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Extraction of anthocyanins and polyphenols from black rice (*Oryza sativa* L.) by modeling and assessing their reversibility and stability

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ABSTRACT

This study was aimed the extraction of total flavonoids, anthocyanins and phenolics, as well as the antioxidant activity of black rice (*Oryza sativa*) and to study the stability in relation to pH, light and copigmentation. Variations in temperature (10–50 °C), time (20–80 min), and solid–solvent ratio (1:15–1:45) were studied using a Box–Behnken design. The regression models were significant ($P < 0.001$) and determination coefficients $\geqslant 0.900$. Extraction at 34.7 °C for 80 min using a solid:solvent ratio of 1:30 rendered an extract with 51.26 mg 100 g^{-1} of flavonoids, 116.58 mg 100 g^{-1} of anthocyanins, 520.17 mg 100 g^{-1} of phenolics and 46.50% inhibition of the DPPH radical. A decrease in the color intensity was observed when pH values were changed while anthocyanins were reversible in the process of protonation/deprotonation. The addition of glucose, phytic and gallic acids in the optimized extract exposed to light displayed an intermolecular copigmentation. The main anthocyanin identified in black rice was cyanidin-3-glucoside.

1. Introduction

Rice (*Oryza sativa L.*) pigmented varieties, such as black rice, have a higher content of phenolic compounds as compared to white rice (Yawadio, Tanimori, & Morita, 2007). Mira, Massaretto, Pascual, and Márquez (2009) found that the total phenolic content was four times higher in pigmented rice than in non-pigmented varieties. Red and purple rice varieties showed higher total phenolics, flavonoids and antioxidant activity than those of light colored varieties, such as white and brown varieties (Min, Gu, Mcclung, Bergman, & Chen, 2012; Shao, Xu, Sun, Bao, & Beta, 2014; Walter et al., 2013).

The name 'black rice' is related to the accumulation of anthocyanins, mainly cyanidin-3-glucoside (Escribano-Bailón, Santos-Buelga, & Rivas-Gonzalo, 2004; Lee, 2010). Abdel-Aal, Young, and Rabalski (2006) verified that black rice has the highest content of total anthocyanins (327.60 mg 100 g^{-1}) among all of the studied colored grains. Frank, Reichardt, Shu, and Engel (2012) determined levels between 22.30 and 140.30 mg 100 g^{-1} of cyanidin-3-glucoside in black rice samples. The total anthocyanin content of the subspecies *indica* and *japonica* ranged from 32.40 to 50.30 mg 100 g^{-1} and 121.30 to 155.90 mg 100 g^{-1}, respectively. Sompong, Siebenhandl-Ehn, Linsberger-Martin, and Berghofer (2011) found that the total anthocyanin content in black rice ranged from 109.50 to 256.60 mg 100 g^{-1}.

Anthocyanins extracted from black rice, especially cyanidin and peonidin-3-glucoside, showed an *in vitro* inhibitory effect on cancer cell proliferation (Chen et al., 2006), a high protection of endothelial cells from oxidative stress events (Zhang et al., 2006) and a considerable protection against angiogenesis induced by vascular endothelial growth factor (Tanaka et al., 2011), while *in vivo* protocols using rats have shown that pigmented rice varieties reduce the risk of atherosclerosis (Xia et al., 2006) and efficiently reduce the total cholesterol, low-density cholesterol, and total triacylglycerol contents of rats fed a high-cholesterol diet (Zawistowski, Kopec, & Kitts, 2009). In this sense, anthocyanins extracted from plants may be suitable substitutes for synthetic dyes because of their attractive bright color and water solubility, which allows them to be incorporated into foods (Bridle & Timberlake, 1997; Nontasan, Moongngarm, & Deeseenthum, 2012), enhancing their possible health effects in humans. However, there are some limitations on the technological application of black rice extracts in food products and beverages, such as their chemical stability with respect to pH, temperature and light (Francis, 1989).

For extraction of anthocyanins the most commonly used solvents are methanol, ethanol, water acidified with acetic acid and ethanol in hydrochloric acid medium (Francis, 1989;

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Pereira-Caro, Watanabe, Fujimura, Yokota, & Ashihara, 2013; Phetpornpaisan, Tippayawat, Jay, & Sutthanut, 2014; Shao et al., 2014). Ethanol and organic acids are desirable because they are less toxic than methanol and hydrochloric acid (Escribano-Bailón et al., 2004). Factors such as type and volume of solvent, temperature and time have a significant influence on the extraction process. The combination of these factors and the determination of optimal conditions are important in order to obtain a suitable yield of anthocyanins. From a practical standpoint, research using random extraction time, concentrations of solvents, and temperatures has been conducted (Phetpornpaisan et al., 2014; Shao et al., 2014) to extract anthocyanins and other phenolic compounds from black rice. To overcome this serious analytical limitation, the use of mathematical models that describe accurately the isolated and combination effects of different factors (i.e., time, temperature, volume of solvents) seems to be a most promising approach to obtain a functional extract from black rice. Response surface methodology (RSM) is a method used to develop and optimize processes and products (Bassani, Nunes, & Granato, 2014) and it can be used to determine the best conditions for the extraction of chemical compounds from natural products (Granato, Calado, & Jarvis, 2014; Granato, Grevink, Zielinski, Nunes, & van Ruth, 2014).

No detailed studies were found in the literature regarding the parameters used in the process of the extraction of anthocyanins from black rice using ethanol/citric acid. Thus, this study was aimed at extracting and modeling, through RSM, the total flavonoids, anthocyanins and phenolics, as well as the antioxidant activity of black rice (*O. sativa* L.) and to study the stability of the pigments in relation to pH, light and copigmentation.

2. Materials and methods

2.1. Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent, glucose, cyanidin-3-glucoside, phytic acid, gallic acid and Amberlite® XAD7HP were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). HPLC grade ethanol UV/HPLC was purchased from Vetec (Rio de Janeiro, Brazil). Citric acid monohydrate was purchased from Reagen (Paraná, Brazil). All other chemicals and reagents were of analytical grade and ultrapure water was used in all experiments.

