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Response surface methodology for optimisation of total polyphenol content and antioxidant activity of extracts from *Maytenus macrocarpa* bark by means of ultrasound-assisted extraction

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Abstract

The aim of this study was to optimise the ultrasound-assisted extraction of the total polyphenol content (TPC) and antioxidant activity from *Maytenus macrocarpa* bark by means of response surface methodology (RSM). The effect and interactions of temperature, time, particle size, solid:solvent ratio and water:ethanol ratio were analysed by using a fractional factorial design type 2^{5-1}. The most significant factors were: temperature, particle size and time. The RSM was applied to the optimisation of the TPC and two total antioxidant activities [Ferric reducing antioxidant power (FRAP) and 2,2 -azino-bis (3-ethylbenthiozoline-6-sulphonic acid) (ABTS)] as response variables. Four polynomial models were applied; the quadratic model was the most adequate one, with an adjusted R^2 value of 0.9422. *M. macrocarpa* has a considerable TPC that contributes to its antioxidant activity. The best results from the analysis of correlations were found in the FRAP versus TPC and ABTS versus FRAP, with a Pearson's *r* coefficient of 0.961 and 0.953, respectively.

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Introduction

Maytenus macrocarpa (Ruiz & Pav.) Briq, commonly known as "Chuchuhuasi" or "chuchuwaso" is a tree belonging to the *Celastraceae* family of the genus *Maytenus* Molina, from which about 445 species of this genus have been identified (Garden 2017). This genus is mainly found in tropical and subtropical zones of America, particularly Bolivia, Colombia, Peru and Ecuador (McKenna et al. 2011). Native to the Amazon region, this plant is used for its medicinal properties such as anti-rheumatic, analgesic, anti-inflammatory, anti-diarrhoeal, anti-pyretic, anti-ulcerogenic and anti-parasitic (Gonzalez et al. 1982; Rommel et al. 2016; Sanz-Biset and Canigueral 2011; Stagegaard et al. 2002). In a review written by Niero et al. (2011), the authors summarised the ethnopharmacological, chemical and pharmacological properties of plants of the *Maytenu*s genus but focused on those growing in Brazil. Moreover, in a recent study, the pharmacological properties and characterisation of the chemical compounds in the extracts of several *Maytenus* species were evaluated for their application to inflammatory diseases (Veloso et al. 2017). Regarding the *M. macrocarpa* bark, in a research project carried out by Piacente et al. (2006), a set of pentacyclic triterpenes were isolated and their anti-HIV activity in infected cells was tested. The authors reported that the 22α-hydroxy-12-en-3-oxo-29-oic acid was the most active compound.

The revised literature evidences a growing interest in the possibilities of the pharmacological applications of the *Maytenus* genus. These medical properties are usually also associated with the antioxidant activity.

At present, studies on the antioxidant potential of the *Mateynus* genus are insufficient in number, particularly on *M. macrocarpa*. Bruni et al. (2006) investigated the antioxidant and radical scavenging activity in the hydroalcholic extracts of bark obtained by a conventional method. In this study, the DPPH (1,1-diphenyl-2-picrylhydrazyl) and the β-carotene bleaching tests were determined.

In this regard, several studies published recently show interest in the use of forest biomass as a source of bioactive compounds from a biorefinery perspective (Aroso et al. 2017; Rosdiana et al. 2017; Santos et al. 2017; Todaro et al. 2017). Biorefinery is becoming an interesting option as part of environmentally friendly chemistry development and the search for natural bioresource products such as wood and tree residues in order to exploit them in an integrated manner (Todaro et al. 2017).

On the other hand, there are various extraction techniques that may be employed in the process; conventional (Soxhlet, maceration or refluxing), microwave, supercritical fluid and ultrasound techniques are the most frequent ones for the extraction of TPC from solid samples of different plants. The advantages and disadvantages of these methods were reviewed by Khoddami et al. (2013). The ultrasound-assisted extraction is one of the fastest, simplest and least expensive methods and with it, a wide range of solvents may be used (Khoddami et al. 2013; Lee and Lin 2007; Vilkhu et al. 2008). The high extraction yields obtained through ultrasound may be at the breakdown of cell walls, which allows for a better washing out of the cell contents and increasing bioavailability of micronutrients whilst retaining a natural-like quality (Veggi et al. 2013; Vinatoru 2001).

The ultrasound-assisted extraction of polyphenols has been applied to a great variety of natural sources, such as organic wastes, fruits, root, peel, seed and bark (Deng et al. 2015; Galvan d'Alessandro et al. 2012; Ghitescu et al. 2015; Veggi et al. 2013; Velmurugan and Muthukumar 2012; Wang et al. 2008). This technique is frequently combined with some statistics tool to achieve optimum yields.

There are several parameters which influence extraction yields of bioactive compounds from natural sources (Gan and Latiff 2011). The concentration and type of solvent, solid:solvent ratio, temperature, time and particle size are the most researched ones, and those studies almost always involve a preliminary work to adjust and select parameters. Nevertheless, the research projects are usually limited to a parametric variability study before optimisation or have a maximum of three factors that are selected directly and an optimisation technique is applied (Aroso et al. 2017; Deng et al. 2015; Ghitescu et al. 2015; Nazir et al. 2017; Pompeu et al. 2009). This could justify the importance of developing a study that considers at least five variables for a broader understanding of their effects and interactions on the extraction process. It is possible to study the effect of several variables and their interactions by employing a factorial or screening design (Crespo et al. 2017).

