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Extraction of bioactive compounds and free radical scavenging activity of purple basil (*Ocimum basilicum* L.) leaf extracts as affected by temperature and time

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ABSTRACT

In the current study, response surface methodology (RSM) was used to assess the effects of extraction time and temperature on the content of bioactive compounds and antioxidant activity of purple basil leaf (*Ocimum basilicum* L.) extracts. The stability of anthocyanins in relation to temperature, light and copigmentation was also studied. The highest anthocyanin content was 67.40 mg/100 g extracted at 30 °C and 60 min. The degradation of anthocyanins with varying temperatures and in the presence of light followed a first-order kinetics and the activation energy was 44.95 kJ/mol. All the extracts exposed to light showed similar half-lives. The extracts protected from light, in the presence of copigments, showed an increase in half-life from 152.67 h for the control to 856.49 and 923.17 h for extract in the presence of gallic acid and phytic acid, respectively. These results clearly indicate that purple basil is a potential source of stable bioactive compounds.

Key words: anthocyanins, extraction, pigments, response surface methodology.

INTRODUCTION

Purple basil (*Ocimum basilicum* L.) is one of the species that belongs to a group of medicinal and aromatic plants of economic value worldwide, especially in Brazil where it is vastly used in traditional dishes, in the folk medicine, and various pharmaceutical applications (Rosas et al. 2004, Lee et al. 2005). The purple basil and belongs to the Lamiaceae family and its chemical composition is composed of an appreciable amount of phenolic compounds, especially flavonoids (Capecka et al. 2005, Carvalho-Filho et al. 2006). Nguyen et al. (2010) studied leaves of three cultivars of purple basil, namely Dark Opal, Sweet Thai, and Genovese, and observed that the basil treated with a higher potassium rate (5.0 mM) resulted in an increase in the concentration of total phenolic compounds and antioxidant activity as compared to the basil treated with a lower potassium rate (1.0 mM).

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Flavonoids are the major class of phenolic compounds and include the pigments called anthocyanins which are responsible for the color of many plants (Holton and Cornish 1995, Wu and Prior 2005, Escribano-Bailón et al. 2004). Phippen and Simon (1998) studied different varieties of basil and found that the yield of anthocyanins ranged from 6.49 to 18.78 mg/100 g wet basis. These authors identified a total of fourteen acylated and glycosylated anthocyanins. Anthocyanins found in high concentrations in purple basil are: cyanidin based *p*-coumaryl and malonyl acids; cyanidin based *p*-coumaryl acid; cyanidin based *p*-coumaryl acid; peonidin 3-(*p*-coumaryl glucoside)-5-glucoside; cyanidin based *p*-coumaryl and malonyl acids.

Studies have shown that anthocyanins extracted from different botanical sources present beneficial health effects on humans, such as the reduction of inflammatory, circulatory, and coronary diseases (Bettini et al. 1985, Vincieri et al. 1992, Waterhouse 1995), *in vitro* anti-cancer properties (Lee et al. 2013), protection of neuronal cells (Pascual-Teresa 2014), *in vitro* and *in vivo* antioxidant properties (Kim et al. 2014, Szymanowska et al. 2015), and lipid-lowering effects (Zawistowski et al. 2009), anti-inflammatory and antimicrobial (Usman et al. 2013). Anthocyanin-rich extracts from purple potatoes, *Solanum tuberosum* L. cv Vitelotte noire (Solanaceae) had been shown to present anti-proliferative activity against the colon cancer cells Caco-2, SW48 and MCF7, MDA-MB-231 breast cancer cells, antimicrobial activity against *Bacillus cereus, Escherichia coli* and *Pseudomonas aeruginosa* and antioxidant activity after in vitro simulated gastrointestinal digestion (Ombra et al. 2015). As anthocyanins can be used as colorants in the food industry in replacement to synthetic dyes (Bridle and Timberlake 1997, Patil et al. 2009, Nontasan et al. 2012), there is a need to study new sources of such compounds and to assess their stability toward pH, light, temperature, oxygen, enzymes, metalic ions, and copigments (Heredia et al. 1998).

The solvents most commonly used in anthocyanin extraction are methanol, ethanol, water acidified with acetic acid and ethanol added with hydrochloric acid (Shao et al. 2014, Phetpornpaisan et al. 2014, Pereira-Caro et al. 2013, Francis 1989). However, extraction procedures using ethanol and organic acids are desirable from a public health and industrial perspectives because they are not as toxic as methanol and hydrochloric acid (Escribano-Bailón et al. 2004, Pedro et al. 2016). Factors such as the type and volume of solvent, temperature and extraction time have a significant influence on the extraction of bioactive compounds from different matrices, so the use of mathematical models that describe accurately the isolated and combined effects of different factors (*i.e.*, time, temperature, solvent volume) seems to be a most promising approach to obtain a functional extract from purple basil (Shao et al. 2014). In this sense, response surface methodology (RSM) can be used to assess the effects of different experimental factors (independent variables, *i.e.*, time and temperature of extraction) on the content of chemical markers and bioactivity of plant extracts as they are the main contributors to the extraction and stability of bioactive compounds (Saha et al. 2011, Zhang et al. 2013, Granato et al. 2014a, Uddin et al. 2015, O'Shea et al. 2015).