2.2. Preparation of samples

The samples of black rice (*O. sativa* L.) were acquired commercially in Ponta Grossa, PR, Brazil, from the 2012 harvest. The rice was ground in an analytical mill to 10 mesh (QUIMIS-6298A21). Then, the samples of rice flour were distributed in low density polyethylene containers and sealed. All samples were stored immediately at 8 °C after milling until analysis.

2.3. Extraction of anthocyanins

A Box–Behnken design was used to evaluate the effect of the combinations of three independent variables (extraction temperature, time, and solid–liquid ratio) in the extraction of the bioactive compounds and free-radical scavenging activity. The values for temperature were 10, 30 and 50 °C, while an extraction time of 20, 50 and 80 min and solid–liquid ratios of 1:15, 1:30, and 1:45 were studied. These values were established from earlier experiments and studies (data not shown). The response variables were: total flavonoids, anthocyanins, phenolics and *in vitro* antioxidant activity (DPPH assay). The experimental design presented seventeen combinations (Table 1), including five replicates of the central point in order to estimate pure error and to assess the lack of fit of the proposed models. All the experiments were performed randomly and in triplicate.

The anthocyanins were extracted from the black rice according to Lees and Francis (1972), with some modifications. One gram of finely ground black rice was added to a mixture of ethanol and citric acid 1.0 mol L^{-1} in the ratio 80:20. The extraction was performed in a thermostated cell, protected from light, under constant

Table 1
The Box–Behnken design with experimental data for the extraction of bioactive compounds from black rice.

Assays	Independent variables Original and coded			Response variables*			
	Temperature (°C)	Time (min)	1:*X* (mL)	Flavonoids (mg 100 g^{-1})	Anthocyanins (mg 100 g^{-1})	Phenolics (mg 100 g^{-1})	AA** (% of inhibition)
1	10(−1)	50(0)	45(+1)	44.10 ± 0.37^{d}	97.82 ± 0.52^{h}	432.70 ± 2.35^{k}	39.33 ± 0.97^{g}
2	10(−1)	20(−1)	30(0)	39.82 ± 0.75^{e}	95.86 ± 1.13^{h}	375.35 ± 0.51^{l}	39.46 ± 0.67^{g}
3	10(−1)	80(+1)	30(0)	38.16 ± 1.06^{e}	85.56 ± 2.90^{i}	360.85 ± 2.67^{m}	35.52 ± 0.79^{h}
4	10(−1)	50(0)	15(−1)	31.71 ± 0.94^{f}	74.34 ± 1.26^{j}	339.68 ± 1.70 ^{n}	29.13 ± 0.48^{i}
5	30(0)	80(+1)	15(−1)	43.47 ± 0.83^{d}	102.12 ± 2.18^{g}	496.99 ± 1.70^{g}	41.08 ± 0.99^{g}
6	30(0)	20(−1)	15(−1)	50.15 ± 0.84^{c}	113.72 ± 1.22^{c}	440.41 ± 0.94^{j}	42.63 ± 1.13^{ef}
7	30(0)	80(+1)	45(+1)	53.59 ± 1.07^{b}	116.76 ± 1.58^{b}	513.13 ± 1.76^{e}	48.08 ± 2.24^{b}
8	30(0)	20(−1)	45(+1)	44.70 ± 1.11^{d}	107.46 ± 0.83^{cd}	526.53 ± 1.42^{d}	41.18 ± 0.45^{fg}
9	50(+1)	50(0)	15(−1)	55.39 ± 0.52^{a}	109.95 ± 0.77^{def}	484,01 ± 0.25^{h}	42.53 ± 1.33^{ef}
10	50(+1)	50(0)	45(+1)	55.14 ± 0.86^{ab}	108.71 ± 1.78^{ef}	525.10 ± 3.09^{d}	41.50 ± 1.01^{f}
11	50(+1)	80(+1)	30(0)	54.73 ± 1.48^{ab}	110.45 ± 2.93^{de}	515.41 ± 2.08^{e}	44.15 ± 0.66^{de}
12	50(+1)	20(−1)	30(0)	53.90 ± 0.19^{ab}	110.72 ± 2.41^{de}	502.87 ± 1.54^{f}	41.84 ± 1.57^{f}
13(C)	30(0)	50(0)	30(0)	54.43 ± 1.95^{ab}	120.40 ± 3.12^{a}	479.94 ± 0.89^{i}	51.78 ± 1.69^{a}
14 (C)	30(0)	50(0)	30(0)	49.95 ± 1.93^{c}	118.65 ± 0.63^{ab}	514.78 ± 0.23^{e}	50.82 ± 0.40^{a}
15 (C)	30(0)	50(0)	30(0)	50.34 ± 1.27 ^{c}	113.67 ± 0.76^{c}	540.64 ± 0.67^{a}	46.41 ± 2.27^{bc}
16 (C)	30(0)	50(0)	30(0)	49.85 ± 1.03^{c}	112.72 ± 0.56^{cd}	537.12 ± 1.00^{b}	46.05 ± 0.67^{cd}
17(C)	30(0)	50(0)	30(0)	49.97 ± 1.06^{c}	112.62 ± 1.71^{f}	533.13 ± 2.18^{c}	47.57 ± 0.10^{bc}
P (Brown–Forsythe)***				0.88	0.57	0.82	0.93
P (ANOVA)****				< 0.001	< 0.001	< 0.001	<0.001

(C) = central points.
Different letters in the same column represent results with statistical difference, according to the Fisher's LSD test ($P \leqslant 0.05$).
* Values are expressed as mean ($n = 3$).
** Antioxidant activity.
*** Value obtained according to the Brown–Forsythe test.
**** Value obtained by one-factor analysis of variance (ANOVA).

stirring. The extraction time, temperature and solid–liquid ratio were determined according to the experimental design. After processing, the extract was filtered (qualitative Whatman #1 paper) and the residue and filter paper were rinsed using the solvent of extraction until completing a volume of 50 mL. The extract was stored at −20 °C in polyethylene bottles, protected from light.

