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Optimizing the extraction of phenolic compounds from *Bixa orellana* L. and effect of physicochemical conditions on its antioxidant activity

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For this work, the surface response methodology was applied in order to optimize the extraction using solvents of phenolic compounds present in *Bixa orellana* L. seeds through a central composite design that evaluated the effect of the factors: extraction time (36 to 60 h) and the solvent ml/g seed ratio (2/1 to 4/1), for the response variable total phenol content extracted from the seed. The levels on the factors that maximized the response variable were: extraction time of 60 h, and the solvent/seed ratio of 4 ml/g. For the extracts obtained at these conditions the effect of pH, and the solid content was evaluated for the antioxidant activity and the total phenols content. The results in this case showed that for higher pH values, the content of total phenols increased as well as the antioxidant activity, being a pH of 6.67 and solid content of 13.62°Brix, the optimal conditions for these variables.

Key words: *Bixa orellana* L., bioactive compounds, antioxidant activity, response surface methodology, central composite design, total phenols.

INTRODUCTION

During the past years, an increasing thoughtful consumption of chemical additives in food has been reported, and as a result, great interest for researching on the use of natural additives. In that sense, a major goal are plant and animal derivates that allow the keeping of sensorial quality and prolong the shelf life of several foods without compromising the consumer's health (Mathenjwa et al., 2012; Xu et al., 2007).

Plants are rich in bioactive compounds that have therapeutical beneficial effects against certain cronic diseases; currently, it has been reported that natural bioactive substances such as phenolic compounds, carotenoids, phytosterols and phytoestrogens, exhibit many health benefits, including excelent antioxidant properties among others (Do Prado et al., 2014; Kunyanga et al., 2012; Venugopalan and Giridhar, 2012).

Phenolic compounds are widely distributed within vegetables and are part of the secondary metabolites in them. They vary from simple molecules as phenolic acids, phenylpropanoids and flavonoids to highly polym

erized compounds such as lignins, melanins, and tannins. Flavonoids are the more wide spread subgroup

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and they exhibit a broad range of physiological properties, such as antimicrobial, anti-inflammatory, anti-allergenic, antiviral, anticarcinogenic and antioxidant activity (Soobrattee et al., 2005; Williams et al., 2004; Zhang et al., 2014; Žugić et al., 2014).

The antioxidant activity of the phenolic compounds are very related to its capacity to eliminate free radicals, donate hydrogen atoms or electrons, or chelate metal cations. The structure of the phenolic compounds determine the scavenging of radicals and the chelating activity of the metals (Rice-Evans et al., 1997). For instance, in the phenolic acids, the antioxidant activity depends on the number and position of the hydroxyl groups in relation to the carboxyl functional group (Balasundram et al., 2006).

Previous studies have found that ethanolic extract of *Bixa orellana* L. has antimicrobial activity against some microorganisms of interest for the food industry as well as antioxidant activity (Fleischer et al., 2003; Silva et al., 2010; Venugopalan and Giridhar, 2012; Viuda et al., 2012). However, there is no studies for the optimization of the extraction process in function of phenolic compounds content in *B. orellana* L. Futhermore, there is no report showing the influence of solid content and the pH on the phenolic compounds content and its antioxidant activity.

In contrast to the traditional extraction techniques, the response surface methodology (RSM), as statistical experimental protocol optimizes processing parameters in an efficient way and allow to interpret easily the effect of the variables and their interaction (Bachir et al., 2014; Yolmeh et al., 2014). This methodology has been broadly used for optimization of phenolic compounds (Aguirre et al., 2013; Savic et al., 2013; Yang et al., 2009).

Here, using this methodology, an optimization for an extraction process that yields high amounts of phenolic compunds, which correlates with some biological properties such as its antioxidant activity was shown (Liu et al., 2012; Rockenbach et al., 2011; Viuda et al., 2012; Zhang et al., 2014; Žugić et al., 2014). This study evaluated the effect of extraction time (36 to 60 h) and the solvent (ml)/g seed ratio (2/1 to 4/1), over the response variable: total phenols content (TP) in extracts from *B. orellana* L. seeds. Likewise, the effect of solid content (SS) and solution pH on the antioxidant activity for the extracted compounds were also assesed.