Still there is a clear lack of studies regarding the evaluation of the effects of the main factors that influence the extraction of bioactive compounds from purple basil, and the evaluation of the stability of anthocyanins toward temperature, light and copigmentation. Based on this fact and taking into account the importance of new sources of natural colorants that can be applied in pharmaceutical, medical and food products, the objective of this study were to evaluate the effect of time and temperature on the extraction of bioactive compounds (total flavonoids, total phenolic content, and anthocyanins) and antioxidant activity of purple basil leaves using response surface methodology, and to evaluate the stability of anthocyanins toward temperature, light and copigmentation.

MATERIALS AND METHODS

Chemicals

Gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteau's phenol reagent, phytic acid (dipotassium salt) were acquired from Sigma Aldrich (St. Louis, USA). Ethyl alcohol (UV/HPLC grade, Vetec, São Paulo, Brazil), monohydrate citric acid (Reagen, Curitiba, Brazil), HCl, Na_2CO_3, KOH and KCl (Merck, Germany) were of analytical grade. The solutions were prepared using ultrapure water (Millipore).

Preparation of Samples

Purple basil (*Ocimum basilicum* L.) samples were acquired in Ponta Grossa, PR, Brazil. The leaves were washed with distilled water, crushed and stored in low-density polyethylene sealed containers and protected from light, at 8 °C.

Experimental Design and Extraction of Bioactive Compounds

A 2^2 factorial design (two factors and two levels coded as +1 and -1) added with a central point was employed assess the effects of extraction time and temperature on the content of bioactive compounds and antioxidant activity of the purple basil extracts. The factors (independent variables) were extraction time (20, 40 and 60 min) and temperature (10, 20 and 30 °C), assuming -1, 0, and +1 as coded values (Table I).

TABLE I
Total phenolic content, flavonoids, anthocyanins and *in vitro* antioxidant capacity of *Ocimum basilicum* L. leaf extracts obtained with different temperature and extraction times.

Temperature (°C)	Time (min)	Total anthocyanins (mg/100 g)	Total flavonoids (mg/100 g)	Total phenolic content (mg/100 g)	DPPH (% inhibition)
10 (-1)	20 (-1)	$21.81^{e} \pm 0.28$	$40.49^{d} \pm 0.20$	$272.54^{d} \pm 0.68$	$43.00^{d} \pm 0.28$
10 (-1)	60 (+1)	$36.85^{d} \pm 0.04$	$58.66^{c} \pm 2.88$	$404.71^{c} \pm 0.69$	$54.11^{c} \pm 0.64$
30 (+1)	20 (-1)	$47.10^{c} \pm 0.50$	$74.85^{b} \pm 5.42$	$464.30^{bc} \pm 33.44$	$66.99^{b} \pm 1.79$
30 (+1)	60 (+1)	$64.70^{a} \pm 1.04$	$106.23^{a} \pm 10.38$	$688.22^{a} \pm 53.85$	$77.21^{a} \pm 0.47$
20 (0)	40 (0)	$52.48^{b} \pm 1.68$	$79.81^{b} \pm 2.83$	$508.43^{b} \pm 16.98$	$69.43^{b} \pm 3.41$
p-value (Bartlett test)[a]		0.15	0.17	0.03	0.21
p-value (ANOVA/Welch)[b]		<0.0001	0.0001	<0.0001	<0.0001

Values are expressed as means ± standard deviation (n = 3); [a]Probability values obtained by the Bartlett test for homogeneity of variances; [b]Probability values obtained by one-way analysis of variances (homoscedastic data) or Welch-ANOVA (heteroscedastic data). Different letters in the same column represent statistically different results according to the Fisher LSD test ($p \leq 0.05$).

The response variables were: total anthocyanins, flavonoids, total phenolic content and *in vitro* antioxidant activity toward the DPPH radical. As the temperature is one of the most important factors affecting the stability of anthocyanins in natural extracts, in the current work we used only a range between 10 and 30 ºC. All experiments were performed in triplicate, including the extraction procedure.

The bioactive compounds were extracted from the purple basil according to the procedures described by Lees and Francis (1972), with some modifications. For that purpose, 1 g of leaf was macerated and added to a mixture of ethanol and citric acid 1.0 mol/L (v=10 mL; 80:20 v/v) and the extraction was carried out according to the experimental design in a thermostated cell, protected from light, under constant stirring. Then, the content was qualitatively filtered (Whatman #1 paper) and the residue and filter paper were

washed until a volume of 25 mL was achieved. The extracts were stored at -20 °C, for fifteen days, until analysis.

