REGULAR ARTICLE

Optimization of phenolic compounds extraction with antioxidant activity from açaí, blueberry and goji berry using response surface methodology

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ABSTRACT

The aim of this study was to determine the best extraction conditions of phenolic compounds present in açaí, blueberry and goji berry fruits using the response surface methodology (RSM). The phenolic compounds profile by high-performance liquid chromatography, antioxidant activity was also determined. A factorial 2^3 design was used to analyze the effect of the solvent (ethanol and water), time (30 and 60 min) and temperature (30 °C and 60 °C) on the extraction of total phenolic compounds (TPC) and activity antioxidant (AA). The variables time and temperature had a positive effect on antioxidant activity (AA) in their highest levels, 60 min and 60 °C, respectively. The solvent ethanol 800 g/mL was more efficient in TPC extracting with AA in all matrices. Rutin was present in high amounts in blueberry and goji berry, and the myricetin in açaí. The açaí showed higher *in vitro* antioxidant activity when extracted at 60 °C for 60 min. The high correlation coefficient (0.98) of global response (GR) showed that we can find out single and global response in research with multiple dependent variables. The GR analysis indicated the highest values of the TPC and AA when the fruits were extracted at 60°C for 60 min using ethanol as solvent and it was very useful for simplifying and improving the phenolic compounds extraction performance.

Keywords: Antioxidant activity; Extraction conditions; Factorial design; HPLC/DAD; Phenolic compounds

INTRODUCTION

The food function goes beyond only nurture (Moraes et al., 2007; Kang et al., 2012; Silva et al., 2014), but may also be related to disease prevention (Castrejón et al., 2008; Paz et al., 2015), leading the consumer to increasingly opt for healthier foods (Swieca, 2015). Fruits can be natural sources of antioxidants such as vitamins (Oliveira et al., 2011; Leong and Oey, 2012), carotenoids (Souza et al., 2014; Valdivielso et al., 2015) and phenolic compounds (Kang et al., 2012; Paz et al., 2015) with antioxidant properties (Castrejón et al., 2008; Koca and Karadeniz, 2009; Kang et al., 2012). The antioxidant activity of these compounds may act at different stages in the oxidation process, lowering free radicals concentration, chelating ions and even decomposing primary products and leading to non-radical compounds (Lobo et al., 2010; Perera et al., 2016) which help balance the immune system (Kang et al., 2012).

Açaí and blueberry stand out mainly for the presence of red pigments called anthocyanin (Su and Chien, 2007, Kang et al., 2011, Rodrigues et al., 2011; Gordon et al., 2012). In addition to anthocyanins, blueberry is an excellent source of quercetin, kaempferol, myricetin, procyanidins, catechin, epicatechin, resveratrol and vitamin C, which contribute to antioxidant activity and bring health benefits to people (Rodrigues et al., 2011; Norberto et al., 2013). The Amazon açaí is widely consumed in Brazil but is already part of the eating habits of vast majority of the world's population. Its fruit exhibit pharmacological and medicinal properties mainly anticarcinogenic (Choi et al., 2017; Wang et al., 2016), anti-inflammatory (Favacho et al., 2011; Kang et al., 2011) Kang et al. (2012) and antimicrobial (Shen et al., 2014; Belda-Galbis et al., 2015) activities. Amazon açaí, in addition to its nutritional qualities, is of great importance for the development of the Amazon region (Gordon et al., 2012).

Goji berry, a fruit originated from Asian countries such as China and India, has been used for many years in herbal medicine (Carnés et al., 2013; Donno et al., 2015). This fruit is considered a functional food of great importance for the China and has become increasingly common in the Europe and North America (Li et al., 2007, Dong et al., 2009; Carnés et al., 2013). Goji berry has gained prominence in recent years in the scientific community due to its anti-inflammatory (Potterat, 2010; Nardi et al., 2016), antioxidant (Amagase and Farnsworth, 2011; Donno et al., 2015) and antitumor (Wawruszak et al., 2016) activities. In addition, the Gojy berry may be effective in prophylaxis of diseases, such as diabetes, and cardiovascular diseases (Kulczyński and Gramza-Michalowska, 2016).

Given the economic and nutritional importance of these species, some studies regarding the extraction process and optimization of extraction conditions are necessary. Obtaining biologically active compounds involves many factors and the experimental design is an adequate methodology to experimentation, which allows the reduction in the number of the tests without prejudice to the quality of the information. Thus, the objective of this study was to investigate the influence of different factors (time, temperature and nature of solvent) on phenolic compounds extraction present in açaí, blueberry and goji berry fruits, through RSM. The chromatographic profile analysis and antioxidant activity of the best extract condition were aim of this work, too.