2.4. Analyses of total flavonoids, anthocyanins, and total phenolics

Flavonoids and anthocyanins from the black rice were quantified by UV–Vis spectrophotometry (Shimadzu UV-1800), at $\lambda = 374$ nm and $\lambda = 535$ nm, respectively. The content of flavonoids and anthocyanins were determined using Eqs. (1) and (2), respectively (Lees & Francis, 1972). The content of total flavonoids was expressed as mg quercetin equivalents (CE) per 100 g of rice and total anthocyanins expressed as mg cyanidin-3-glucoside equivalents (CGE) per 100 g dry weight sample (mg CGE 100 g^{-1} DW).

$$\text{Total flavonoids (TF)} = \frac{A_{374\text{nm}} \times \text{dilution factor}}{76.6} \quad (1)$$

$$\text{Total anthocyanins (TA)} = \frac{A_{535\text{nm}} \times \text{dilution factor}}{98.2} \quad (2)$$

Total phenolic content was determined using the Folin–Ciocalteu method (Huber & Rupasinghe, 2009; Singleton, Orthofer, & Lamuela-Raventos, 1999), with slight modifications. To a 5.0 mL flask, 3.0 mL of ultrapure water, 250 μL of Folin–Ciocalteu reagent 0.2 N and 250 μL of properly diluted sample were added. The solution was stirred for 5 min and 250 μL of a 10% Na_2CO_3 solution (w/v) were added, and the volume was completed with ultrapure water. The mixture was incubated at 25 °C in a water bath (99-20 MQBTC) for 60 min. The absorbance was recorded at 761 nm using a spectrophotometer (Shimadzu 1800, Japan). The content of phenolic compounds was determined from the standard curve of gallic acid (10–70 μmol L^{-1}, $y = 0.01816x - 0.01015$; $R^2 = 0.9982$). The results were expressed in mg of gallic acid equivalents per 100 g dry weight sample (mg GAE 100 g^{-1} DW).

2.5. Determination of free-radical scavenging activity

The antioxidant activity was determined according to Brand-Williams, Cuvelier, and Berset (1995), with slight modifications. Kinetic assays of DPPH· with different concentrations of the extracts were carried out to determine the reaction time. A total of 1.5 mL of an ethanolic solution of DPPH (2.2316×10^{-4} mol L^{-1}), 200 μL of sample, and 1.8 mL of ethanol were added to a test tube to a final volume of 3.5 mL. The tubes were sealed, shaken and incubated for 60 min in the dark at room temperature (25 ± 1 °C). The absorbance was recorded at $\lambda = 517$ nm and the ability of extracts to scavenge the DPPH· was calculated using Eq. (3).

$$\text{Scavenging ability (\%)} = \frac{(A_{517\text{nm of control}} - A_{517\text{nm of sample}})}{A_{517\text{nm of control}}} \times 100 \quad (3)$$

2.6. Stability studies of the optimized anthocyanin extract

2.6.1. Effect of pH

A spectrophotometric titration using a deprotonation–protonation process was performed and monitored at $\lambda = 516$ nm. The extract obtained using the optimum conditions (item 2.7) was diluted using ultrapure water until it reached an absorbance of 1.0 at 516 nm. The pH was recorded in pH meter (B-474 Micronal, Brazil). This was placed in a thermostated cell (Denver Instrument Titrator 280, USA) at 25 °C (MQBTC 99-20), protected from light, and kept under constant agitation. Aliquots of KOH 1 mol L^{-1} were added to the extract and every change of 0.20 in pH, the absorbance was recorded at 516 nm until a pH 7.20 was reached.

2.6.2. Effect of light and copigmentation

This study was conducted according to the procedures outlined by Amr and Al-Tamimi (2007) and Awika (2008), with modifications. Four solutions of optimized extract were prepared using sodium citrate/citric acid buffer solution at a concentration of 0.1 mol L^{-1} and pH 3.6. One of the solutions was considered as control (absence of copigments) and in the other glucose, phytic acid and gallic acid were added at a concentration of 7.5×10^{-4} mol L^{-1}. Each solution was divided into three flasks with 15 mL each, covered with plastic wrap and exposed continuously to the direct light of a white fluorescent lamp (20 W) in a closed wooden chamber. The temperature inside the chamber was maintained at 32 ± 2 °C. The absorbance was recorded every 60 min at $\lambda = 516$ nm until a 50% decrease in the initial absorbance was achieved. The rate constant for degradation of anthocyanins (k) and its half-life ($t_{1/2}$) (Reyes & Cisneros-Zevallos, 2007) were calculated using Eqs. (4) and (5), respectively.

$$\ln (A_t)/A_0 = -k \times t \quad (4)$$

$$t_{1/2} = \ln 2/k \quad (5)$$

2.7. Partial purification and HPLC analysis of anthocyanins

The crude extracts were purified following the procedures outlined by Zhang, Fu, and Zhang (2011), with modifications. A glass column (1.0 cm × 20.0 cm) was filled with 6.0 g of Amberlite® XAD7HP resin. Then, 1.5 g of lyophilized anthocyanins extract was solubilized with 5 mL of an aqueous formic acid solution at a concentration of 1.22 mol L^{-1}. The solution was washed twice with 15 mL of ethyl acetate at 8 °C to remove nonpolar compounds. The resulting solution was inserted in the column and eluted slowly with 150 mL of ultrapure water to remove sugars and aliphatic acids. Anthocyanins extracts were eluted with either 30 mL of a 50% acidified water–ethanol solution (P50) or 30 mL of acidified absolute ethanol, P100 (Andersen, 1988). The volume was reduced by means of a rota evaporator at 50 °C and reduced pressure.