Total Anthocyanins, Flavonoids and Total Phenolic Content

Total content of anthocyanins (λ = 535 nm) and flavonoids (λ = 374 nm) from the purple basil were quantified by UV-Vis spectrophotometry (Shimadzu UV-1800) using Equations 1 and 2, respectively and were expressed as mg cyanidin-3-glucoside equivalents (CGE) per 100 g of fresh tissue and mg quercetin equivalents (CE) per 100 g of fresh tissue.

$$\text{Total anthocyanins (TA)} = \frac{A_{535nm} \text{ x dilution factor}}{98.2} \quad (1)$$

$$\text{Total flavonoids (TF)} = \frac{A_{374nm} \text{ x dilution factor}}{76.6} \quad (2)$$

The Folin-Ciocalteu assay (Singleton et al. 1999) was used to assess the total hydrophilic phenolic content of purple basil leaf extracts. Briefly, 3.0 mL of ultrapure water, 250 µL of Folin-Ciocalteu reagent 0.2 N and 250 µL of diluted purple basil leaf extract were added to a test tube and the solution was agitated for 5 min. Then, an aliquot of 250 µL of a 10% Na_2CO_3 solution (w/v) was added, and the volume was completed to 5.0 mL with ultrapure water. After 60 min reaction, the absorbance was registered at λ= 761 nm using a spectrophotometer (Shimadzu 1800, Japan). For quantification purposes, an analytical curve of gallic acid (10 to 70 µmol/L, $y = 0.01816x - 0.01015$; $R^2 = 0.9982$) as constructed and the total phenolic content was estimated. Data were expressed as mg of gallic acid equivalents per 100 g of fresh tissue (mg GAE/100 g).

Determination of Antioxidant Activity

The free radical scavenging activity toward DPPH radical was quantified according to a previously described method (Brand-Williams et al. 1995), with modifications. The reaction time was based on a kinetic assay of DPPH with different concentrations of the extracts. Briefly, an aliquot (1.5 mL) of an ethanolic solution of DPPH (2.2316 x 10^{-4} mol/L), 200 µL of sample, and 1.8 mL of ethyl alcohol were added to a test tube to a final volume of 3.5 mL. After 60 min reaction at 25 ± 1 °C, the absorbance was registered at λ = 517 nm and the DPPH inhibition of purple basil extracts was calculated using Equation 3.

$$\text{DPPH inhibition (\%)} = \frac{(A_{517nm \text{ of control}} - A_{517nm \text{ of sample}}) \text{ x } 100}{A_{517nm \text{ of control}}} \quad (3)$$

Stability Studies of the Anthocyanin Extract Effect of Temperature, Light and Copigmentation

The anthocyanins extract was diluted with ultrapure water according to the Lambert-Beer' law (maximum absorbance of 1.0 at λ = 516 nm) and the changes in pH was recorded using a previously calibrated pH meter (MICRONAL B-474). For that purpose, 8 mL of this solution were transferred into a capped test tube protected from light and then tubed were immersed in a water bath (Microquímica MQBTC 99-20) at different temperatures (60, 70, 80 and 90 °C). For each temperature, the absorbance was recorded at a maximum wavelength (λ = 516 nm) on times 0, 30, 60, 90, 120, 150 and 180 min. Using these data, the percentage of color degradation (% R), rate constant for anthocyanins degradation (k), half-life time ($t_{1/2}$),

and activation energy (E_A) were calculated using Equations 4, 5, 6 and 7, respectively (Reyes and Cisneros-Zevallos 2007).

$$\% R = (A_t/A_0) \times 100 \quad (4)$$

$$\ln (A_t/A_0) = -k \times t \quad (5)$$

$$t_{1/2} = \ln 2 / k \quad (6)$$

$$\ln k = - E_A/RT + \ln A \quad (7)$$

whereby: A_t = final absorbance; A_0 = initial absorbance; R = 8,314462 J K^{-1} mol^{-1}; T = absolute temperature (K).

The effect of light and copigmentation on the stability of anthocyanins was conducted according to the procedures described by Amr and Al-Tamimi (2007) and Awika (2008), with modifications. Three solutions of extract were prepared using a sodium citrate/citric acid buffer solution at 0.1 mol/L and pH 3.6. One of the solutions was considered the control (absence of copigments) and in the other phytic acid and gallic acid were added at a concentration of 7.5 x 10^{-4} mol/L. 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 (T = 32 ± 2 °C). The absorbance was recorded every 60 min at λ = 535 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 and Cisneros-Zevallos 2007) were calculated using Equations 5 and 6, respectively.

Statistical Analysis

Results are expressed as means followed by the standard deviation (n = 3). Differences among the five treatments were highlighted by one-way analysis of variances and means were compared using the Fisher's least significant difference test (LSD) after checking the homoscedasticity by the Bartlett's test. When data were heteroscedastic, Welch analysis of variances was applied (Nunes et al. 2015) Linear regression analysis was carried out to assess, quantitatively, the correlation between responses. Probability values below 5% were considered significant for inferential analysis (Granato et al. 2014a).