RSM is a combination of a statistical and mathematical tool widely used for improving and optimising processes, in which one or more dependent variables are related to several independent variables, called factors. This technique allows the researcher to obtain a mathematical model, which is capable of describing the behaviour of the process (Pompeu et al. 2009; Whitcomb and Anderson 2004). The RSM method is based on adjusting mathematical models (linear, quadratic and cubic functions and others) to the experimental results generated from the design of experiment. The verification of the model is done by means of variance analysis (Witek-Krowiak et al. 2014). Currently, there are several research papers that report on the use of RSM for optimisation of the extraction process of phenolic compounds from various natural sources (Deng et al. 2015; Ghitescu et al. 2015; Karacabey and Mazza 2010; Silva et al. 2007).

Detailed knowledge of optimising the extraction processes of the extracts by means of ultrasound-assisted extraction from *M. macrocarpa* bark can contribute, on the one hand, towards a better understanding of the effect of the main parameters and of the operation conditions of the process itself; on the other hand, it demonstrates the potential of this species as a source of bioactive compounds for industrial applications. Thus, the aim of this study was to optimise total polyphenol content and antioxidant activity by means of ultrasound-assisted extraction from *M. macrocarpa* bark in order to better use this valuable resource from a biorefinery perspective.

Materials and methods

Samples

Maytenus macrocarpa bark was collected from Tena in Ecuador in March 2017 and identified by Dr. David Neil in the Herbarium of the Universidad Estatal Amazónica

(ECUAMZ), Puyo, Ecuador. A voucher (David Neill-18244) specimen was deposited in the herbarium and recorded on the website www.tropicos.org.

Prior to experiments, samples were stabilised in a stove for 48 h at a temperature of 45 °C and reduced to small chips of different particle sizes (0.5, 1.75 and 3.0 mm) in accordance with ASTM-E1757-01 (2007) using a set of Tyler mesh sieves. The moisture content of the bark samples was determined according to ASTM-E871-82 (2006). This value was used to calculate the initial mass of the samples on a dry basis (m_{0_dry}) prior to the extraction process. After the extraction and filtration process, the solid residue was placed in a stove at 103 °C for 24 h until constant weight (m_{R_dry}) was achieved. From these two masses and Eq. (1), the Y_{TEC} was calculated.

All chemicals and solvents were of analytical grade and purchased from Sigma-Aldrich.

Extracts

Extracts were obtained by ultrasound-assisted extraction methods using a Bransonic Ultrasonic Bath CPXH Series model. In this research, ethanol was used as the extraction solvent according to the results reported by Wang et al. (2008). In their study, they found significant differences in the total phenolic content of the extracts for various solvents. The hydroalcoholic extracts contained the highest total phenolic content. Further, ethanol is less toxic, cheaper and can easily be recovered. 5 g of samples were placed in a 100-ml glass beaker and the corresponding solvent mixture was added. After being sonicated at the conditions defined in the design of experiment for each run, the mixtures were filtered through Whatman paper No. 4 under vacuum conditions and stored in amber glass bottles at 4 °C until use. Moreover, all the analyses were carried out on the days subsequent to the extraction to avoid any change in the samples due to prolonged storage.

Determination of TEC and TPC

The total extractable content (TEC) was determined by a gravimetric method and expressed as a percentage of the extracted mass per 100 g of dry weight (d.w).Y_{TEC} was calculated as:

$$\mathrm{TEC(g/100g\ d.w)} = \frac{m_{0\_dry} - m_{R\_dry}}{m_{0\_dry}} * 100 \quad (1)$$

where m_{0_dry} and m_{R_dry} are the initial mass and mass after the extraction on a dry basis, respectively.

Determination of TPC in the extracts was carried out in a Genesys 10 UV scanning spectrophotometer using Folin–Ciocalteu reagent (Singleton and Rossi 1965), in accordance with the procedure described previously by Baqueiro-Peña and Guerrero-Beltrán (2017) with slight modifications according to the experimental requirements. Briefly, 40 µl of each hydroalcoholic extract and 500 µl of the Folin–Ciocalteu reagent were added into 10-ml volumetric flasks covered with aluminium foil. The mixture was

left to stand for 8 min before adding 500 µl of Na_2CO_3 (10%). The solution was then adjusted with distilled water to a final volume of 10 ml and mixed thoroughly. After 120 min of incubation in a dark environment at room temperature (25 °C), the absorbance was measured at a wavelength of 765 nm. In the same way, a blank using 40 µl of water, instead of sample, was prepared.

The total phenolic content was calculated using a standard curve of gallic acid in a concentration range of 2–10 mg/l. An adjusted value of $R^2 = 0.9951$ was obtained. The total polyphenolic concentration was expressed as grams of gallic acid equivalents per 100 g of dry weight of the bark sample (g GA eq./100 g d.w.). The following equation was employed:

$$\text{Abs} = 0.0734x - 0.0028 \tag{2}$$

$$\text{TPC}\ (g\ \text{GA eq.}/100\ \text{g d.w.}) = \left(\frac{\text{Abs} - c}{m}\right) * \text{DF}_T * \frac{100}{df * m_0} \tag{3}$$

where Abs is the measured absorbance of the sample, c is the intercept (-0.0028), m is the slope (0.0734), DF_T is the total dilution factor (6.25×10^{-3}; 1.25×10^{-2}; 1.8×10^{-2}) for the solid:liquid ratios of 1:5; 1:10; and 1:15, respectively, m_0 is the initial mass of the sample and df is the drying factor (0.9591) calculating the phenolic compound content on a dry basis.