MATERIALS AND METHODS

Reagents and equipments

The 2,2-Azinobis (3-ethylbenzothiazoline-6-sulfonic) (ABTS) and Trolox (6-hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid) reagents were acquired from Sigma-Aldrich®; Folin Ciocalteau, ethanol and metanol were acquired from Merck®. Tannic acid was acquired from Carlo Erba reagent®. The absorbance was measured in a UV-1700 spectrophotometer (Shimadzu®, Japan).

Sample preparation

The seeds were collected from plants grown at a particular location at the municipality of San Luis, Antioquia located at 1050 masl, and were identified and classified by El Herbario in Universidad de Antioquia as *B. orellana* L., red variety. The seeds were dried in a conventional stove at 37±0.2°C for 48 h.

Extraction process optimization of phenolic compounds from seeds

The dried seeds were put through a process of extraction with ethanol 95% to 4±0.2°C. A central composite design (CCD) was done in order to evaluate the effect of the process variables: extraction time (T: 36 to 60 h) and solvent-seed ratio (SSR: 2/1 to 4/1) expressed as millilitre of solvent per gram of seed. The response variable: total phenols content (TP), expressed as mg of tannic acid per g of extract ($mg_{TA}.g^{-1}$). The extract was filtered using filter paper (Whatman, 595-1/2, 110 mm diameter) and then the solvent was eliminated using a vacuum concentrator Vacufuge®.

Effect of pH and solid content in the amount of phenolic compounds and the antioxidant activity of the extract

The effect of the variables pH (3 to 8) and solid content (8 to 20°Brix) were evaluated through a new CCD for the content of phenolic compounds and the antioxidant activity of the extract using the optimized extraction process conditions previously obtained.

Determination of total phenols content (TP)

Here, 1 ml of methanol was added to 5 mg of extract, 10 µl of this solution was filled up to 500 µl with distilled water. 250 µl of the Folin-Ciocalteu (1:1) reagent were added to the standard and the samples. Sonification was done during 5 min and then, 1250 µl of Na_2CO_3 20% were added. The mixture was left at room temperature for 2 h in darkness. Finally, absorbance was read at 725 nm as compared to a tannic acid curve and the results were expressed as mg of tannic acid per gram of extract (mg_{TA}/g extract). All the measurements were made by sixtiplicate (Singleton and Rossi, 1965).

Antioxidant activity of the extract by ABTS method

Radical scavenging activity against the ABTS radical was determined through the method described previously (Re et al., 1999). One milliliter of the ABTS* solution was mixed with 100 µl of the extract or the Trolox standard, then it was incubated at 30°C for 30 min in darkness; afterwards, the absorbance was read at 730 nm. Acqueous solutions of Trolox concentrations (between 0 and 400 µM) were used for the calibration. The results are expressed as micromoles of Trolox equivalents per gram of extract (μmol_{TE}/g).

Antioxidant activity of the extract by ferric reducing antioxidant power (FRAP) method

For establishing the iron's capacity as a reducing agent, the methodology was previously described (Pulido et al., 2000). Briefly, 900 µl of FRAP reagent (with 2,4,6-Tripyridyl-s-Triazine [TPTZ], $FeCl_3$ and sodium acetate buffer) freshly prepared and heated up to 37°C was mixed with 90 µl of distilled water and 30 µl of the standard Trolox and 30 µl of the sample were added, respectively. They were then incubated at 37°C for 30 min. Afterwards, the absorbance was read at 595 nm. The aqueous solutions of Trolox

Table 1. Central composite design for *Bixa orellana* L. seeds extraction.