The partially purified anthocyanins extracts (P50 and P100) were subjected to HPLC analysis according to Zhang et al. (2011), with modifications. The equipment (model Alliance 2695, Waters, Milford, MA, USA) had a 2998 photodiode PDA (Waters, Milford, MA, USA) quaternary pump detectors, and a Symmetry C18 column (Waters, Milford, MA, USA) with dimensions of 4.6 × 150 mm, 3.5 μm kept at 20 °C. The extracts were pre-filtered using a 0.22 μm membrane and 20 μL of sample were injected into the HPLC system. The mobile phase consisted of solvent A (4.5% formic acid aqueous solution) and solvent B (acetonitrile) and the gradient elution was: 3% B at 0 min, 22 min 22% 35% 31 min 85% in 40 min and 85% at 41 min. Followed by an isocratic run to 100% B in 5 min and reconditioning the column, 3% B in 10 min. The flow rate was 1.0 mL min^{-1}. Anthocyanins were identified and quantified at $\lambda = 515$ nm using the DAD detector by comparing the retention time with the standard of cyanidin-3-glucoside in the concentration range from 3.03 to 12.10 mg L^{-1} ($y = 41622x + 39470$; $R^2 = 0.9952$).

2.8. Statistical analysis

The data obtained from the experimental design were evaluated using the Anderson–Darling normality test. For data that did not follow a normal distribution, they were transformed by the Box–Cox technique. Subsequently, the homogeneity of variances

was tested for all variables by the Brown–Forsythe test (Action Software version 2.5, Estatcamp, Campinas, Brazil). One-factor analysis of variance (ANOVA) was performed to determine significant differences among treatments, while the least significance difference Fisher test was used to compare the means among treatments (Granato, Calado, et al., 2014). When the probability value was below 0.05, the response variable was subjected to multiple linear regression analysis using the response surface methodology (RSM), in which the response function (Y) was partitioned into linear, quadratic and interactive components. Data were modeled using Eq. (6):

$$Y = \beta_0 + \sum_{i=1}^{k} B_i X_i + \sum_{i=1}^{k} B_{ii} X_i^2 + \sum_{i>j}^{k} B_{ij} X_i X_j \quad (6)$$

where Y is the predicted response; β_0 denotes the model intercept; β_i, β_{ii} and β_{ij} represent the coefficients of the linear, quadratic and interactive effect, respectively; X_i, and X_j are the coded independent variables; and k is equal to the number of the tested factors ($k = 3$). The Pearson correlation coefficients were calculated using the mean values ($n = 17$) to determine the degree of association between the pairwise variables. Regression analysis based on the ordinary least-square method and the lack-of-fit test ($P_{\text{lack of fit}}$) were also performed. The value of $P_{\text{lack of fit}}$ was used to verify the adequacy of the model, and models with $P_{\text{lack of fit}} > 0.05$ indicated that the model could fit the experimental data adequately. The mathematical model only included the regression coefficients that showed statistical significance ($P \leqslant 0.05$). The appropriateness of the model was also evaluated by the % of total explained variance (R^2 and R^2_{adj}) and by the model statistical significance (P-value model) formally tested by ANOVA (Granato, Calado, et al., 2014; Granato, Grevink, et al., 2014).

In order to maximize the extraction of total flavonoids, anthocyanins, phenolics and the *in vitro* antioxidant activity measured by the DPPH assay of the black rice extract, the regression models were submitted to a multiresponse optimization approach according to the procedures outlined by Derringer and Suich (1980). Statistical analyses were performed using Statistica 7.0 software (StatSoft Inc. South America, Tulsa, USA).

3. Results and discussion

3.1. Response surface methodology

Average values for the content of flavonoids, anthocyanins, phenolics and antioxidant activity are shown in Table 1. All responses were homoscedastic as formally checked by the Brown–Forsythe test ($P \geqslant 0.05$), while significant differences were detected for all response variables using one-factor ANOVA ($P < 0.05$). These requirements are essential for the application of response surface methodology (Granato, Calado, et al., 2014; Granato, Grevink, et al., 2014).

The flavonoid content varied statistically ($P < 0.001$) from 31.71 (assay 4) to 55.39 (assay 9) mg 100 g^{-1}. The multiple regression analysis showed that the model was significant ($P < 0.001$) and adequately adjusted the experimental data, presenting $P_{\text{lack of fit}} = 0.48$ and $R^2_{\text{adj}} = 0.90$, which means that the proposed multiple regression model was able and suitable to explain 90% of the variance.

The effect of temperature (x_1), solvent ratio (x_2) and the interaction of solvent ratio and time (x_2x_3), using the linear model in Table 2, led to an increase in the flavonoid content. Temperature (x_1^2), in the quadratic model, and the interaction between temperature and ratio of solvent (x_1x_2), in the linear model, tended to significantly decrease the flavonoid content.

Anthocyanin content varied significantly ($P < 0.001$) from 74.34 (assay 4) to 120.40 mg 100 g^{-1} (assay 13). Multiple regression analysis showed that the model was significant ($P < 0.001$) and adequately adjusted the experimental data with $P_{\text{lack of fit}} = 0.76$ and explained 90% of variance in the data. The effect of

Table 2
Regression coefficients of the polynomial models (RSM) for the extraction of total flavonoids, anthocyanins, total phenolic content and free-radical scavenging activity towards DPPH*.

Parameters	Regression coefficient	Standard error	−95% CI	+95% CI	*F*-value	*P*-value
Total flavonoids (mg 100 g^{-1})						
Constant	49.605	0.687	48.093	51.118	72.205	0.0001
x_1	8.171	0.729	6.567	9.775	11.214	0.0001
x_1^2	−2.987	1.001	−5.191	−0.782	−2.982	0.0125
x_2	2.101	0.729	0.497	3.705	2.884	0.0149
x_1x_2	−3.160	1.030	−5.428	−0.892	−3.066	0.0107
x_2x_3	3.892	1.030	1.624	6.161	3.777	0.0031
Anthocyanins (mg 100 g^{-1})						
Constant	115.053	1.512	111.684	118.422	76.099	0.0001
x_1	10.781	1.345	7.784	13.779	8.014	0.0001
x_1^2	−13.707	1.852	−17.833	−9.581	−7.402	0.0002
x_2	3.827	1.345	0.830	6.825	2.845	0.0017
x_2^2	−4.339	1.852	−8.465	−0.214	−2.343	0.0411
x_1x_2	−6.180	1.902	−10.419	−1.941	−3.248	0.0087
x_2x_3	5.225	1.902	0.986	9.464	2.746	0.0206
Total phenolic content (mg 100 g^{-1})						
Constant	498.265	7.894	481.212	515.319	63.121	0.0001
x_1	77.136	8.373	59.048	95.224	9.213	0.0001
x_1^2	−43.984	11.507	−68.844	−19.125	−3.822	0.0021
x_2	21.819	8.373	3.731	39.907	2.606	0.0217
Antioxidant Activity (% *inhibition*)						
Constant	47.860	1.286	45.081	50.638	37.212	0.0001
x_1	3.322	1.144	0.850	5.795	2.903	0.0123
x_1^2	−6.785	1.575	−10.188	−3.382	−4.307	0.0008
x_2^2	−3.785	1.575	−7.188	−0.382	−2.403	0.0319