Total antioxidant activity

There is no simple universal method with which antioxidant capacity can be measured accurately and quantitatively in foods, botanicals, nutraceuticals and other dietary supplements. In this study, the radical scavenging methods were selected using the ABTS and FRAP assays, because they are commonly employed methods for antioxidant capacity measurement due to the simplicity of the assays, instrumentation required and robustness, meaning that the assays can be performed rapidly and the results are reproducible. Furthermore, both methods are comparable (Prior et al. 2005).

Ferric reducing antioxidant power, FRAP

The FRAP assay was freshly prepared prior to adding 2.5 ml of 10 mmol/l TPTZ (2,4,6-tripyridyl -*s*-triazine) in 40 mmol/l HCl and 2.5 ml of 20 mmol/l $FeCl_3 \cdot 6H_2O$ to 25 ml of 300 mmol/l acetate buffer (pH = 3.6) solution, shaken and then warmed at 37 °C for 30 min. This is in accordance with what was stipulated by Thaipong et al. (2006), with slight changes.

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standards were used to prepare a calibration curve (concentration range from 0.1 to 1 mg/l), and the results were expressed as mg of Trolox equivalent per 100 g of d.w. of sample. It can be determined from the following equation:

$$\text{Abs} = 0.1879x \tag{4}$$

$$\text{FRAP(g Trolox eq./100 g d.w.)} = \left(\frac{\text{Abs} + c}{m}\right) * \text{DF}_T * \frac{100}{df * m_0} \quad (5)$$

where c is the intercept (0), m is the slope (0.1879), DF_T is the total dilution factor (1.67×10^{-2}) for the solid:liquid ratio of 1:10 calculating phenolic compounds content on a dry basis.

ABTS free radical scavenging assay

The ABTS assay was carried out by means of the procedure reported by Baqueiro-Peña and Guerrero-Beltrán (2017), with some modification. Radical formation: 3.3 mg of sodium persulphate and 19.4 mg of ABTS were placed in an amber bottle wrapped with aluminium foil, 5 ml of distilled water was added, thoroughly mixed and stored in the dark at room temperature for 16 h. Once the $ABTS^+$ radical was formed, a 1:10 dilution with absolute ethanol was prepared. Subsequently, 20 µl of the diluted $ABTS^+$ radical was placed in a spectrophotometer cell for reading of the initial absorbance (A_i) at a wavelength of 754 nm. Therefore, 20 µl of sample was added, thoroughly mixed, allowed to react for 7 min and the final absorbance (A_f) was measured.

The standard curve was performed using Trolox solutions in the range of 0–0.016 mg ($R^2 = 0.9911$). The antioxidant activity of extracts was expressed as g of Trolox equivalents per 100 g d.w through the following equations:

$$\text{Inhibition}\,(\%) = \left(\frac{A_i - A_f}{A_i}\right) * 100 \quad (6)$$

$$I\,(\%) = 0.1637 * x \quad (7)$$

$$I\,(\text{g Trolox eq./100 g d.w.}) = \left(\frac{I - b}{m}\right) * \text{DF} * \frac{100}{df * m_0}. \quad (8)$$

Statistical analysis

The experimental planning of this study was performed in two stages. The first stage was by means of a two-level factorial design (TLFD)-type fractional (2^{5-1}), with two replicates and three central points, which allowed for the evaluation of the model's curvature. In this way, the influence of the five factors was considered (solid:liquid ratio, ethanol proportion, particle size, temperature and time) on the extraction yield expressed as total polyphenol content. Daniel's half-normal plot method of effects was used for determining the significant effects, in accordance with Whitcomb and Oehlert (2007). This allowed for the identification of significant variables for the extraction yield. The experimental planning for the fractional factorial design is presented in Table 1, which also includes the response variables.

Table 1 Fractional factorial design (2^{5-1}) setting in the original and coded form of the independent variables (A, B, C, D and E) and experimental results of TEC and TPC yields