The effects of extraction time and temperature on the content of total phenolic content, flavonoids, anthocyanins, and antioxidant activity (DPPH assay) were assessed by means of the response surface methodology (Alvarez et al. 2015). For that purpose, the responses [E(y)] were described as a function of the extraction time (x_1), temperature (x_2), and their linear interaction (x_1x_2) using Equation: $E(y) = \beta_o + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2$, whereby β_o is a constant, and β_1, β_2 and β_{12} are regression coefficients. When the regression coefficient was not significant ($p > 0.10$), it was removed from the initial model and experimental data were refitted to provide the final multiple regression model and to generate 2-dimensional contour plots for the responses (Rebouças et al. 2014). All analysis were performed using Action v. 2.8 (Statcamp, Brazil) and Statistica v. 7 (Statsoft, USA) software.

RESULTS AND DISCUSSION

Bioactive Compounds and RSM Modeling

The concentration of total anthocyanins, flavonoids, total phenolic compounds and *in vitro* free radical scavenging activity toward DPPH radical is shown in Table I. It is possible to observe that the time and

temperature of extraction impacted significantly in all response variables ($p < 0.0001$). The *Ocimum basilicum* L. leaf extracts presented a high content of total phenolics (272 – 688 mg/100 g) in which about 14.49 to 16.12% are made of total flavonoids (40.49 – 106.23 mg/100 g). It is important to note that the content of anthocyanins ranged from 21.81 and 64.70 mg/100 g, representing from 8.0 to 10.3% of the total phenolic content (w/w fresh tissue).

Higher contents ($p < 0.05$) of total anthocyanins, flavonoids, total phenolic content and antioxidant activity were obtained when the extraction temperature and time were higher (t = 60 min and T = 30 °C) and this was corroborated by means of inferential statistical analysis (ANOVA/Welch-ANOVA followed by Fisher's least significance difference test). When purple basis was extracted using t = 40 min and T = 20 °C, high contents of bioactive compounds and antioxidant activity were also obtained, however, lower ($p < 0.05$) than those obtained with t = 60 min and T = 30 °C. On the other hand, when purple basil was extracted with using T = 10 °C and t = 20 min, the lowest values of all bioactive compounds and free radical scavenging activity were observed, indicating a poor extraction of antioxidant compounds.

After checking statistical differences among treatments, the main purpose of the current research work was to assess the quantitative effects of the response variables (extraction time and temperature) on the antioxidant compounds from purple basil leaves. For this purpose, multiple linear regression analysis based on the response surface methodology was used and results are shown in Table II and the contour plots generated using the experimental data are shown in Figure 1. Both extraction time and temperature were highly significant (*t*-value = 21.32 and 34.70, $p < 0.001$, respectively) in the extraction of total anthocyanins and the model was deemed significant ($R^2 = 0.897$ and adjusted $R^2 = 0.872$).

TABLE II

Regression coefficients obtained by multiple regression analysis (response surface methodology) to assess the effects of time and temperature of extraction of total anthocyanins, flavonoids, total phenolic content, and antioxidant activity of *Ocimum basilicum* L. leaf extracts.

Parameters	Regression coefficient	Standard error	*t*-value	*p*-value	-95% confidence limit	+95% confidence limit
Total anthocyanins						
Mean	2.418	1.131	2.138	0.076	-0.349	5.185
(1)Temperature (°C)	1.328	0.038	34.697	<0.001	1.234	1.422
(2)Time (min)	0.408	0.019	21.315	<0.001	0.361	0.455
R^2	0.897					
Adjusted R^2	0.872					
Total flavonoids						
Mean	31.753	3.988	7.961	<0.001	21.994	41.513
(1)Temperature (°C)	0.925	0.246	3.758	0.009	0.322	1.527
1 by 2	0.028	0.004	6.846	<0.001	0.018	0.038
R^2	0.916					
Adjusted R^2	0.895					
Total phenolic content						
Mean	147.421	48.659	3.029	0.023	28.357	266.485
(1)Temperature (°C)	7.294	2.188	3.333	0.016	1.940	12.648
(2)Time (min)	2.157	1.094	1.972	0.096	-0.520	4.834
1 by 2	0.115	0.048	2.344	0.057	-0.005	0.234
R^2	0.946					
Adjusted R^2	0.923					

TABLE II (continuation)

Parameters	Regression coefficient	Standard error	*t*-value	*p*-value	-95% confidence limit	+95% confidence limit
Free radical scavenging activity toward DPPH						
Mean	28.598	2.220	12.879	<0.001	23.165	34.031
(1)Temperature (°C)	1.177	0.075	15.663	<0.001	0.993	1.361
(2)Time (min)	0.266	0.037	7.094	<0.001	0.175	0.358
R^2	0.865					
Adjusted R^2	0.831					