MATERIAL AND METHODS

Samples
Samples of açaí, blueberry and goji berry were obtained in the Municipal Market of Curitiba - Paraná, Brazil. The fruits were lyophilized (Liotop - L1019, São Paulo, Brazil) and ground in an analytical mill and stored at -12ºC.

Preparation of extract and experimental design
The 2^3 factorial design was used to evaluate the effect of solvent, temperature and time over phenolic compounds extraction. The design was composed of eight trials performed in triplicate. Independent variables were: ethanol 800 g/L and pure water as solvent (variable X1), extraction time of 30 min and 60 min (variable X2), and extraction temperature of 30 °C and 60 °C (X3 variable) while dependent variables were antioxidant activity (AA) and total phenolic compounds (TPC).

The extraction of antioxidant compounds were performed according to Ribeiro et al. (2007). Samples containing 3 g of lyophilized fruits were subjected to the extraction process with 30 mL of solvent in a shaker (Solab SL222,

Piracicaba, Brazil) according to the experimental design shown in Table 1. The extracts were transferred to Falcon tube and centrifuged at 447 x g for 15 min (Hermle Z 200 A, Wehingen, Germany). After filtrating, the supernatants were stored in a freezer at -12 °C. Each extraction was performed in triplicate amounting to 24 trials for each fruit.

Total phenolic compounds assay
Total phenolic compounds (TPC) were quantified by the Folin-Ciocalteu method described by Singleton et al. (1999) using gallic acid as the standard.

Anthocyanin assay
Total anthocyanin content (TAC) was determined using the pH-differential method (Giusti and Wrolsted, 2001).

Antioxidant activities
Four methods were used to determine the antioxidant capacity of the samples: DPPH (2,2-diphenyl-1-picryl-hydrazyl) radical scavenging, ABTS (2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)), coupled oxidation of β-carotene and linoleic acid and ferric reducing antioxidant power (FRAP).

DPPH radical scavenging assay
Measurement of DPPH scavenging activity was performed according to the methodology described by Brand-Williams et al. (1995). The results were expressed as EC_{50} (concentration required to obtain a 50% antioxidant effect) and µmol Trolox/g sample. Absorbances were read in spectrophotometer (Bel Photonics 2000, Piracicaba, Brazil) at 517 nm and the tests were performed in triplicate.

ABTS (2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)) assay
The antioxidant activity by the ABTS method was performed according to Re et al. (1999), in UV/Vis spectrophotometer (Bel Photonics 2000, Piracicaba, Brazil) at 734 nm. Trolox was used as reference and the results of the antioxidant activity were expressed as µmol of trolox equivalent antioxidant capacity (TEAC)/g of sample. All analyses were carried out in triplicate.

Coupled oxidation of β-carotene and linoleic acid assay
The measure of antioxidant activity was determined by the coupled oxidation of β-carotene and linoleic acid according to Ahn et al. (2004). Emulsion oxidation was spectrometrically monitored (Bel Photonics 2000, Piracicaba, Brazil) and the absorbance was read at 470 nm, at time zero (t = 0) and subsequently after every 20 min, until the characteristic color of β-carotene disappeared in the control reaction (t = 100 min). The antioxidant

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Table 1: Factor delineation for total phenolic compounds and antioxidant activity in açaí, blueberry and goji berry

Assay	X1	X2	X3	Açaí		Blueberry		Goji berry	
				TPC (mg GAE/g)	AA (µmol Trolox/g)	TPC (mg GAE/g)	AA (µmol Trolox/g)	TPC (mg GAE/g)	AA (µmol Trolox/g)
A1	Water (-1)	30 (−1)	30 (−1)	18.68[c]	33.93[e]	6.01[e]	9.18[c]	15.14[e]	8.89[g]
A2	Ethanol (+1)	30 (−1)	30 (−1)	14.46[g]	45.73[d]	13.33[c]	30.51[b]	11.77[g]	14.40[d]
A3	Water (-1)	60 (+1)	30 (−1)	19.02[b]	31.00[g]	6.19[e]	8.89[c]	15.74[c]	9.76[e]
A4	Ethanol (+1)	60 (+1)	30 (−1)	15.55[f]	52.44[b]	15.04[b]	32.96[b]	15.18[e]	15.39[c]
A5	Water (-1)	30 (−1)	60 (+1)	17.90[d]	31.87[f]	6.93[e]	10.40[c]	15.49 [d]	9.79[f]
A6	Ethanol (+1)	30 (−1)	60 (+1)	15.60[e]	47.14[c]	15.76[b]	32.87[b]	13.84[f]	16.29[b]
A7	Water (-1)	60 (+1)	60 (+1)	21.31[a]	27.22[h]	8.98 [d]	12.43[c]	21.62[a]	11.86[e]
A8	Ethanol (+1)	60 (+1)	60 (+1)	18.69[c]	57.91[a]	17.91[a]	49.53[a]	16.59[b]	19.40[a]
Pooled SD	-	-	-	2.36	10.91	4.32	13.79	2.77	3.64