Run[a]	Solid:Liquid[b] (w/v) (A)	Water:EtOH (v/v) (B)	Particle size (mm) (C)	Temperature (°C) (D)	Time (min) (E)	Y_{TEC} (g/100 g d.w)	Y_{TPC} (g GA eq./100 g d.w.)
1	1:5(−1)[c]	20:80(+1)	0.5(−1)	60(+1)	30(+1)	15.08	2.55
2	1:5(−1)	20:80(+1)	3(+1)	60(+1)	5(−1)	14.58	1.72
3	1:5(−1)	20:80(+1)	0.5(−1)	30(−1)	5(−1)	13.32	1.62
4	1:15(+1)	20:80(+1)	3(+1)	30(−1)	5(−1)	12.44	0.65
5	1:5(−1)	80:20(−1)	0.5(−1)	30(−1)	30(+1)	12.71	1.70
6	1:5(−1)	80:20(−1)	0.5(−1)	30(−1)	30(+1)	12.44	1.51
7	1:10(0)	50:50(0)	1.75(0)	45(0)	17.5(0)	14.64	2.01
8	1:5(−1)	20:80(+1)	0.5(−1)	30(−1)	5(−1)	12.37	1.40
9	1:15(+1)	80:20(−1)	3(+1)	30(−1)	30(+1)	12.04	1.29
10	1:5(−1)	20:80(+1)	3(+1)	30(−1)	30(+1)	12.06	1.12
11	1:5(−1)	20:80(+1)	3(+1)	60(+1)	5(−1)	12.07	1.45
12	1:5(−1)	80:20(−1)	3(+1)	60(+1)	30(+1)	13.73	2.20
13	1:15(+1)	20:80(+1)	3(+1)	30(−1)	5(−1)	10.66	0.71
14	1:15(+1)	80:20(−1)	0.5(−1)	60(+1)	30(+1)	12.28	1.50
15	1:15(+1)	80:20(−1)	0.5(−1)	30(−1)	5(−1)	13.24	1.85
16	1:15(+1)	80:20(−1)	3(+1)	60(+1)	5(−1)	13.38	1.30
17	1:15(+1)	20:80(+1)	0.5(−1)	60(+1)	5(−1)	16.33	2.82
18	1:15(+1)	20:80(+1)	3(+1)	60(+1)	30(+1)	16.72	2.45
19	1:15(+1)	20:80(+1)	0.5(−1)	60(+1)	5(−1)	15.79	2.66
20	1:5(−1)	80:20(−1)	3(+1)	60(+1)	30(+1)	14.72	2.47
21	1:15(+1)	80:20(−1)	3(+1)	60(+1)	5(−1)	11.71	1.19
22	1:10(0)	50:50(0)	1.75(0)	45(0)	17.5(0)	14.48	2.25
23	1:15(+1)	20:80(+1)	3(+1)	60(+1)	30(+1)	15.42	2.22
24	1:5(−1)	20:80(+1)	3(+1)	30(−1)	30(+1)	12.45	1.49
25	1:15(+1)	20:80(+1)	0.5(−1)	30(−1)	30(+1)	15.17	2.49
26	1:15(+1)	20:80(+1)	0.5(−1)	30(−1)	30(+1)	15.67	2.39
27	1:5(−1)	80:20(−1)	3(+1)	30(−1)	5(−1)	9.495	0.98
28	1:5(−1)	80:20(−1)	3(+1)	30(−1)	5(−1)	11.16	0.94
29	1:15(+1)	80:20(−1)	3(+1)	30(−1)	30(+1)	13.1	1.89
30	1:15(+1)	80:20(−1)	0.5(−1)	60(+1)	30(+1)	13.55	1.94
31	1:15(+1)	80:20(−1)	0.5(−1)	30(−1)	5(−1)	14.73	1.49
32	1:5(−1)	80:20(−1)	0.5(−1)	60(+1)	5(−1)	13.85	2.30
33	1:5(−1)	80:20(−1)	0.5(−1)	60(+1)	5(−1)	13.42	2.06
34	1:5(−1)	20:80(+1)	0.5(−1)	60(+1)	30(+1)	14.38	2.53
35	1:10(0)	50:50(0)	1.75(0)	45(0)	17.5(0)	14.25	2.22

[a]Non-randomised

[b]Liquid: this refers to the blend of water and ethanol

[c]Values in parenthesis are the coded forms of variables in the experimental design

The assays were performed under homogeneous conditions (repeated series of data, in a short period of time, by the same analyst, under the same conditions: the same instrument and laboratory). Therefore, the precision of the assays (TPC, FRAP and ABTS) was checked in terms of repeatability, according to Horwitz and Albert (2006). For more details, see *Supplementary Material* 1. A number of professional and technical organisations have implemented the use of the HorRat value as an appropriate criterion for interlaboratory as well as intralaboratory variability (Horwitz and Albert 2006).

In the second stage, four polynomial models (linear, two-factor interactions (2FI), quadratic and cubic) for evaluation of the interaction between the significant factors and the process yield were analysed. The RSM-type Box–Behnken design (BBD) was employed to determine the optimum values of the extraction time, the particle size and the extraction temperature related to response yields of total phenolic content (Y_{TPC}). A second-order polynomial equation was applied, as follows:

$$Y_{TPC} = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} x_i x_j \quad (9)$$

where β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively. The various x_i, x_j values are the independent

Table 2 RSM—Box–Behnken setting in the original and coded form of the independent variables (X_1, X_2 and X_3) and experimental results of TPC, FRAP and ABTS

Run[a]	Temperature (X_1) (°C)	Time (X_2) (min)	Particle size (X_3) (mm)	TPC (g GA eq./100 g d.w.)	FRAP (g Trolox eq./100 g d.w.)	ABTS (g Trolox eq./100 g d.w.)
1	30(−1)	5(−1)	1.75(0)	0.43	0.80	4.91
2	45(0)	17.5(0)	1.75(0)	1.22	1.58	7.34
3	60(+1)	17.5(0)	0.5(−1)	2.96	2.37	8.38
4	45(0)	17.5(0)	1.75(0)	1.46	1.65	7.61
5	45(0)	5(−1)	0.5(−1)	0.86	1.18	5.87
6	45(0)	17.5(0)	1.75(0)	1.21	1.43	6.95
7	45(0)	5(−1)	3(+1)	0.69	1.02	5.40
8	45(0)	30(+1)	3(+1)	1.98	1.57	7.33
9	60(+1)	17.5(0)	3(+1)	2.38	1.99	7.97
10	45(0)	30(+1)	0.5(−1)	2.47	2.16	8.00
11	30(−1)	17.5(0)	0.5(−1)	0.94	1.27	6.44
12	45(0)	17.5(0)	1.75(0)	1.21	1.43	7.01
13	60(+1)	5(−1)	1.75(0)	1.08	1.51	7.15
14	60(+1)	30(+1)	1.75(0)	2.92	2.22	8.15
15	45(0)	17.5(0)	1.75(0)	1.55	1.53	7.31
16	30(−1)	17.5(0)	3(+1)	0.85	1.03	5.69
17	30(−1)	30(+1)	1.75(0)	0.87	1.18	6.04

[a]Non-randomised

variables affecting the response Y_{TPC}, and k is the number of variables (Kiran et al. 2016).

