimization of the variables was assessed using the quadratic mathematical model within the experimental range studied. The best conditions identified for the extraction of

anthocyanins were as follows: 47.2% methanol in water, a temperature of 75.5 °C, a pressure of 200 atm, 90 s purge, a pH of 3.01, and 50.2% flushing. In this case, the pressure was set at its maximum value in order to favor the release of anthocyanins into the extraction solvent. In an effort to find a compromise between the extraction and loss of anthocyanins, an optimum temperature of 75.5 °C was selected. An acidic medium was found to be optimum for the extraction of anthocyanins because these compounds can be degraded rapidly in neutral and alkaline conditions to give their constituent phenolic acids (Seeram et al. 2001; Woodward et al. 2009). The explanation that higher anthocyanin recoveries can be achieved at pH from 1.0 to 3.0 can be found in literature due to the more stability of anthocyanins (Barnes et al. 2009; Cai et al. 2016).

Regarding phenolics extraction, the best conditions were 74.6% methanol, a temperature of 99.4 °C, a

Fig. 2 Three-dimensional response surface plots for the graphical representation of the influence of temperature and solvent composition on **a** total anthocyanins (TA) and **b** total phenolic compounds (TP) extraction
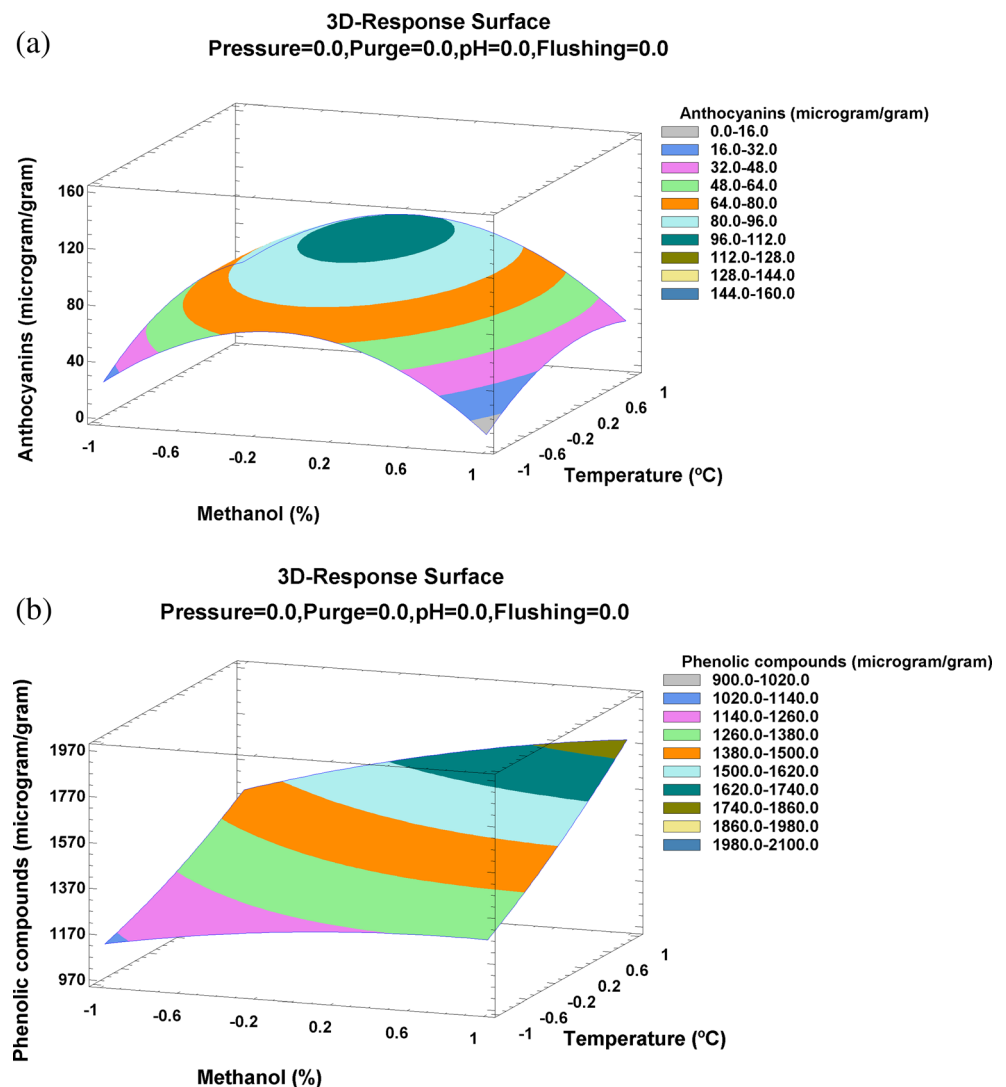
pressure of 100 atm, 90 s purge, a pH value of 7, and 100% flushing. For these compounds, a high temperature value and a neutral pH ensures the maximum extraction of phenolic compounds in methanol. A possible explanation could be that, although anthocyanins are phenolic compounds, they are present at low levels (< 10%) in the overall mixture. Moreover, the products obtained after anthocyanins had degraded at neutral pH and high temperatures could be other phenolic compounds and these would form part of the total phenolic composition (Carrera et al. 2012). The conditions described above were therefore used for further experiments.