x_1 = temperature, x_2 = solvent ratio, x_3 = time.
* Significant variables with level of probability of 0.05; CI – 95% of confidence interval.

temperature (x_1), solvent ratio (x_2) and the interaction between solvent ratio and time (x_2x_3), shown in the linear model in Table 2, led to an increase in the anthocyanin content. The quadratic effect of temperature (x_1^2) and solvent ratio (x_2^2), together with the linear interaction of these factors (x_1x_2), tended to decrease the anthocyanin content.

The phenolic content varied statistically ($P < 0.001$) from 339.68 (assay 4) to 540.64 mg 100 g^{-1} (assay 15). Multiple linear regression analysis showed that the model was significant ($P < 0.001$) and adequately adjusted the experimental data with $P_{\text{lack of fit}} = 0.24$ and explained 86% of variance in the data. The effect of temperature (x_1) and solvent ratio (x_2) shown in the linear model in Table 2, led to an increase in the phenolic content. The quadratic effect of temperature (x_1^2) tended to decrease the phenolic content.

The antioxidant activity varied statistically ($P < 0.001$) from 29.13 (assay 4) to 51.78% (assay 13). Multiple regression analysis showed that the model was significant ($P < 0.001$) and adequately adjusted the experimental data with $P_{\text{lack of fit}} = 0.31$, explaining 72% of the variance in the data. The temperature (x_1) in the linear model showed a significant and positive effect on increasing the antioxidant activity. The temperature (x_1^2) and the solvent ratio (x_2^2) showed a significant effect and tended to decrease the antioxidant activity.

For all the response variables, temperature and solvent ratio were the factors that had the greatest effect, as shown in Table 2. The surface responses (Fig. 1A–D) show the effects of extraction time, temperature and solvent ratio on the response variables. The rise in temperature and solvent ratio provided the highest extraction of total flavonoids, anthocyanins, phenolics, and rendered the highest antioxidant activity. Cacace and Mazza (2003) observed that temperature and solvent ratio significantly affected the extraction of anthocyanins from black currants. Fan, Han, Gu, and Chen (2008) observed that the extraction temperature was the main parameter influencing the yield of anthocyanins from purple sweet potato. Increasing the proportion of solvent increases the concentration gradient, which increases the diffusion of solid compounds in the solvent. Raising the temperature increases the diffusion coefficient and the solubility of the compounds increases. However, temperatures above 50 °C may degrade anthocyanins (Cacace & Mazza, 2003).

Most studies available in the literature regarding black rice report the extraction of anthocyanins with methanol and hydrochloric acid. In the present study, ethanol and citric acid were used because they present lower toxicity as compared to methanol and hydrochloric acid (Escribano-Bailón et al., 2004). The anthocyanin content in a food extract may be affected by the process