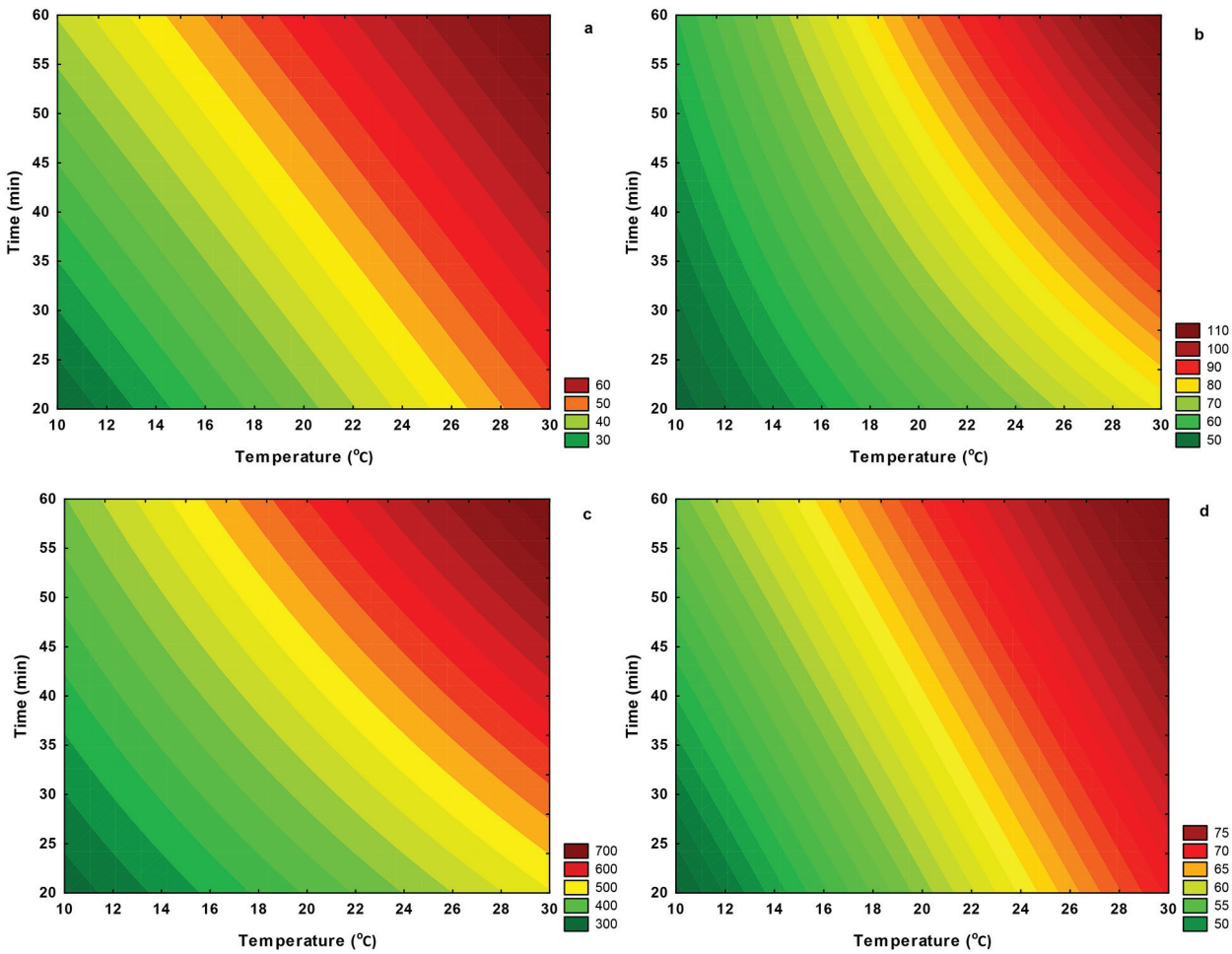

Figure 1 - Contour plots to project the experimental data obtained for the extraction of total anthocyanins **(a)**, total flavonoids **(b)**, total phenolic content **(c),** and free radical scavenging activity **(d)** of *Ocimum basilicum* L. extracts. All data are expressed as mg/100 g. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

For the total flavonoids content, the effect of temperature (*t*-value = 3.758, $p = 0.009$) and the linear interaction between time and temperature (*t*-value = 6.846, $p < 0.001$) were the parameters that influenced the response, while the effect of time (*t*-value = 1.409, $p = 0.208$) was not important. This model also presented a high coefficient of determination ($R^2 = 0.916$ and adjusted $R^2 = 0.895$), implying that more than 90% of data variability was explained by the multiple linear regression model (Bassani et al. 2014).

The higher the extraction time (t-value = 1.972, p = 0.096) and, more importantly the temperature (t-value = 3.333, p = 0.016), the higher the content of total phenolic compounds. The interaction between these two factors also influenced positively the extraction of phenolic compounds from purple basil (t-value = 2.344, p = 0.057). The regression model explained up to 95% of data variability, indicating the suitability of RSM in modeling the extraction of total phenolic content in the range studied.

The free radical scavenging activity toward DPPH radical was also modeled by means of RSM and results clearly indicated the statistical significance of extraction time (t-value = 7.094, $p < 0.001$) and temperature (t-value = 15.663, $p < 0.001$) on the response. Interestingly, the interaction of both independent variables did not affect significantly (t-value = -0.293, p = 0.780) the antioxidant activity of purple basil extracts under the conditions adopted in the current work. The proposed multiple regression model presented $R^2 = 0.865$ and adjusted $R^2 = 0.831$, indicating it was possible to model the effects of time and temperature on such a chemical response.