Kinetics of the Extraction Process

Among the different variables that can affect an extraction process, extraction time has been identified as having a significant effect on the extraction of anthocyanins and phenolic compounds from different matrices (Carrera et al. 2012; Setyaningsih et al. 2015; He et al. 2016). In order to evaluate the kinetics in this study, several extractions were carried out under the optimum PLE conditions. Extraction times of 2, 5, 10, 15, 20, and 25 min were used for the extraction of mulberry samples and these tests were carried out in duplicate. The results for the recovery of anthocyanins and total phenolic compounds for different extraction times are shown in Fig. 3. A similar trend can be observed for both types of compounds. An increase in the extraction of compounds with time was observed until a maximum was reached at 10 min. Degradation clearly occurred after 10 min of extraction and this was more marked for the anthocyanins content. The high temperatures could be the cause of the degradation of anthocyanins and phenolics (Kechinski et al. 2010; Fischer et al. 2013). Therefore, a period of 10 min was

selected as the optimum extraction time for the PLE process for both anthocyanins and phenolics.

Intra-Day Repeatability and Inter-Day Repeatability

Intra-day and inter-day repeatability were considered to evaluate the precision of the PLE method. Intra-day repeatability was determined by carrying out 12 extractions under the same conditions within 1 day, and inter-day repeatability was assessed by carrying out nine more extractions, each on two consecutive days. A total of 30 extractions were performed under the optimum extraction conditions on three consecutive days. Results were expressed by the coefficient of variation (CV) of means, with a range of ± 10% considered as the acceptable limit by the AOAC (AOAC 1998). Intra-day repeatability was found to be 3.87% for anthocyanins and 4.03% for phenolic compounds. Inter-day repeatability was found to be 4.20% for anthocyanins and 4.38% for phenolic compounds. Both results are consistent with an excellent precision of less than 5.0%.

Application to Real Samples

The suitability of the new PLE methods was evaluated by the extraction of seven mulberry marmalades purchased from different commercial markets in Spain. The samples were extracted under the optimum conditions developed for the PLE methods. The quantification of the samples was accomplished by UHPLC and the Folin–Ciocalteu spectrophotometric method for the determination of the four anthocyanins and the total phenolic compounds, respectively. The analyses on real samples were performed in triplicate. The amounts of anthocyanins and total phenolic compounds extracted by PLE from the mulberry marmalades and also from the mulberry fruit are shown in Table 4. It can be observed that the

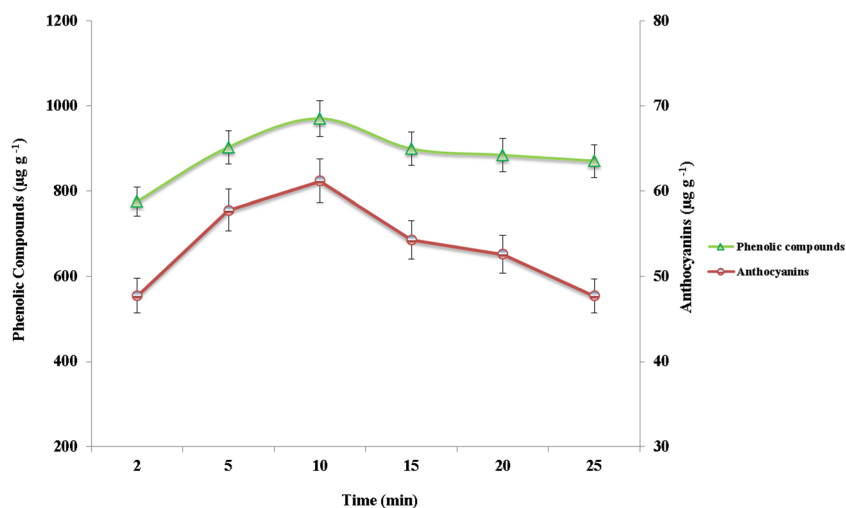Fig. 3 Recovery of anthocyanins and total phenolic compounds using different extraction times

Table 4 Concentrations of total anthocyanins and total phenolic compounds ($n = 3$) in different types of mulberry marmalades and mulberry pulp through PLE and UAE