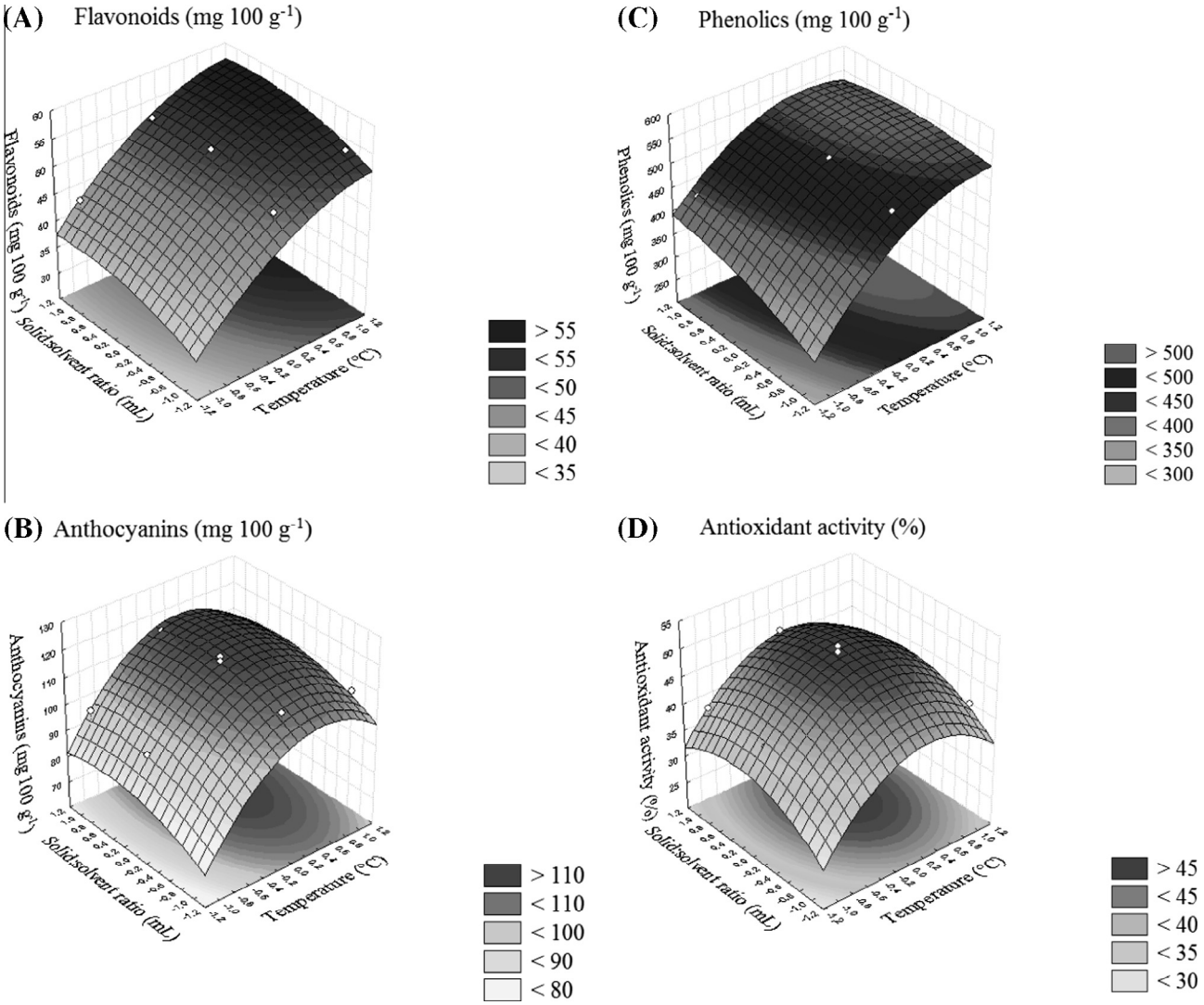

Fig. 1. Effect of temperature and solvent ratio of the content of total flavonoids (A), anthocyanins (B), phenolics (C) and antioxidant activity (D).

of extraction, solvent type and its concentration in a solution, time and temperature of extraction, degree of maturation and variety of the food, among others (Cacace & Mazza, 2003). Comparing the content of anthocyanins in black rice with other grains, the value obtained for the black rice (120.4 mg 100 g^{-1}) was higher than that of amaranth, 90.83–103.6 mg 100 g^{-1} and quinoa, 120.4 mg 100 g^{-1} (Pasko et al., 2009).

After modeling the extraction of compounds and the antioxidant activity of black rice extracts, a simultaneous optimization based on the desirability function was performed aiming at maximizing the content of bioactive compounds and antioxidant capacity of black rice extracts. The final results suggested that the condition of 80 min of extraction, at 34.7 °C, at a ratio of 1:30 (solid:solvent) could be considered suitable to obtain the optimized solution for this combination of variables. The overall desirability function obtained for this solution was 0.9587. These new process conditions were applied to the extraction of total flavonoids, anthocyanins, total phenolics and free-radical scavenging activity towards the DPPH radical. The observed and predicted mean values, along with the computed absolute errors (AE), were: total flavonoids (observed: 51.26 mg 100 g^{-1}; predicted: 52.05 mg 100 g^{-1}; AE = −3.58%); total anthocyanins (observed: 116.58 mg 100 g^{-1}; predicted: 116.82 mg 100 g^{-1}; AE = −0.64%); total phenolics (observed: 520.17 mg 100 g^{-1}; predicted: 519.37 mg 100 g^{-1}; AE = 1.53%); and antioxidant activity (observed: 46.50% inhibition; predicted: 48.27% inhibition; AE = −4.71%). Once the predicted and observed values were found to be very similar (low absolute errors, that is, below 10%), it is possible to infer that the RSM models were deemed significant, robust and can be considered predictive under the range studied.

There was a significant ($P < 0.001$) positive correlation between antioxidant activity with flavonoids ($r = 0.7655$), anthocyanins ($r = 0.9391$) and phenolics (0.7372). The structure of the flavonoids contains two aromatic rings linked by three carbon atoms, the so-called flavylium cation (Lee, Umano, Shibamoto, & Lee, 2005). The anthocyanins derive from the flavylium cation, and contain one or more hydroxyl substituents including other functional groups (sugars, for example). The presence of hydroxyl groups provides antioxidant properties for the donation of hydrogen atoms for reactive species, with the formation of stable products. Therefore, the higher the anthocyanin content, the higher the antioxidant activity (Zhang, Shao, Bao, & Beta, 2015). Similarly, Kim et al. (2014) studied the chemical composition and antioxidant activity (ABTS, FRAP, and DPPH) nine varieties of pigmented rice (*O. sativa* L.) and verified that cyanidin-3-glucoside, peonidin-3-glucoside, proanthocyanidin dimers, proanthocyanidin trimers, and catechin presented a high and significant correlation with the antioxidant data. In a recent study conducted by Jun, Shin, Song, and Kim (2015), ferulic acid (178.3 μg g^{-1}) was the major phenolic compound in a fraction of the ethyl acetate extract from black rice bran and the antioxidant activity (ABTS, DPPH, and reducing power) was closely correlated to the total content of phenolic compounds in the organic extracts.

3.2. Anthocyanin stability

3.2.1. Effect of pH

The pH is the factor that most affects the color of anthocyanins in solution, and this is due to the shift of reversible or irreversible equilibrium structure of these compounds (Mazza & Brouillard, 1987). Fig. 2A shows the behavior of anthocyanins with increasing pH and deprotonation associated with the change in the molecular structure of these compounds (Torskangerpoll & Andersen, 2005). A decrease in absorbance of 50% was observed at pH 3.15. According to Francis (1989) pH values above 3.0 are associated with