The selection of the independent variables was based on the results of the preliminary analysis (TLFD). The experimental conditions are shown in Table 2. TLFD and BBD were carried out through the software Design Expert version 10.0.3 (Stat Ease, USA).

Validation of RSM model

For the validation of the model, the values of the coefficients of the adjusted-R^2 and predicted R^2 were determined and analysed. Validity for each experimental run was obtained, and adequacy of the model was evaluated by analysis of variance (ANOVA) (Nazir et al. 2017; Pompeu et al. 2009).

Results and discussions

Factors affecting the extraction of TEC and TPC yields using an ultrasound method

In the preliminary experiments of the present investigation, the effect of five factors on the ultrasound-assisted extraction was studied: solid:solvent ratio, water:ethanol ratio, particle size, temperature and ultrasonic time. TEC and TPC from *M. macrocarpa* bark as response variables were considered. The range values for each factor are shown in Table 1. The TEC yield from 9.49 to 16.72% was obtained for the whole range of studied variables (see Table 1). These results are close to those reported by other authors using ultrasound-assisted extraction methods. Some of

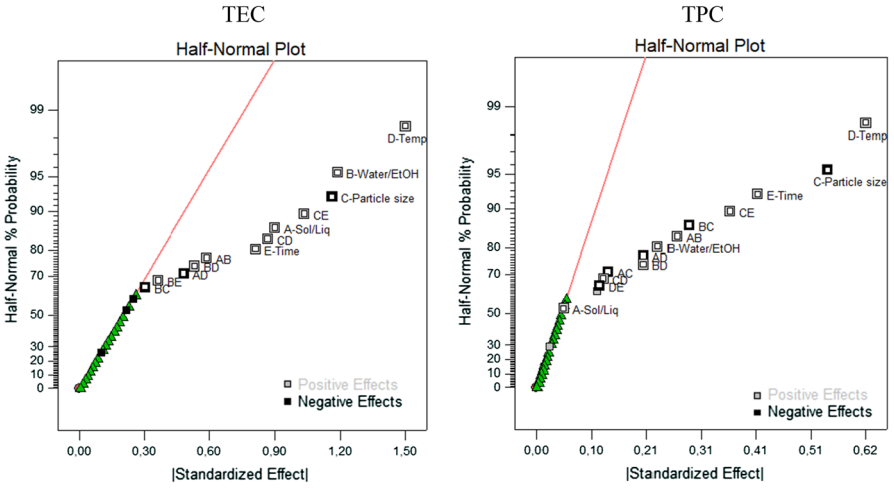

Fig. 1 Half-normal versus effect plots for TEC and TPC

their results were even slightly higher at 14% (80% ethanol) from *F. religiosa* bark (Ashraf et al. 2016), and 15.8 and 15.5% (70% ethanol) for the laboratory and semi-pilot plant scale from spruce bark (Veggi et al. 2013). However, regarding TPC, the minimum value obtained was 0.65 and the maximum was 2.82 g GA eq./100 g d.w., which will be discussed later.

Figure 1 shows the estimation of the standardised effects on the TEC and TPC. Taking into consideration that the most significant variables correspond to higher values of standardised effects according to Whitcomb and Oehlert (2007). It can be observed that the factors A, B, C, D and E and the interactions of CD and CE have the most influence on the TEC yield. The temperature, water:EtOH ratio and particle size produced the greatest effect, in that order. The remaining factors were also significant, but the effect was less. As for TPC, four factors and their interactions were significant: B, C, D, E, AB, AD, BC, BD and CE, whilst A (solid:liquid ratio) is insignificant with p value > 0.05 (see Table 3). This is in agreement with results reported by Pompeu et al. (2009) where the maximum phenolic compounds extraction was achieved with a solid:liquid ratio of 1:2 (with a water: EtOH 50%) from fruits of *Euterpe oleracea*. With this solid:liquid ratio value, the extraction yield remained almost constant.

Temperature remains the most significant factor, while particle size and sonication time are the effects which follow in terms of influence on the extraction of

Table 3 ANOVA for the factorial model for TPC extraction

Source	Sum of squares	*df*	Mean square	*F* value	*p* value Prob > *F*	
Model	10.57	14	0.76	22.09	< 0.0001	Significant
A-Sol/Liq	0.020	1	0.020	0.59	0.4514	
B-Water/EtOH	0.41	1	0.41	12.07	0.0025	
C-Particle size	2.36	1	2.36	69.17	< 0.0001	
D-Temp	3.03	1	3.03	88.69	< 0.0001	
E-Time	1.37	1	1.37	40.11	< 0.0001	
AB	0.55	1	0.55	16.17	0.0007	
AC	0.14	1	0.14	4.18	0.0550	
AD	0.32	1	0.32	9.38	0.0064	
BC	0.65	1	0.65	18.98	0.0003	
BD	0.32	1	0.32	9.38	0.0064	
BE	0.10	1	0.10	2.99	0.0999	
CD	0.13	1	0.13	3.67	0.0706	
CE	1.05	1	1.05	30.64	< 0.0001	
DE	0.11	1	0.11	3.23	0.0882	
Curvature	0.40	1	0.40	11.82	0.0028	Significant
Residual	0.65	19	0.034			
Lack of fit	4.928	1	4.928	0.14	0.7150	Not-significant
Pure error	0.64	18	0.036			
Cor total	11.62	34				

polyphenols. The decreasing influence of factor B (water:EtOH ratio) on the extraction of polyphenols in comparison to TEC may be because the polyphenols are more soluble in water than in ethanol (Veggi et al. 2013). Ethanol concentrations greater than 70% begin to inhibit the polyphenols extraction process (Du et al. 2010; Lazar et al. 2016). In both response variables, the particle size was the only factor with a negative effect; the other factors had a positive effect.