Marmalade Sample	Total anthocyanins ($\mu g\ g^{-1}$)		Total phenolic compounds ($\mu g\ g^{-1}$)	
	PLE	UAE*	PLE	UAE*
M-1	20.3[a]	18.84[b]	245.30[a]	234.60[a]
M-2	ND	ND	448.63[a]	419.83[b]
M-3	0.62[a]	0.60[a]	493.06[a]	467.06[b]
M-4	0.81[a]	0.78[a]	400.11[a]	348.51[b]
M-5	2.41[a]	2.32[a]	316.13[a]	306.13[a]
M-6	5.98[a]	5.59[b]	210.07[a]	190.67[b]
M-7	27.7[a]	26.10[a]	326.46[a]	315.64[a]
Mulberry	164.53[a]	160.42[a]	2186.09[a]	1916.37[b]

Different letters in the same line for each kind of compounds indicate that the means differ significantly by Tukey's test ($p < 0.05$)

ND, not detected as it was below the detection limit

*These values correspond to the total anthocyanins and total phenolic compounds extracted in the same samples under the optimum conditions developed for the UAE methods in our previous work (Espada-Bellido et al. 2017)

anthocyanin contents extracted from the marmalades were in the range 0.62–27.7 $\mu g\ g^{-1}$ whereas the anthocyanins found in the mulberry fruit reached a value of 164.5 $\mu g\ g^{-1}$. Regarding the phenolics content extracted from the marmalades, the range was between 210.07–493.06 $\mu g\ g^{-1}$ whereas phenolics found in the mulberry fruit reached a value of 2186.09 $\mu g\ g^{-1}$. These results suggest that both anthocyanins and phenolic compounds in mulberry marmalades are present at a considerably lower level than those in the whole mulberry fruit. The low concentration of anthocyanins found in the marmalade samples could be due to the thermal process and/or to the storage conditions, both of which could cause significant anthocyanin degradation (Lee et al. 2002). It is known that thermally treated foods (juices, marmalades, etc.) are frequently subjected to long-term storage prior to consumption and this can lead to changes in the physical and nutritional properties (Sui et al. 2016). The influence of long-term storage on the subsequent loss of anthocyanins has recently been highlighted for different fruits and jams (Amaro et al. 2013; Fracassetti et al. 2013; Reque et al. 2014). Besides, matrix effect could have a huge importance in the extraction, since marmalades have high quantities of sugars and other ingredients that could greatly affect the extraction. Marmalades rarely have a percentage greater than 40% of fruit content, with the rest of the sample consisting of sugars and other additives. To complete the study, the results obtained using PLE were compared to those achieved by UAE methods analyzing the same samples under the optimum conditions achieved in a previous work (Espada-Bellido et al. 2017). In general terms non-significant differences were obtained between UAE and PLE for

anthocyanins extraction efficiency. Only two samples showed small differences extracting more anthocyanins by PLE. However, most of the analyzed samples showed significant differences as far as total phenolic compounds are concerned. Although three of the analyzed samples did not show statistical differences, a larger extraction of phenolic compounds was observed in all of them. Therefore, an improvement in the extraction of total phenolic compounds was observed if PLE was employed. Several authors have already shown the most extraction efficiency for the recovery of total phenolics in different vegetal matrices through PLE (Casazza et al. 2010; Cai et al. 2016; Machado et al. 2017). This fact can be explained due to the high pressures and temperatures employed which can enhance solubility and extraction efficiency of these compounds (Barba et al. 2016; Machado et al. 2017). However, anthocyanins are extremely sensible and their degradation can be enhanced by the combination of high pressure and temperature affecting their recovery in a negative way (Corrales et al. 2008; Verbeyst et al. 2010, 2011; Machado et al. 2017). In our previous studies, milder conditions were employed for the extraction of anthocyanins by UAE compared to PLE (48 vs 75.5 °C). Thus, the increased of solubility could be canceled out by the thermal degradation of the anthocyanins being the possible reason for the non-significant differences between both UAE and PLE extraction methods. It is noteworthy to mention that PLE optimum conditions for anthocyanins decreases solvent consumption (47.2% of methanol in PLE vs 76% of methanol in UAE) for the extraction process. Therefore, the results of the present study demonstrate that PLE can be considered a powerful tool for the extraction of total phenolic compounds from mulberries and an efficient alternative for the extraction of anthocyanins.

Conclusion

Fast extraction processes of bioactive compounds such as anthocyanins and total phenolics from mulberry (*Morus nigra*) pulp have been developed. Pressurized-liquid extraction (PLE) was used. Temperature and solvent composition were found as the most significant factors for the extraction procedure. Only a period of 10 min was necessary for the optimum extraction for both anthocyanins and phenolics. Excellent intra-day repeatability and inter-day repeatability were found giving values less than 5.0%. The developed methods were successfully applied to several commercial mulberry marmalades samples. PLE results were compared to those achieved by UAE methods. PLE optimum conditions for anthocyanins decreases solvent consumption and showed better total phenolics results compared to UAE. Therefore, the proposed PLE method proved to be a good strategy for extracting polyphenols and an efficient alternative for the extraction of anthocyanins from mulberries.

Compliance with Ethical Standards

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