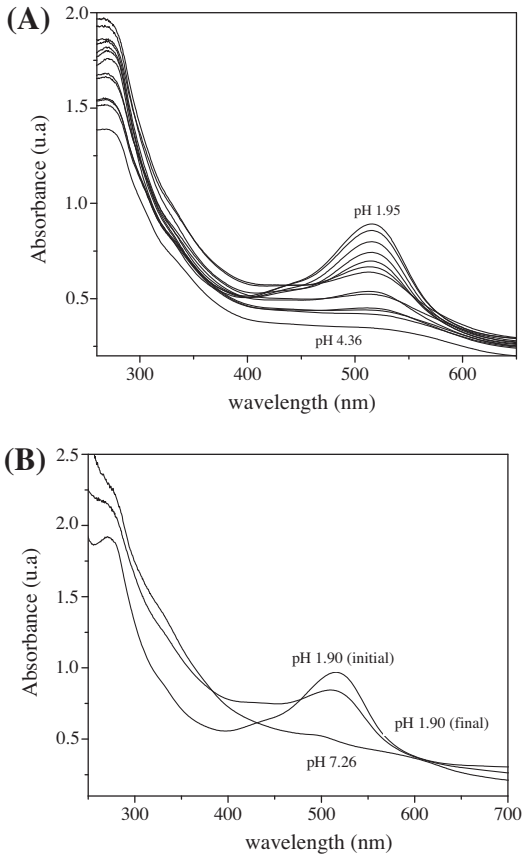

Fig. 2. Effect of pH on the absorbance (A) and reversibility (B) of the anthocyanins from black rice.

decreased anthocyanin stability and with the onset of structural degradation of these compounds. As the pH increases, the conversion of flavylium cation into the quinoidal form begins. This compound is unstable and the nucleophilic attack by water molecules results in the development of pseudobase carbinol and chalcone (Bąkowska, Kucharska, & Oszmiański, 2003). Similarly, Sui, Dong, and Zhou (2014) evaluated the stability of cyanidin-3-glucoside and cyanidin-3-rutinoside from black rice, in an aqueous system, with varying pH values (2.2–6.0) and temperatures (100–165 °C) and observed that the stability of the two anthocyanins decreased gradually with increasing pH.

The presence of glycosylated and acyl groups prevents the opening of the heterocyclic ring of the molecule and the formation of unstable compounds, such as aldehydes and ketones (Torskangerpoll & Andersen, 2005). Fig. 2B shows the process of deprotonation–protonation of the anthocyanins extract from black rice. A solution of the extract was titrated to pH 7.26 and then made to reverse titration to pH 1.90. The reverse spectrophotometric titration showed an increase in maximum absorbance with decreasing pH, indicating reversibility of structural anthocyanin in black rice. From this titration, it is hypothesized that there was no breakage of the anthocyanins structure. This is a favorable point for the use of these compounds when ingested, both in natural and isolated forms since significant variations in pH occur during the digestive process and these variations probably will not alter the beneficial effect of these compounds. The study of the reversibility of anthocyanins with the change in pH is an important factor for industrial applications and for the availability of these compounds for nutraceutical purposes.

Table 3
Effects of light and copigments the rate constant (k) and half-life ($t_{1/2}$) on the stability of anthocyanins from black rice.

Extract	k (h^{-1})	R^{2a}	$t_{1/2}$ (h)	P-value[b]
Control (no pigment)	0.049	0.998	14.060	<0.001
Glucose	0.045	0.997	15.576	<0.001
Phytic acid	0.044	0.997	15.718	<0.001
Gallic acid	0.042	0.998	16.503	<0.001

[a] Coefficient of Determination.
[b] Descriptive level (significance).

3.2.2. Effect of light and copigmentation

Table 3 shows the rate constants (k) and half-life time ($t_{1/2}$) in the degradation reaction of anthocyanins from black rice copigments and in the presence of light. It was found that after 12 h, extracts exposed to light with the addition of glucose and phytic and gallic acids, showed an increase in half-life compared to control. The half-life for the control was 14.06 h, whereas with the addition of glucose, phytic acid and gallic the $t_{1/2}$ was 15.58, 15.72 and 16.50 h, respectively. The increase in half-life of the extract shows the effect of intermolecular copigmentation. In this type of interaction van der Waals forces and hydrophobic effects predominate, resulting in "stacking" between the anthocyanin molecule and the copigment (Dangles, Wigand, & Brouillard, 1992). Copigments are rich in pi (Π) systems and are capable of associating with the flavylium ion. This combination protects the anthocyanin molecule from nucleophilic attack of water and thereby increases its stability (Mazza & Brouillard, 1987). Among copigments added to extract, gallic acid provided the biggest increase in half-life time. This may be related to the structure which differs from phytic acid and glucose once it presents an aromatic ring and planar structure. The planar structure of gallic acid allows greater protection for the anthocyanin molecule by stacking below and above the plane. Gauche, Malagoli, and Luiz (2010) observed that anthocyanins from Cabernet Sauvignon grapes (*Vitis vinifera* L.) had greater color stability in the presence of organic acids. Likewise, Gris, Ferreira, Falcão, and Bordignon-luiz (2007) found that the half-life of anthocyanins from Cabernet Sauvignon grapes increased in the presence of caffeic acid as compared to the control (absence of copigments).

The interaction between copigments and anthocyanins leads to the formation of a complex *pi–pi* (Π–Π) with changes in spectral properties of the *flavylium* cation molecules. The spectral changes cause an increase in the maximum absorbance (hyperchromic effect) and the maximum wavelength (bathochromic effect) (Gauche et al., 2010). Table 4 shows the bathochromic and hyperchromic effects of extract of anthocyanins in the presence of organic acids and glucose as compared to the control (absence of copigments). All the extracts in the presence of copigments showed an increase in the wavelength of maximum absorption at time 0 as compared to the control (no pigments). The hyperchromic effect observed is due to interaction between the flavylium cation of the anthocyanin molecule and copigments added to the extract (glucose and organic acids) (Gauche et al., 2010).

Table 4
Variations in the wavelength of maximum absorption ($\Delta\lambda$) and maximum absorbance (Δabs) of the extracts in the presence and absence of copigments.

Extract	λ_{max} (nm)	$\Delta\lambda$ (nm)	Abs_{max}	Δabs
Control (no pigment)	513.60	0.00	0.89	0.00
Glucose	516.55	2.95	0.92	0.03
Phytic acid	514.90	1.30	0.94	0.05
Gallic acid	514.60	1.00	0.91	0.02

3.3. Partial purification and identification of anthocyanins

In the UV–Vis spectrum, two bands of maximum absorption were identified for both extracts: one at a wavelength of 274 nm to the extract eluted with 50% ethanol solution and one band at 278 nm to the extract eluted with absolute ethanol. These bands clearly indicate the presence of hydroxyl groups attached to the aromatic ring (phenolic compounds). Likewise, one band at 524 nm was verified to the extract eluted with 50% ethanol solution and one band at 535 nm to the extract eluted with absolute ethanol, thus indicating the remarkable presence of anthocyanins. The difference in maximum absorption of the two extracts is due to the solvent; however, these data suggest that both extract presented the same anthocyanin.