One of the most important advantages of using RSM is to provide useful regression equations that can be applied to predict certain responses based on pre-selected factors. In other words, if the equations generated are statistically significant (p-value of regression coefficients are below the stipulated α-value) and the equations can explain more than 70% of data variability ($R^2 > 0.70$), it is possible to assume the model can be used to predict the response on the basis of specific values of the factors (independent variables). In our case, all regression models generated presented $p < 0.10$ and $R^2 > 0.80$. So, if one considers the extraction of total anthocyanins conducted at T = 27 °C and t = 47 min, the mean theoretical value would be 57.46 mg/100 g (confidence interval at ± 95% = 56.37 – 58.54 mg/100 g). The same is applied to the other variables: if one wishes to have an idea of the experimental value to be obtained when the extraction of total flavonoids is conducted at T = 18.8 °C and t = 36 min, the mean concentration of flavonoids should be 68.15 mg/100 g and the confidence interval at ± 95% would be 64.21 and 72.08 mg/100 g. Clearly, it would be more valid if the analysis goes back to the laboratory and perform the experiment under the simulated conditions (T and t) and compare the experimental and theoretical values. If both results are in agreement (relative standard error below 10%), then the regression model can be regarded as significant and predictive (Granato et al. 2014b).

Similarly to what was observed in the current study, Bassani et al. (2014) used a 3^2 design of experiment aimed to model the effects of extraction time (5, 7.5, and 10 min) and temperature (60, 75, 90 °C) on the total content of phenolic compounds and total flavonoids content as well as on the antioxidant capacity (DPPH assay) of water-soluble extracts from roasted leaves of *Ilex paraguariensis* and authors obtained high R^2 values: 0.989 (antioxidant activity), 0.997 (total phenolic content), and 0.999 (total flavonoids). Pedro et al. (2016) used a Box–Behnken design to assess the effects of temperature (10 – 50 ºC), time (20-80 min), and solid-solvent ratio (1:15 – 1:45 w/v) on the extraction of total phenolic content, total flavonoids content, total anthocyanins, and *in vitro* antioxidant activity of extracts from *Oryza sativa* L. (black rice). Experimental data were subjected to RSM modeling and authors were able to identify the factors (alone and in combination) that affected the extraction of bioactive compounds and presented regression equations based on RSM to explain the results.

Using linear regression analysis, it was possible to analyze the correlation between the antioxidant activity toward DPPH radical and the total phenolic content (Figure 2a), flavonoids (Figure 2b), and total anthocyanins (Figure 2c). Not surprisingly, all correlations were highly significant ($p < 0.05$) from the statistical standpoint, showing the importance of such compounds in scavenging the free radicals in the

reaction medium. The proposed equations can be used experimentally (inside the range of the study) to have an idea of the antioxidant activity toward DPPH radical in terms of the content of bioactive compounds. For example, if one wishes to estimate the antioxidant activity of a purple basil extract containing 350, 87.50, or 53.90 mg/100 g of total phenolic content, total flavonoids, and total anthocyanins, respectively using the exact extraction conditions employed in the current work, one would obtain, theoretically, 52.17, 70.50, and 69.85% of DPPH inhibition, respectively.