Table 3 presents the results of ANOVA for a factorial model of TPC. The "Curvature F value" of 11.82 implies there is significant curvature (as measured by the difference between the average of the centre points and the average of the factorial points) in the design space. The curvature test is significant, which suggests that optimisation can be investigated.

The factorial model fitted well with an R^2 value of 0.942. In addition, the predicted R^2 of 0.8058 is in reasonable agreement with the adjusted R^2 of 0.8995 with a smaller difference of 0.2 (Anderson and Whitcomb 2016). Non-significant lack of fit is good as it means that the model is adequate for representing the experimental data.

According to the results of the factorial design, the highest effect factors were selected in order to extract the largest amount of polyphenols. In addition, the Pareto chart and Bonferroni limit were employed in order to reinforce this selection (Anderson and Whitcomb 2016). As shown in Fig. 2, the D, C and E effects are above the Bonferroni limit and are certainly significant. When considering C and E, one must take into account their interaction, which is also highly significant. On the basis of this analysis, the following factors were selected: temperature, particle size and sonication time for RSM optimisation in the TPC extraction. Similar results were reported by Deng et al. (2015) in an optimisation study on ultrasound-assisted

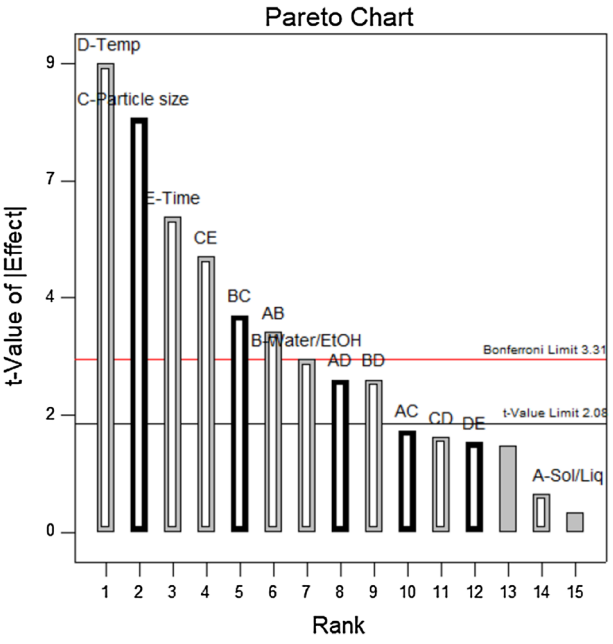

Fig. 2 Pareto chart for t value of effects for TPC extraction

extraction from sugar apple (*Annona squamosa* L.*)* peel. The water:EtOH ratio had an intermediately significant effect above the "*t* value limit" and very close to the Bonferroni limit; for this reason, the highest level was considered (20:80 v/v) in the RSM runs. In terms of the solid:liquid ratio, the minor value (1:5) was selected purely for reasons of costs, since it shows that it did not influence the extraction process.

Response surface methodology analysis

The aim of the optimisation was to find the finest combination values of the three independent variables with the greatest influence within the studied range in order to maximise the TPC for ultrasound-assisted extraction. Four models were analysed, and the best statistical results were obtained from the quadratic model that significantly improved the adjustment coefficients of the model (see Table 4). Therefore, the second-order polynomial equation was selected with an R^2 value of 0.9747. It can be interpreted that 97.47% of the total variation on the TPC extraction was attributed to the factors studied. Adjusted R^2 (0.9422) and predicted R^2 (0.7506) show reasonable agreement with a difference of less than 0.2, as was suggested by Anderson and Whitcomb (2016). An insignificant lack of fit with a p value > 0.05 is good for the model, which suggests that the model is adequate for the experimental data at a 95% confidence level (Whitcomb and Anderson 2004).

The predicted TPC values for the quadratic model and values measured in the laboratory were compared and are shown in Fig. 3. The graph distribution verifies the model's adequacy to cover the whole range of the data analysed. Thus, it is implied that the model can be applied successfully. The polynomial equation in terms of coded factors obtained from a regression analysis can be described as follows:

$$\begin{aligned} \mathrm{TPC} = {} & 1.33 + 0.78\mathrm{A} + 0.65\mathrm{B} - 0.17\mathrm{C} + 0.35\mathrm{AB} - 0.12\mathrm{AC} \\ & - 0.078\mathrm{BC} + 0.14\mathrm{A}^2 - 0.15\mathrm{B}^2 + 0.31\mathrm{C}^2. \end{aligned} \tag{10}$$

From the coded terms of the second-order quadratic equation, it may be concluded that the linear effects of temperature (A) and time (B), and the interaction between both independent variables (AC) on the TPC of the extracts were positive and significant with p value < 0.01, whilst the quadratic term of higher influence was the particle size (C^2) with a p value < 0.001.