T (h)	SSR	TP ($mg_{TA}.g^{-1}$)
48.00	3:1	36.31 ± 1.99
31.03	3:1	28.44 ± 1.05
48.00	3:1	36.68 ± 3.60
64.97	3:1	39.10 ± 3.20
48.00	4.4:1	42.95 ± 0.14
60.00	4:1	41.90 ± 3.73
36.00	4:1	41.43 ± 4.77
36.00	2:1	32.49 ± 2.46
48.00	3:1	36.10 ± 1.61
48.00	3:1	36.30 ± 2.80
48.00	3:1	36.02 ± 2.33
48.00	1.6:1	35.65 ± 0.20
60.00	2:1	37.86 ± 1.88

concentrations (between 0 and 500 µM) were used for calibration. The results were expressed as micromoles Trolox equivalent per extract gram ($\mu mol_{TE}/g$).

Response surface methodology (RSM)

The experimental results obtained from CCD were adjusted to a polynomial of the Equation 1 using Design-Expert software, 7.1.6 version (Stat-Ease, EE.UU).

$$TP\ (mg_{TA}.g^{-1}) = \beta o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1} \sum_{i=1} \beta_{ij} \quad (1)$$

where β_o is the constant coefficient; β_i, β_{ii}, β_{ij} are the coefficients for the linear quadratic, and the interaction terms, respectively; x_i and x_j are the independent variables; and ε is the error.

The resulting mathematical model was optimized in order to determine the levels of the factors that provide the maximum value for the response variable. Under these optimal conditions, the following tests of the study were made.

Statistical analysis

The developed model and the statistical significance of the regression coefficients were tested using the analysis of variance (ANOVA). The interaction between the independent variables and their effect in the result were studied through an analysis of the surface response graphics. The variance homogeneity assumptions was validated using the Breusch-Pagan test, the error independence with the Durbin-Watson test, and the normality using Shapiro-Wilk test.

RESULTS AND DISCUSSION

Effect of the T and SSR factors on the response variable TP in the extraction process of phenolic compounds from *B. orellana* seeds

Table 1 shows randomly experimental data of CCD with the results for the response variable TP expressed as mg of tannic acid per g of extract ($mg_{TA}.g^{-1}$) in each run.

ANOVA results are shown in Table 2 where the *p* value is found for each factor; it also shows its statistical significance on the response variable. It is noticeable that SSR and T present a significant statistical effect ($p<0.0001$) for its linear and quadratic term with negative and positive signs, respectively. Moreover, it was found that the interaction between SSR and T is significant (value p=0.02), with a negative sign. Whereas, the effect of SSR in its quadratic term presents a significant effect ($p<0.0001$) with a positive sign for all the amount of TP extracted. From the ANOVA, TP model is obtained as presented in Equation 2.

$$TP(mg_{TA}.g^{-1}) = 27.37 - 4.58 * SSR + 0.32 * T - 0.047 * SSR * T + 1.57 * SSR^2 \quad (2)$$

Figure 1 shows the surface response graphic that represents the effect of T and SSR on the amount of total phenols (TP) extracted from the seeds. There are observable ascending tendencies for the TP content in the upper levels for the independent variables that are in line with the findings in model 2.

Table 2. Central composite design ANOVA for the extraction process of phenolic compounds present in *B. orellana L* seeds.

Source of variation	Sum of squares	Degree of freedom	Mean squared	F value	p-value
Model	88.72	4	22.18	304.61	<0.0001
SSR	38.53	1	38.53	529.14	<0.0001
T	15.50	1	15.50	212.93	<0.0001
SSR.T	0.72	1	0.72	9.94	0.020
SSR^2	15.91	1	15.91	218.45	<0.0001
R^2			0.99		

Table 3. Optimal predicted and experimental values for the extraction optimization of the *Bixa orellana L* seeds.