The detection of anthocyanins present in both extracts was performed using the HPLC at 515 nm and only one peak was observed, corresponding to cyanidin-3-glucoside (Fig. 3). The cyanidin-3-glucoside contents of the extracts eluted with 50% ethanol solution

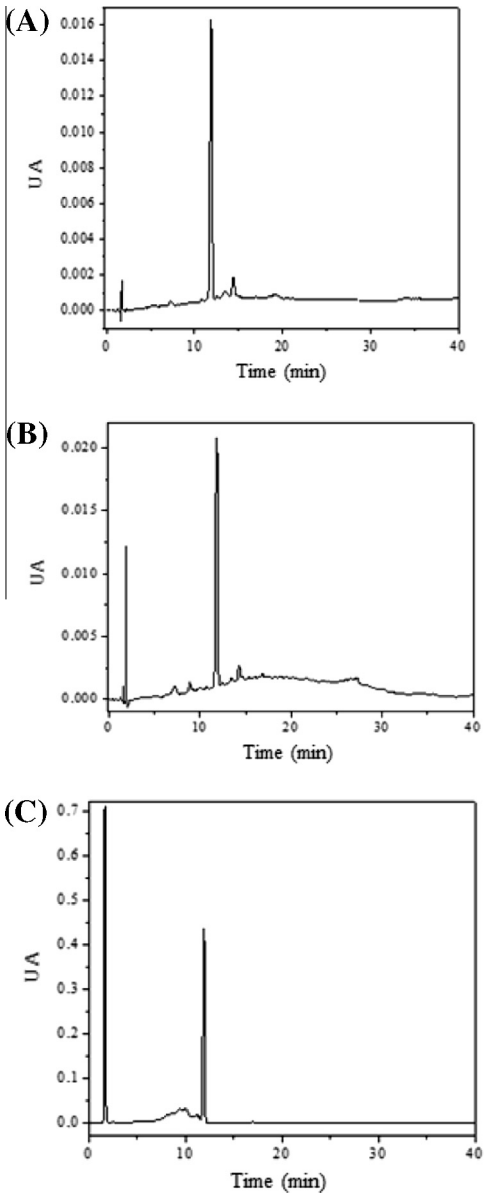

Fig. 3. HPLC chromatograms of partially purified extracts from black rice: extract eluted with 50% acidified ethanol, P50 (A), extract eluted with absolute ethanol, P100 (B), and a standard solution of cyanidin-3-glucoside (C).

and pure ethanol were 50.32 ± 0.02 and 10.03 ± 0.03 μg g^{-1}, respectively. According Escribano-Bailón et al. (2004) and cyanidin-3-glucoside is the major anthocyanin, ranging from 80% to 100% of total contents in pigmented rice cultivars. According to Abdel-Aal et al. (2006), cyanidin-3-glucoside corresponds to 80% to 100% of the total content of anthocyanins in black rice. Yawadio et al. (2007) identified cyanidin-3-glucoside and peonidin-3-glucoside in black rice, while Sompong et al. (2011) confirmed that cyanidin-3-glucoside and peonidin-3-glucoside are the most abundant anthocyanins in black rice extracts, with contents ranging from 19.4 to 140.8 mg 100 g^{-1} and 11.1 to 12.8 mg 100 g^{-1}, respectively. So, considering the retention time and the wavelength in which anthocyanin was detected compared with the standard, it is possible to confirm that cyanidin-3-glucoside is the main anthocyanin in black rice.

According to guideline 94/36/EC/1994 from the Council of the European Union, food colorants are defined as substances which add or restore color in a food, and include natural sources which are normally not consumed as a foodstuff as such and not normally used as a characteristic ingredient in food. The Scientific Committee on Food SCF evaluation made a couple of exemptions from this extended toxicological testing for natural color extracts, to which no formal acceptable daily intake (ADI) value could be allocated, provided that their intake as a food colorant would not result in an ingestion substantially differing from the amounts likely to be ingested from the normal consumption of the foods in which they occur (European Parliament, 1994). In this regard, crude and purified extracts from black rice obtained by ethanol and citric acid may represent a suitable and natural alternative to synthetic food colorants. In this sense, Loypimai, Moongngarm, Chottanom, and Moontree (2015) developed a natural food colorant from black rice bran by using ohmic heating assisted extraction and data showed the powder extract was rich in bioactive compounds, especially anthocyanins. Nontasan et al. (2012) applied a black rice extract in a powder form containing 37.7 mg g^{-1} of total phenolic content and 9.48 mg g^{-1} of total anthocyanins in a flavored yogurt (0.6% w/w) and verified the product presented a pleasant purplish-pink color that remained constant after 21 days of cold storage (4 °C).

4. Conclusion

Response surface methodology was used to evaluate the effect of temperature, time and solid–liquid ratio on the extraction of compounds from black rice. A temperature of 34.7 °C, 80 min extraction and a solid–liquid ratio of 1:30 proved to be the best condition to maximize the total contents of flavonoids, anthocyanins, phenolics and *in vitro* antioxidant activity of a black rice extract. The use of citric acid–ethanol is noteworthy because it is a non-toxic solvent system that could be used to extract bioactive and pigmented compounds from black rice, and the extracts could be used by the food industry as a natural colorant.

The process of protonation and deprotonation showed that the extract rich in anthocyanins obtained from black rice has reversible structural changes as a response to pH changes. The stability of the extract exposed to light via intermolecular copigmentation increased when glucose, phytic acid, and/or gallic acid were added in the system. Cyanidin-3-glucoside was the main anthocyanin identified in black rice. In a possible industrial application of the extract, the stability parameters must be taken into account for better preservation of the sensory aspects of products.

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