Table 4 Summary of the evaluation of the four polynomial models analysed in the optimisation of TPC from *M. macrocarpa* bark

Source	Sequential p value	Lack of Fit p value	Adjusted R^2	Predicted R^2	
Linear	< 0.0001	0.0567	0.8249	0.7139	
2FI	0.1247	0.0823	0.8644	0.6522	
Quadratic	0.0309	0.2862	0.9422	0.7506	Suggested
Cubic	0.2570		0.9570		

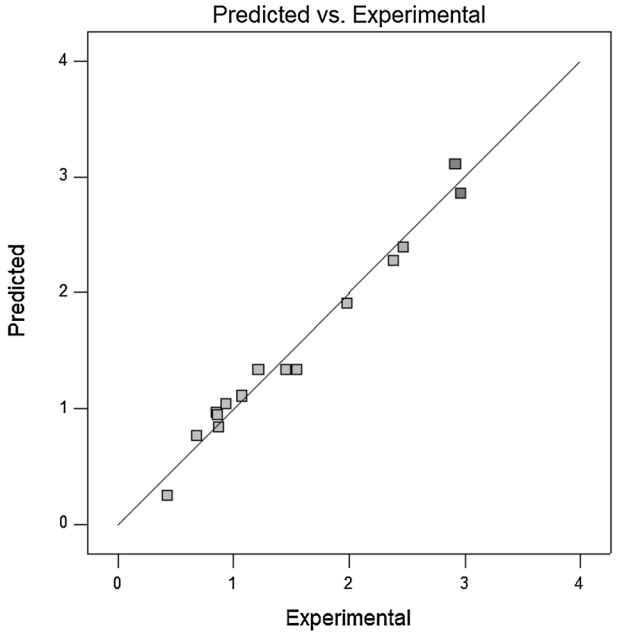

Fig. 3 Relationship between experimental and predicted TPC of *M. macrocarpa* bark

Optimisation of the ultrasound-assisted extraction of TPC and antioxidant activity from *M. macrocarpa* bark

The 3D graphic surface presented in Fig. 4 shows the relationship between the TPC and the variables studied, as well as with the antioxidant activities assayed. As can be seen, the most influential factor on the TPC extraction was temperature followed by sonication time and particle size, with *F* values of 177.17, 94.13 and 6.14, respectively. One observed an increase in TPC extraction with an increase in temperature. This effect was more pronounced with longer sonication time and minor particle size. This could be because as temperature rises, the diffusivity and solubility of the polyphenols is increased due to the fact that the viscosity of the extracts is decreased, improving the mass transfer and accelerating the extraction process (Lazar et al. 2016). Similar effects were reported by Wang et al. (2008). However, Deng et al. (2015) found that at temperature values above 60 °C, some of the thermolabile phenolic compounds began to degrade, resulting in a decrease in the phenolic content. At the particle size range studied, this factor had a greater influence at the highest temperature level. This influence was expected since a larger contact surface brings with it a higher exposure between the biomass and the extraction solvent. Furthermore, the action of the ultrasonic irradiations is most efficient, due to the penetration process in cell walls taking place at a higher rate (Galvan d'Alessandro et al. 2012; Lazar et al. 2016).

The results obtained for TPC during the optimisation of the process of the *M. macrocarpa* bark phenolic extraction varied between 0.43 and 2.96 g GA

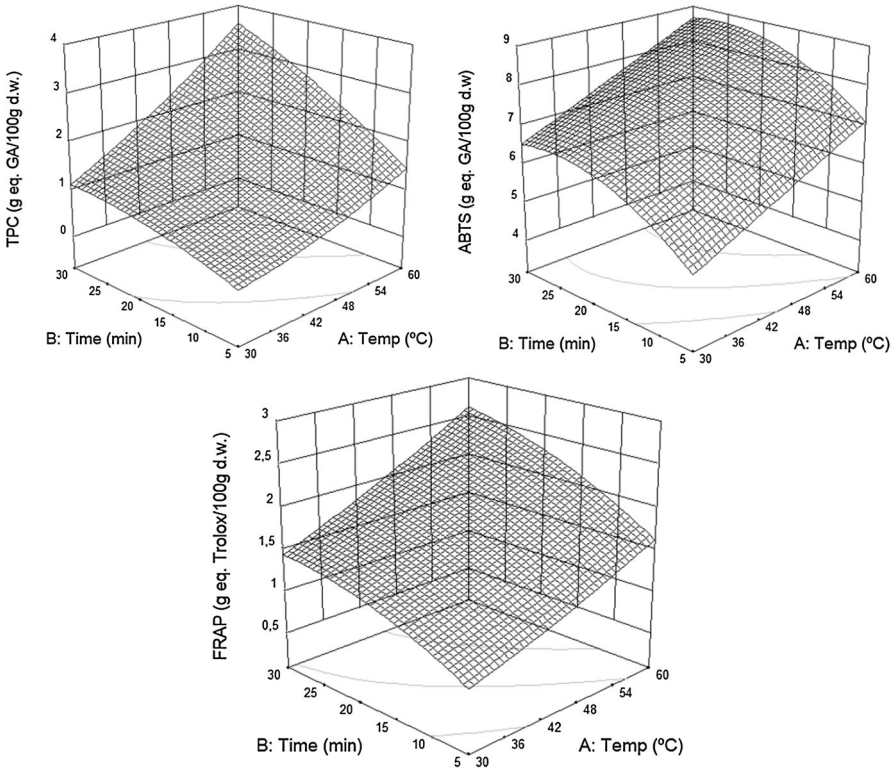

Fig. 4 Response surface plots of extraction for the effects of temperature and sonication time on the TPC, FRAP and ABTS from *M. macrocarpa* bark ethanolic extracts