The experiments were performed in duplicate and the results expressed as mean (n=2); Runs: assay A1 to A8 - combination of solvent, time and temperature; variable x1: solvent; variable x2: temperature (°C); variable x3: time (min); Values followed by different letters in the same column are significantly different (*P*<0.05). TPC: Total phenolic compounds; AA: Antioxidant activity (DPPH method).

activity was determined as percent inhibition relative to control sample.

Ferric reducing antioxidant power (FRAP) assay
Antioxidant activity determination using the FRAP method was performed as described by Pulido et al. (2000). The absorbance was measured in UV/Vis spectrophotometer (Bel Photonics 2000, Piracicaba, Brazil) at 593 nm. Aqueous solutions of ferrous sulfate were used for calibration, and the results were expressed as µmol of Fe^{2+}/g of the sample. All analyses were carried out in triplicate.

HPLC-DAD-UV-Vis profile
The extracts obtained under optimum conditions as indicated by the factorial design were rotary evaporated (Fisatom® 802, Sao Paulo, Brazil) and lyophilized (Liotop - L1019, São Paulo, Brazil). Aliquots 10 µL at 0.1 g/mL of extract concentration were injected into a chromatograph coupled to a photodiode array (PDA) detector (Varian, 920-LC, Walnut Creek, US) using a C18 RP (250 x 4,6 mm, 5 µm) column. For the fractionation, a binary gradient system was used in which the mobile phase "A" consisted of ultrapure water and phase "B" consisted of acetonitrile, both containing 0.2 mL/L acetic acid. The gradient started with 5 % of B up to 95% of B in 36 min and returned to the initial condition. Total analysis time was 45 min at a flow rate of 1 mL/min and the temperature during the analysis was maintained at 30 °C. Calibration curves and linear regression based on the peak areas were used to identify and quantify peaks corresponding to the phenolic compounds. The identification was performed by comparison of retention times and absorption in ultraviolet at wavelengths of 280 nm and 320 nm. These calibration curves were obtained using external standards of catechin, caffeic acid, ferulic acid, epicatechin, coumaric acid, gallic acid, rutin, and myricetin. All standards were dissolved in phase "B" at the following concentrations:

2 µg/mL; 4 µg/mL; 8 µg/mL; 16 µg/mL; 30 µg/mL. These concentrations were used afterwards for obtaining the limit of quantification (LQ) of 0.35 µg/mL and the detection limit (LD) of the equipment of 0.12 µg/mL employing standards of gallic acid, vanillic acid, caffeic acid, coumaric acid, and ferulic acid according to Oldoni et al. (2015). The content phenolic compounds were expressed for each compound in µg/g of sample. Determination of the phenolic compounds by HPLC was performed in triplicate.

Statistical analysis
The set of data and contents of total phenolic compounds derived from the factorial design were analyzed by response surface methodology (RSM). The anthocyanin content and the antioxidant activity were carried out by principal component analysis (PCA). The data were processed by one-way analysis of variance (ANOVA). The averages were compared by Tukey test, considering the significance level of 95% (p<0.05), using the STATISTICA program 8.0 version (StatSoft, USA). Past Software 3.07 developed by Hammer et al. (2001) expressed the reproducibility of the results as Pooled Standard Deviation (Pooled SD). The global response (GR) was carried out over the data set too. GR was calculated according to equation (1):

$$RG = \left[\frac{R(x_1)}{MR(x_1)} + \frac{R(x_2)}{MR(x_2)} + \ldots + \frac{R(x_n)}{MR(x_n)} \right] \quad (1)$$

Where: $R(x_n)$ is the response for each dependent variable, $MR(x_n)$ is the maximum value of response for each dependent variable. All experiments were carried out three times.