Variable	Optimal values
T	59.34 h
SSR	4/1
	TP ($mg_{TA}.g^{-1}$)
Predicted value (RSM)	41.78
Experimental value	44.60 ± 2.05

From Table 3, it is posible to see how SSR has a significant effect on FT ($p<0.0001$) in its linear term and in its quadratic term. The sign difference between the lineal and quadratic terms of SSR (Equation 2) can be explained if it is assumed that at lower values for SSR, the linear term neutralizes the quadratic term, exhibiting a moderate effect, but after a certain value the quadratic term imposes itself and the effect is much stronger, that is, above a certain value and the effect of the solvent becomes evident (Yolmeh et al., 2014). As expected, higher ratios of the solvent improve the solute diffusivity and maximize the extraction yield; however, if the ratio is higher than the optimal value, the excess of solvent has no significant effect over the extraction yield, and as a result it can then be wasted (Chan et al., 2014; Wong et al., 2015).

On the other hand, the T factor also exhibited a significant effect ($p<0.05$) over TP, and according to model 2, there is a positive relation between T and the response showing that an increase in the extraction times will have higher TP content improving the quality of the extraction. This variable also presented a positive effect in other extraction studies (Savic et al., 2013; Sinha et al., 2013; Wong et al., 2015; Yolmeh et al., 2014).

Model optimization and validation

The model obtained for the extraction of the seeds (Equation 2) was put through an optimization process aimed to predict the factors levels that maximize the TP content. The optimal values were validated by comparing predicted values with the experimental ones.

Effect of pH and SS on FT and antioxidant activity of the extract optimized

The extraction of phenolic compounds was performed at the defined conditions for the optimization process described earlier. A new experimental design was used in order to evaluate the SS and pH effect on the TP content ($mg_{TA}.g^{-1}$) and also the antioxidant activity as determined by the ABTS and FRAP methods ($\mu mol_{TE}.g^{-1}$). Table 4 shows the random runs of the new CCD.

Table 5 shows the ANOVA results for CCD in which the pH factor shows a significant statistical effect ($p<0.05$) for its linear and quadratic terms on the response variable. Whereas the SS factor has no effect on the responses. The R^2 values of the models for TP (0.97), ABTS (0.98) and FRAP (0.76) suggesting that the polynomials presented in Equations 3 to 5 rightly represent the existent relationship between the responses and the factors.

$$TP(mg_{TA}.g^{-1}) = -127.16 + 54.90 * pH - 3.80 * pH^2 \quad (3)$$

$$ABTS(\mu mol_{TE}.g^{-1}) = -1520.12 + 661.74 * pH - 46.83 * pH^2 \quad (4)$$

$$FRAP(\mu mol_{TE}.g^{-1}) = -1773.39 + 840.56 * pH - 62.97 * pH^2 \quad (5)$$

The obtained models (Equations 3 to 5) are graphically represented in Figure 2 for each response variable. It can be observed that there is a pH value that maximizes the responses, this is one of the main goals when applying the RSM.

Table 4. CCD for the SS and pH effect on TP and the antioxidant activity by ABTS and FRAP methods.

pH	°Brix	TP ($mg_{TA}.g^{-1}$)	ABTS ($\mu mol_{TE}.g^{-1}$)	FRAP ($\mu mol_{TE}.g^{-1}$)
6	14	58.99	92.50	1009.17
6	14	66.41	696.67	1042.50
9	14	63.72	698.06	917.50
6	14	61.03	713.33	917.50
3	8	2.59	28.61	50.83
6	14	55.73	709.72	1005.00
6	22	61.77	705.28	892.50
3	20	2.59	40.28	242.50
6	14	59.82	704.72	817.50
2	14	4.33	8.61	984.17
8	8	61.33	734.17	517.50
6	6	82.61	723.61	975.83
8	20	67.63	711.94	917.50
6	8	61.38	765.28	1092.50
6	20	57.95	696.94	1017.50

Table 5. ANOVA for TP, ABTS and FRAP of the CCD.