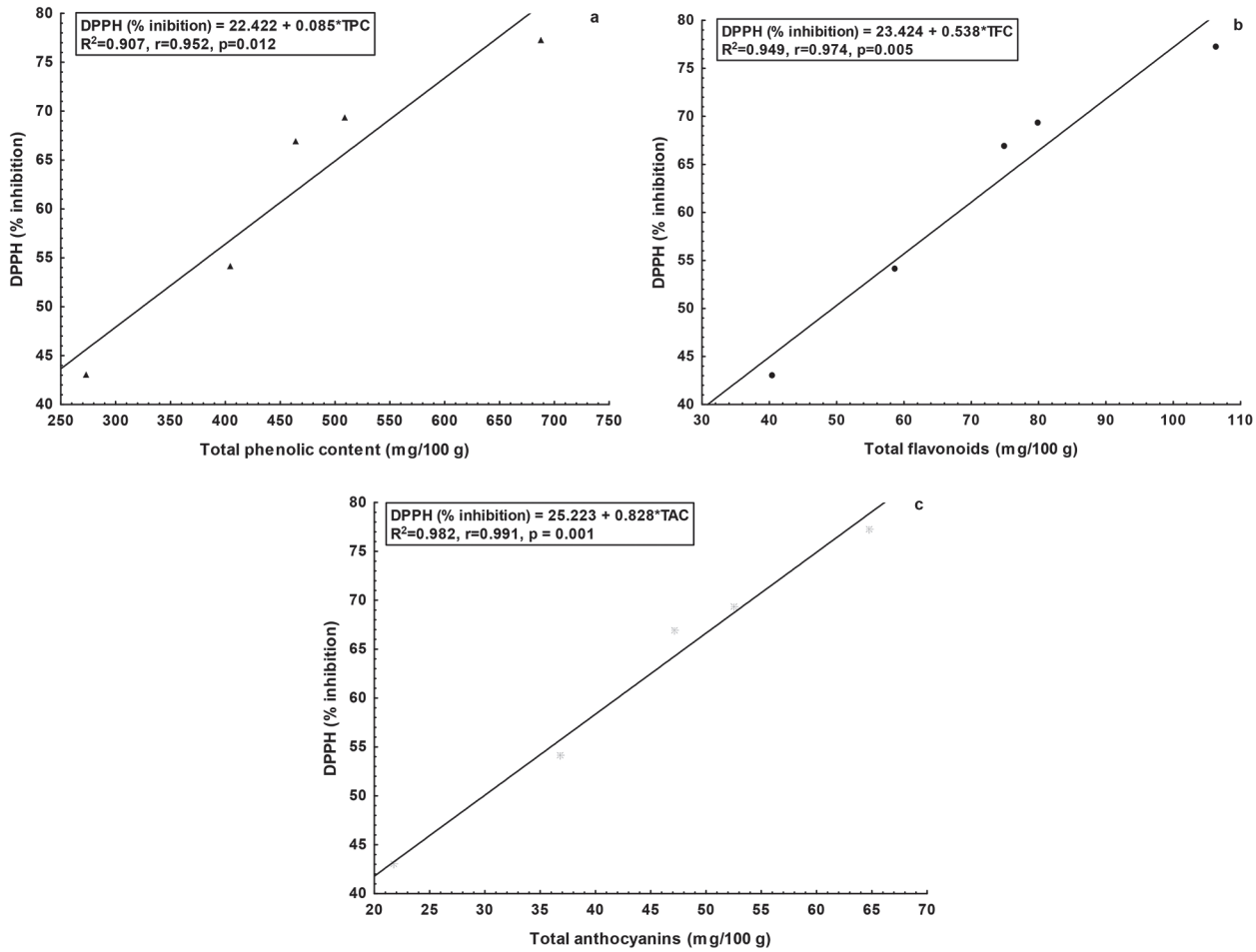

Figure 2 - Regression analysis based on the ordinary least-squares method between the free radical scavenging activity toward DPPH and the total content of phenolic compounds **(a)**, flavonoids **(b)**, and total anthocyanins **(c)**. Note that TPC = total phenolic content, TFC = total flavonoids content, and TAC = total anthocyanins content.

The antioxidant activity of phenolic compounds in a food extract depends on the number and type of functional groups present in the aromatic ring, such as the hydroxyl groups and the position they occupy, either *ortho* or *para*, as well as the presence of other groups. The combination of the non-binding electron pair of the hydroxyl group with the PI system (л) of the aromatic ring strongly activates the *ortho* and *para* positions. Similarly, other functional groups, such as alkyl and methoxyl weakly activate the *ortho* and *para* positions by hiperconjugative effects. These groups favor electron delocalization and stabilize the phenoxyl radical formed on oxidation processes (Rice-Evans et al. 1996).

Stability Study of Purple Basil Anthocyanins Effect of Temperature

From the study of the effect of temperature on the stability of anthocyanins extracted from purple basil, a linear behavior was observed, indicating that the thermal degradation followed first-order kinetics. Table III shows the degradation rate constant (*k*), the half-life time ($t_{1/2}$) and color retention percentage (% R) of the anthocyanins as the temperature increased. These parameters show that the rate of degradation reaction increased when the temperature was raised as a function of exposure time. It is observed that the half-life decreased markedly according to the increase in temperature. Patras et al. (2010) suggested that foods rich in anthocyanins should be manufactured at low temperatures and be stored for reduced periods of time. This provides greater retention of color and stability to the pigment.

TABLE III
Effects of temperature on the degradation of anthocyanins as noted by the first-order rate constant (*k*), half-life time ($t_{1/2}$), and retention of color (R).

Temperature (°C)	*k* (h^{-1})	[a] R^2	$t_{1/2}$ (h)	R (%)	[b] *p*-value
60	0.01288	0.9974	53.82	0.963	<0.0001
70	0.02261	0.9990	30.66	0.935	0.0226
80	0.02515	0.9977	27.56	0.915	<0.0001
90	0.05466	0.9935	12.68	0.887	0.0006

[a] R^2 = coefficient of determination; [b] *p*-value = probability value obtained by analysis of variances.

The activation energy for the reaction of anthocyanins degradation was 44.95 kJ/mol. Mourtzinos et al. (2008) found that the activation energy was 54 kJ/mol for anthocyanins extracted from *Hibiscus sabdariffa* L. According to Silva et al. (2010) excessive heating may promote the loss of anthocyanin color and cause the opening of the aromatic ring chalcones forming colorless compounds (Katsaboxakis et al. 1998, Heredia et al. 1998). Reduced values of activation energy suggest that the anthocyanin extract is susceptible to degradation when temperature is increased (Hou et al. 2013).