eq./100 g d.w. The optimal value of TPC calculated for the model was 3.12 g GA eq./100 g d.w. for ($X_1 = 59.2$ °C, $X_2 = 30$ min and $X_3 = 0.56$ mm). This result is higher than the yield of total polyphenols (1.32 g GA eq./100 g d.w of spruce bark) obtained by Ghitescu et al. (2015) in comparable conditions under ultrasonic irradiation. A similar value of TPC (2.64 g GA eq./100 g d.w) was achieved by Bouras et al. (2015) from *Quercus suber* L. bark in the optimisation process using microwave.

With reference to the behaviour of the two antioxidant activities employed, a similar trend to the TPC was obtained, as shown in Fig. 4. The results indicate an improvement in the antioxidant potential in the extracts with an increase in temperature and sonication time, whilst for larger particle size the antioxidant activity decreased. The results indicated *M. macrocarpa* bark is a promising alternative resource as an antioxidant source based on its potential as a scavenger of radicals.

Correlation between TPC and the different antioxidant capacities tested

The FRAP and ABTS assays, based on two different chemical mechanisms, were considered in order to evaluate the antioxidant potential of the compounds present in *M. macrocarpa* bark extracts. The antioxidant potential of the natural extracts is largely attributed to the polyphenolic content. The correlation between the antioxidant activity values and the TPC was determined by using linear regression. The corrected Pearson coefficient (r) was applied to the evaluation and the results are presented in Fig. 5.

From the analysis of correlations, values above 0.90 were obtained in all cases. The best results were found in the FRAP versus TPC and ABTS versus FRAP, with a Pearson's r coefficient of 0.961 and 0.953, respectively. It must be pointed out that previous studies have reported similar results regarding the relationship between ABTS versus FRAP (Pearson's $r \geq 0.97$) in melanoidins from coffee brew and methanol extract of guava fruit, although this was in antioxidant activity tests of a different reaction mechanism (Delgado-Andrade et al. 2005; Thaipong et al. 2006). Hence, the antioxidant properties assessed by these assays are positively correlated with the phenolic content of the extracts and between themselves.

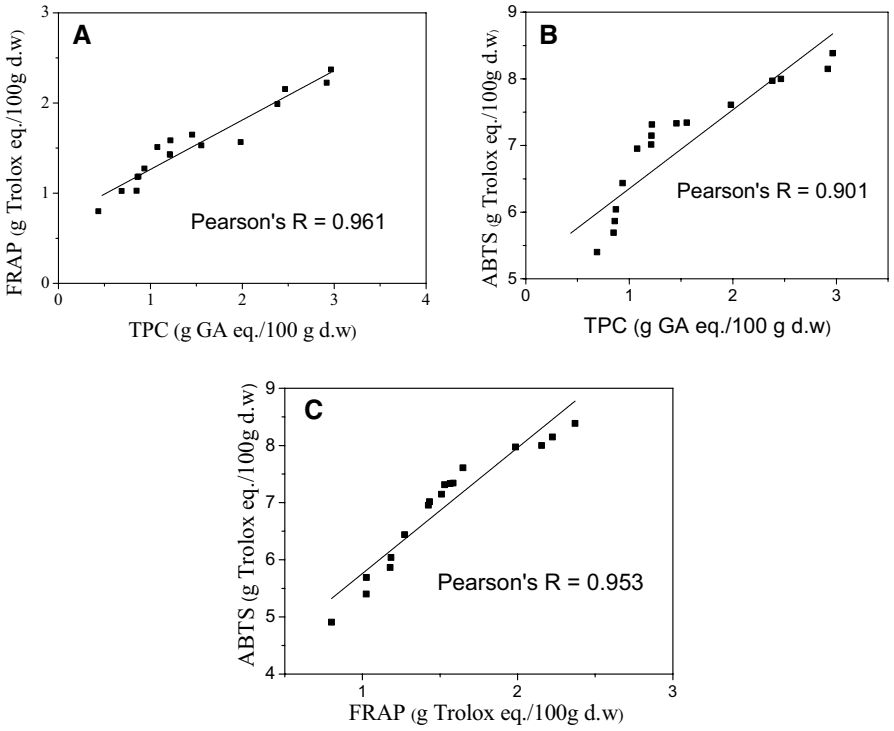

Fig. 5 Correlations between antioxidant activity and TPC of the extracts from *M. macrocarpa*

Conclusion

It was demonstrated by means of a fractional factorial design that the most significant factors in ultrasound-assisted extraction of TPC from *M. macropcarpa* bark were temperature, particle size and sonication time, whilst temperature, water:EtOH ratio and particle size were the most significant for TEC, in that order, respectively. The polynomial model applied to the polyphenol extraction process is adequate for representing the experimental data, with a R^2 adjusted value of 0.9422. *M. macrocarpa* has a considerable phenolic compound content that contributes to its antioxidant activity (FRAP and ABTS assay), with a high correlation coefficient. These results may be interesting from the perspective of finding a possible source of bioactive compounds for pharmacy applications.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflicts of interest.

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