Source (Xi)	TP ($mg_{TA}.g^{-1}$) p-value	ABTS ($\mu mol_{TE}.g^{-1}$), p-value	FRAP ($\mu mol_{TE}.g^{-1}$), p-value
Model	<0.0001	<0.0001	0.0008
pH	<0.0001	<0.0001	0.0008
pH^2	<0.0001	<0.0001	0.0008
R^2	0.97	0.98	0.76

Table 6. Optimal predicted values for the models 3, 4 and 5.

Independent variable		Response variable		
pH	SS (°Brix)	TP ($mg_{TA}.g^{-1}$)	ABTS ($\mu mol_{TE}.g^{-1}$)	FRAP ($\mu mol_{TE}.g^{-1}$)
6.67	13.62	69.96	810.21	1031.25

Table 4 shows that the pH presents a significant effect ($p<0.05$) on TP and the antioxidant activity when evaluated through ABTS and FRAP methods. For the three models (3, 4 and 5), the positive sign in the pH coefficient in the linear term indicates that for a given range of pH the increments of this variable has a positive effect on each one of the reponse variables (TP, ABTS and FRAP), that is, at a higer pH value there is an increase in the TP content, and at the same time the antioxidant activity increases. Nevertheless, the negative sign of the quadratic term for the pH value in Equations 3, 4, and 5 indicates that above certain pH values, there is a negative influence on the three response variables. Accordingly, others studies have shown that the total content of phenolic compounds for all varieties of yam were the highest at pH 5, but gradually decreased with elevated pH (Chen et al., 2008). However, another study made on carrots and mint leaves showed a significant increase in the antioxidant activity with increasing pH values ranging from 4 to 9 (Arabshahi et al., 2007).

The variance analysis also indicates that SS shows no significant effect ($p>0.05$) on TP and the antioxidant activity within the analysed ranges from 8 to 20°Brix; conversely, a study made on carrot juice showed that increments of solid contents (from 11 to 65°Brix) increased the anthocyanin degradation rates (Kırca et al., 2007).

Model optimization

The models obtained when evaluating the pH and SS effect (model 3, 4 and 5) were put through an optimization process for predicting the levels of the factors that maximize the response variables. Table 6 presents the

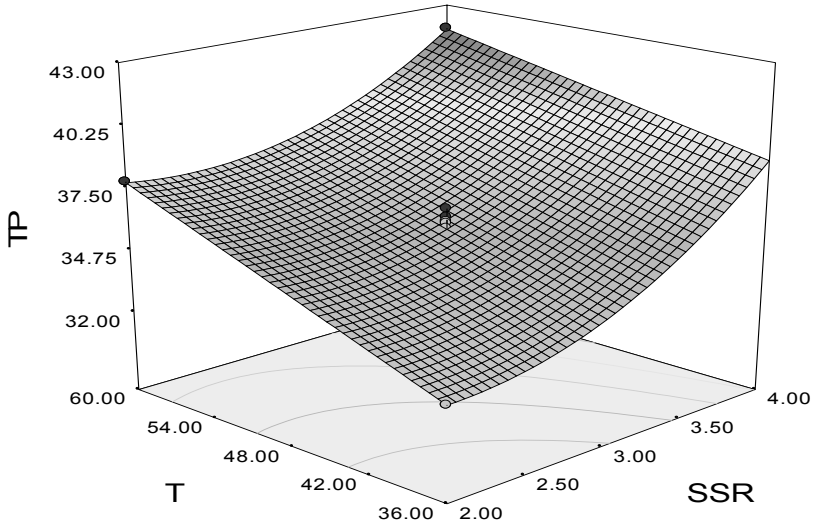

Figure 1. Surface response for the TP content extracted from *Bixa orellana* L. seeds as a function of T and SSR.