Effect of Light and Copigmentation

Table IV shows the rate constants and half-lives for reaction of anthocyanins degradation toward light in the purple basil with or without copigments. It was found that after 24 hours, all extracts exposed to light showed no significant difference in the half-life (control - 17.08 h, phytic acid -15.61 h and gallic acid - 17.20 h). However, all extracts protected from light showed a remarkably higher half-life than the extracts exposed to light. Lima et al. (2005) observed that the anthocyanin extracts from purple cherry showed greater stability when protected from light. Bąkowska et al. (2003) found that the heating and UV irradiation affect negatively the stability of anthocyanins.

The half-life for the extracts protected from light in the presence of phytic and gallic acids was 923.17 and 856.49 h, after 24 hours, respectively, whereas half-life of the control was 152.67 h. The considerable increase in half-life of the extracts in the presence of phytic and gallic acids compared to the control shows the effect of cogpigmentation on the chemical stabilization of anthocyanins. Copigments have *pi* electron and may link with the flavylium ion. This combination protects the anthocyanin molecule against nucleophilic attack by water and consequently increases its chemical stability (Mazza and Brouillard 1990).

The interaction of copigments with the anthocyanins gives the bathochromic effect (increase of the maximum absorbance) and hyperchromatic effect (shift of the peak wavelength) (Bąkowska et al. 2003).

TABLE IV
Effects of light and copigments on the first-order rate constant (*k*) and half-life time ($t_{1/2}$) on the degradation of anthocyanins extracted from *Ocimum basilicum* L. leaf.

Extract	k (h^{-1})	[a] R^2	$t_{1/2}$ (h)	[b] *p*-value
Control (protected)	0.00454	0.9998	152.67	<0.0001
Control (exposed)	0.04058	0.9963	17.08	<0.0001
Phytic acid (protected)	0.00075	0.9969	923.17	<0.0001
Phytic acid (exposed)	0.0444	0.9977	15.61	<0.0001
Gallic acid (protected)	0.00081	0.9985	856.49	<0.0001
Gallic acid (exposed)	0.04029	0.9987	17.20	<0.0001

[a] R^2 = coefficient of determination; [b] *p*-value = probability value obtained by analysis of variances.

The increase in half-life of anthocyanin extracts (protected from light) in the presence of copigments yielded a significant increase in the chemical stability of the extracts. Gauche et al. (2010) observed an increased half-life of anthocyanins from Cabernet Sauvignon grapes added with tannic and gallic acids as compared to the control. Gris et al. (2007) found that a solution containing anthocyanins extracted from Caberbet Sauvignon grapes in the presence of caffeic acid and absence of light presented an increased half-life compared to the control.

Some experimental parameters, such as solvent polarity, temperature, extraction time, and solvent: food matrix ratio are important to extract bioactive compounds from a certain source. The most commonly extraction solvents used are methanol and ethanol in acidic medium. Under these conditions the flavylium cation of anthocyanins (red color) is more stable. Strong acids can hydrolyze the structure of the molecule, while weak acids such as citric and tartaric acids are preferred (Strack and Wray 1989). Methanol is an efficient solvent for extraction of many bioactive compounds, however, its degree of toxicity should be considered, especially by food companies (Escribano-Bailón et al. 2004). Thus, considering the stability of anthocyanins in acidic medium and the toxicity of the solvent, ethanol in the presence of citric acid was used in the current work to extract antioxidant compounds from purple basil. The use of this solvent can facilitate the application of the extract in food products due to its low toxicity.

As a conclusion, RSM was shown to be a suitable statistical approach to model the effects of time and temperature on the extraction of bioactive compounds as well as antioxidant activity from purple basil leaf. As the anthocyanins extract was stable at moderate temperature, the application of such extract in food matrix may be advantageous over synthetic colorants. In this sense, further works should focus on the application of purple basil extracts in beverages and other foods and study their sensory properties and chemical stability during shelf life.

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RESUMO

No presente estudo, a metodologia de superfície de resposta (MSR) foi utilizada para avaliar o efeito de tempo e temperatura de extração no conteúdo de compostos bioativos e atividade antioxidante de extratos de folhas de manjericão

roxo (*Ocimum basilicum* L.). A estabilidade das antocianinas relacionada à temperatura, luz e copigmentação também foi estudada. O maior conteúdo de antocianinas foi 64,70 mg/100 g a 30 °C e 60 min. A degradação das antocianinas com diferentes temperaturas e em presença de luz seguiu uma cinética de primeira ordem e a energia de ativação foi 44,95 kJ/mol. Todos os extratos expostos à luz apresentaram tempos de meia vida semelhantes. Enquanto que os extratos protegidos da luz em presença dos copigmentos apresentaram um aumento do tempo de meia vida: 856,49 h para o ácido gálico e 923,17 h o ácido fítico enquanto que para o controle foi de 152,67 h. Estes resultados indicam claramente que o manjericão roxo é uma potencial fonte de compostos bioativos estáveis.

Palavras-chave: antocianinas, extração, pigmentos, metodologia de superfície de resposta.

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