RESULTS AND DISCUSSION

Total phenolic compounds (TPC) content and antioxidant activity (AA) in fruit extracts after being subjected to

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various treatments (A1 to A8) according to factorial design are summarized in Table 1. Extracts obtained a specific and characteristic coloring for each fruit, differing according to the solvent extractor. These variations can be caused by the diversity of compounds each solvent can extract. It is depending on their polarity, thus providing different colors for each respective extract.

The total average of TPC content in açaí extracts ranged from 14.46 to 21.31 mg GAE/g sample and AA from 27.22 to 57.91 μmol of Trolox/g sample. Açaí TPC content in assays A1 (water, 30 °C, 30 min) and A8 (ethanol, 60 °C, 60 min) did not differ statistically by the Tukey test ($p < 0.05$). The lower TPC content was found in assay A2 (ethanol, 30 °C, 30 min) and significantly differ from the others treatments. The greatest TPC content in the açaí samples was found at A7 treatment (water, 60 °C, 60 min), but with lower AA (Table 1). In this study, the results of TPC and AA from açaí were lower than those reported by Kang et al. (2012) in açaí from Para State, Brazil. The values ranged from 31.2 to 73.0 mg GAE/g sample and 133.40 to 320.30 μmol of trolox/g sample, respectively. However, Paz et al. (2015) showed levels of 1.81 mg GAE/g sample and 1.57 mg of Trolox/g açaí from Brazil's Amazon Forest, for phenolic compounds and antioxidant activity, respectively.

Blueberry extract showed a variation from 6.01 to 17.91 mg GAE/g sample and from 8.89 to 49.53 μmol of trolox/g sample for TPC and AA, respectively. The highest phenolic compounds content found in blueberry was in treatment A8. In this condition (A8 assay) the compounds extracted from blueberry had the highest AA and significantly differed from the other treatments (Table 1). This shows ethanol was the most suitable solvent for the extraction of phenolic compounds in blueberry. Pertuzatti et al. (2014), studied ten blueberry Brazillian cultivars and found small amounts of TPC, those values ranged from 1.62 to 3.45 mg GAE/g of açaí. For other hand, Castréjon et al. (2008) studying blueberry varieties in different ripening stages from Berlin, Germany, found high TPC levels from 17.3 to 52.6 mg GAE/g sample.

The TPC and AA for goji berry extracts ranged from 11.77 to 21.62 mg GAE/g sample and from 8.89 to 19.40 μmol of Trolox/g sample, respectively. The A7 treatment showed the highest TPC and significantly differed from other treatments. The water is the most suitable solvent for phenolic compounds extraction from goji berry, however, the largest AA values for this fruit extract was found in assay A8, with 19.40 μmol of Trolox/g sample (Table 1). Our results showed lower TPC values than found by Donno et al. (2015) in the goji berry from Alzate di Momo in the Northern Italy, who found phenolic compounds levels ranging from 255.87 to 281.81 mg GAE/g sample.

These differences are usually caused by the great diversity of chemical compounds that can be extracted depending on the methodology used.

The wide range of studies related to conditions and extraction methods of phenolic compounds and antioxidant activity makes it difficult to perform an effective comparison. In addition, the large differences in TPC and AA values observed in these studies can be also attributed to different climactic conditions during cultivation of the fruits due to the different regions where each one fruit was planted. The harvest season is also an important factor for comparison.

The analysis of variance (ANOVA) of the dependent variable; TPC and AA from açaí, blueberry and goji berry subjected to extraction conditions presented by the factorial design is shown in Table 2. The factors time, temperature and solvent nature in the extraction of phenolic compounds and AA were significant ($p < 0.05$) for all fruits. The analysis of variance, F_{calc} for all response variables (TPC and AA) was always greater than $F_{tabulated}$ (4.45), and in some cases, this amount was about 80 times greater than $F_{tabulated}$ (Table 2). The lower ratio found in F_{calc} by $F_{tabulated}$ in the analysis of variance was 5.85. These results showed that the empirical data were adequately adjusted to the proposed models.

The global response (GR) showed that the kind of solvent was the mostly independent variable to extract the phenolic compounds from açaí, blueberry and goji berry. The effect estimated for solvent type was 1.34, while for extraction time and extraction temperature were 0.52 and 0.51, respectively. All effect estimated were significant ($p_{value} < 0.0000$). Thus the best conditions for phenolic compounds extraction with antioxidant activity from fruits was found when the ethanol was used as extractor solvent in the extraction temperature at 60°C during 60 minutes (Table 3).