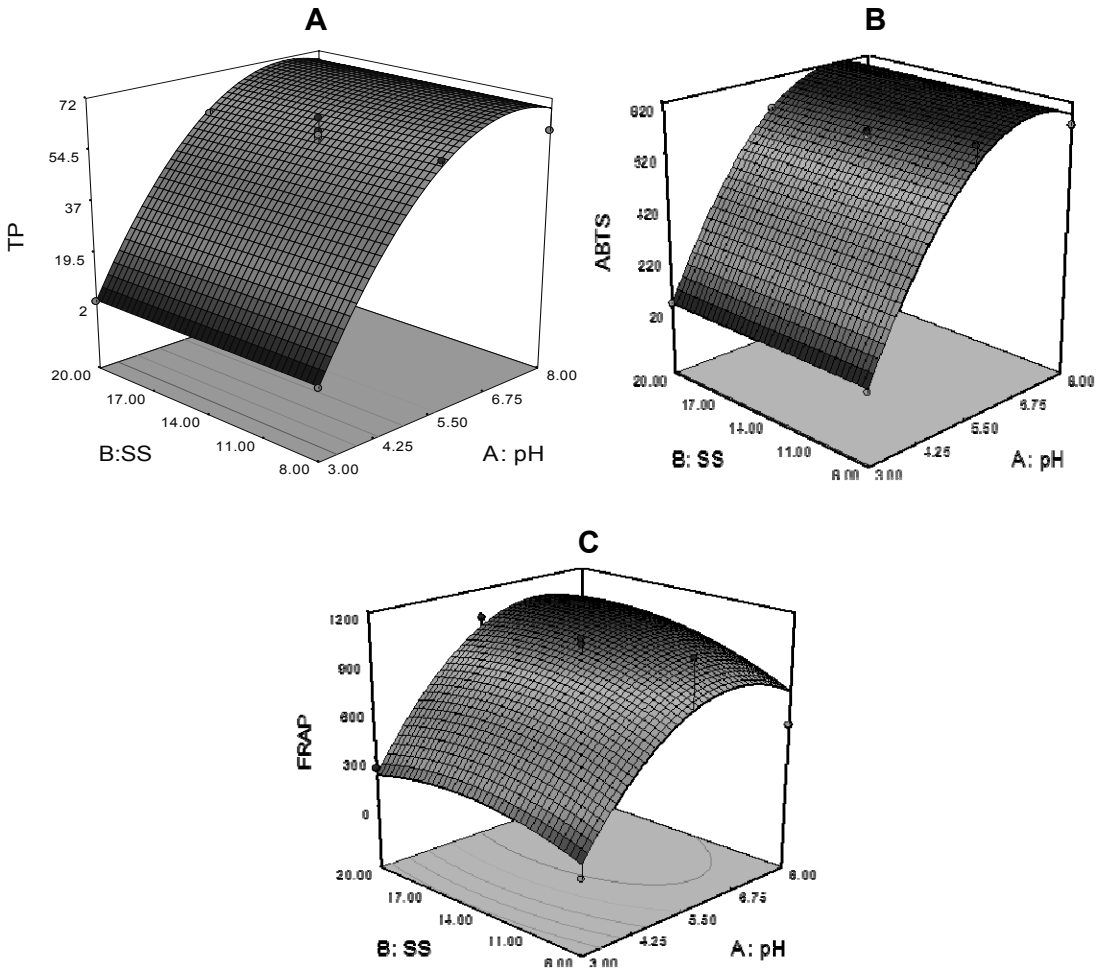

Figure 2. Surface response for (A) TP, (B) ABTS and (C) FRAP, as function of SS and pH, from ethanolic extract of *Bixa orellana* L.

optimal values for independent variables as well as predicted values for response variables.

Conclusions

In this study, it was discovered that higher TP contents (44.60 ± 2.05 $mg_{TA}.g^{-1}$) were obtained at an extraction time of 60 h and a solvent/seed ratio of 4 ml/g of extract. Futhermore, the pH has a significant effect on the TP content and the antioxidant activity, whereas the SS content has no significant effect on the response variables within the evaluated range. These data suggest that RSM is a useful tool for developing predictive models that allow to stablish optimal extraction conditions that enhance obtaining higher TP content extracts.

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Conflict of interest

The authors declare that they have no conflict of interest.

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