The entire data set was adjusted by multiple linear regression and six linear mathematical models were generated established by factorial design and response surface methodology (RSM) (Table 4). The TPC and AA answers were significant at the 5% level for each fruit (Table 4). For each model, we observed a high coefficient correlation varying from 0.81 to 0.98, thus 81 to 98% of data can be explained by the proposed models (Table 4).

Each multiple linear regression model generates a response surface, where the dependent variables (TPC and AA) are shown on the z axis as a function of the independent variables; solvent nature, extraction time and extraction temperature (Figs. 1 to 3). High values TPC from açaí were obtained with pure water as solvent at 60 °C during

Table 2: Analysis of variance of responses of the TPC and AA in fruits subjected to treatments according to a Fractional Design

Source of variation	Açaí/Dependent variable: Phenolic compounds total					Açaí/Dependent variable: Antioxidant activity by DPPH method				
	SS	DF	MS	F_{calc}	*p value*	SS	DF	MS	F_{calc}	*p value*
Solvent (X1)	*59.7242*	*1*	*59.72415*	*39.54818*	*0.000008*	*2442.325*	*1*	*2442.325*	*350.8172*	*0.000000*
Time (X2)	*23.6017*	*1*	*23.60167*	*15.62857*	*0.001026*	*59.633*	*1*	*59.633*	*8.5657*	*0.009414*
Temperature (X3)	*12.4993*	*1*	*12.49927*	*8.27677*	*0.010461*	2.026	1	2.026	0.2910	0.596571
X1;X2	0.0661	1	0.06615	0.04380	0.836708	*187.416*	*1*	*187.416*	*26.9206*	*0.000074*
X1;X3	2.8704	1	2.87042	1.90073	0.185869	*48.932*	*1*	*48.932*	*7.0286*	*0.016800*
X2;X3	*9.6774*	*1*	*9.67740*	*6.40819*	*0.021515*	0.010	1	0.010	0.0014	0.970142
Residue	25.6728	17	1.51016			118.351	17	6.962		
Total	134.1118	23				2858.692	23			

Source of variation	Blueberry/Dependent variable: Phenolic compounds total					Blue berry/Dependent variable: Antioxidant activity by DPPH method				
	SS	DF	MS	F_{calc}	*p value*	SS	DF	MS	F_{calc}	*p value*
Solvent (X1)	*412.4275*	*1*	*412.4275*	*1108.144*	*0.000000*	*4132.223*	*1*	*4132.223*	*730.878*	*0.000*
Time (X2)	*8.8452*	*1*	*8.8452*	*23.766*	*0.000142*	*163.104*	*1*	*163.104*	*28.849*	*0.000*
Temperature (X3)	*19.2425*	*1*	*19.2425*	*51.702*	*0.000002*	*210.326*	*1*	*210.326*	*37.201*	*0.000*
X1;X2	0.0176	1	0.0176	0.047	0.830418	*113.144*	*1*	*113.144*	*20.012*	*0.000*
X1;X3	0.0513	1	0.0513	0.138	0.714928	*75.275*	*1*	*75.275*	*13.314*	*0.002*
X2;X3	0.8932	1	0.8932	2.400	0.139755	*102.308*	*1*	*102.308*	*18.096*	*0.001*
Residue	6.3270	17	0.3722			96.114	17	5.654		
Total	447.8044	23				4892.494	23			

Source of variation	Goji berry/Dependent variable: Phenolic compounds total					Goji berry/Dependent variable: Antioxidant activity by DPPH method				
	SS	DF	MS	F_{calc}	*p value*	SS	DF	MS	F_{calc}	*p value*
Solvent (X1)	*51.8322*	*1*	*51.83220*	*32.09225*	*0.000028*	*245.504*	*1.000*	*245.504*	*276.756*	*0.000*
Time (X2)	*51.8910*	*1*	*51.89100*	*32.12866*	*0.000028*	*16.537*	*1.000*	*16.537*	*18.642*	*0.000*
Temperature (X3)	*41.8968*	*1*	*41.89684*	*25.94070*	*0.000090*	*32.313*	*1.000*	*32.313*	*36.426*	*0.000*
X1;X2	0.2262	1	0.22620	0.14006	0.712853	0.910	1.000	0.910	1.026	0.325
X1;X3	5.4626	1	5.46260	3.38221	0.083437	2.331	1.000	2.331	2.628	0.123
X2;X3	5.4435	1	5.44354	3.37040	0.083932	*5.191*	*1.000*	*5.191*	*5.852*	*0.027*
Residue	27.4567	17	1.61510			15.080	17.000	0.887		
Total	184.2091	23				317.867	23.000			

* Values in italic refer to significant differences, $F_{0.05;2;17}$=3.59, $F_{0.05;3;17}$=3.20, $F_{0.05;5;17}$=2.81; SS: Sum of squares; DF: Degree of freedom; MS: Mean of squares

60 min (Fig. 1a). On the other hand, ethanol (800 g/L) at a temperature of 60 °C and for 60 min was the best extraction condition for phenolic compounds with high antioxidant activity (Fig. 1b).

The best solvent to extract goji berry TPC was pure water (Fig. 2a), however, this solvent was not the best option for extracting phenolic compounds with antioxidant activity, in this case ethanol 800 g/L obtained better results (Fig. 2b). The variables time (60 min) and temperature (60 °C) had a positive effect on TPC and AA (Fig. 2 ab).

The best extraction conditions of blueberry phenolic compounds were ethanol 800 g/L at 60 °C during 60 min, showing the most extreme time and temperature conditions led to an increased TPC extraction (Fig. 3a) with greater AA (Fig. 3b). Generally, this same result was observed with the other fruits. Thus, the optimum extraction condition for phenolic compounds with high antioxidant activity was obtained with the ethanol (800 g/L) at 60 °C during 60 minute of extraction.

Oldoni et al. (2015), evaluated the extraction conditions of phenolic compounds from propolis with different ethanol concentrations, temperature and time. The authors reported that the best condition was found with ethanol (800 g/L) at 70 °C during 45 min of extraction. Melo et al. (2015) evaluated the best ratio of ethanol and extraction time in winery by-products, and concluded that moderate ethanol concentrations (430 g/L and 570 g/L), combined with high temperatures (96 °C) were the best conditions for antioxidants extraction.

The principal component analysis (PCA) was performed on the data set of anthocyanin and antioxidant activity levels. Two major components were identified, with 99.50% of explained variance, PC1 for 88.16% variation, and PC2 for 11.33% (Fig. 4ab). There was the formation of three groups represented by the analyzed fruits (açaí, blueberry and goji berry) and distributed in the quadrants of the PCA scores chart (Fig. 4a).

The first group is represented by açaí (second quadrant Fig. 4b), which has a high antioxidant activity by ABTS,

β-carotene and FRAP. Blueberry extract (third quadrant Fig. 4b) showed high anthocyanin content. The other group, also as sole representative was the goji berry group (quadrants 1 and 4), which showed high EC_{50} values. The two most important dependent variables in groups formation and occurrence were the antioxidant activity by FRAP and EC_{50}, taking into account their commonalities. The least important variable in group classification was anthocyanin content. Groupings of açaí, blueberry and goji berry fruit extracts because of their antioxidant activities and anthocyanin content could be analyzed by PCA. In addition, ANOVA test and PCA confirmed the anthocyanin and antioxidant activity results significantly contributing to improve the knowledge of this three fruits analyzed.

Fruit extracts chemical characterization and antioxidant activity in the best extraction condition

The extracts of the three fruits in the best extraction condition (ethanol at 60 °C and 60 min of extraction), A8 assay, were analyzed for total anthocyanin, AA by four different methods (EC_{50}, FRAP, ABTS and β-carotene) and phenolic compounds profile by HPLC/DAD.

Total anthocyanin content ranged from 4.17 to 874.17 mg/100 g of fruit (Table 5). The highest and lowest anthocyanin concentrations were found in blueberry and goji berry, respectively, with a statistically significant difference (Table 5). In this study the total anthocyanin content of the blueberry (874.17 mg/100 g) was found to be higher than fiveten blueberry Brazillian cultivars studied (40.62 to 378.31 mg/100 g), which were reported by Rodrigues et al. (2011). Our results for AA from açaí was higher than the findings of Yuyama et al. (2011) and Borges et al. (2016), who reported anthocyanin content in açaí from Brazil ranging between 128.40 to 868.91 mg/100 g and 40.6 to 74.2 mg/100 g, respectively. The differences between the values found in this study can be due to methodological differences employed in the extraction of this compound class (Table 5).
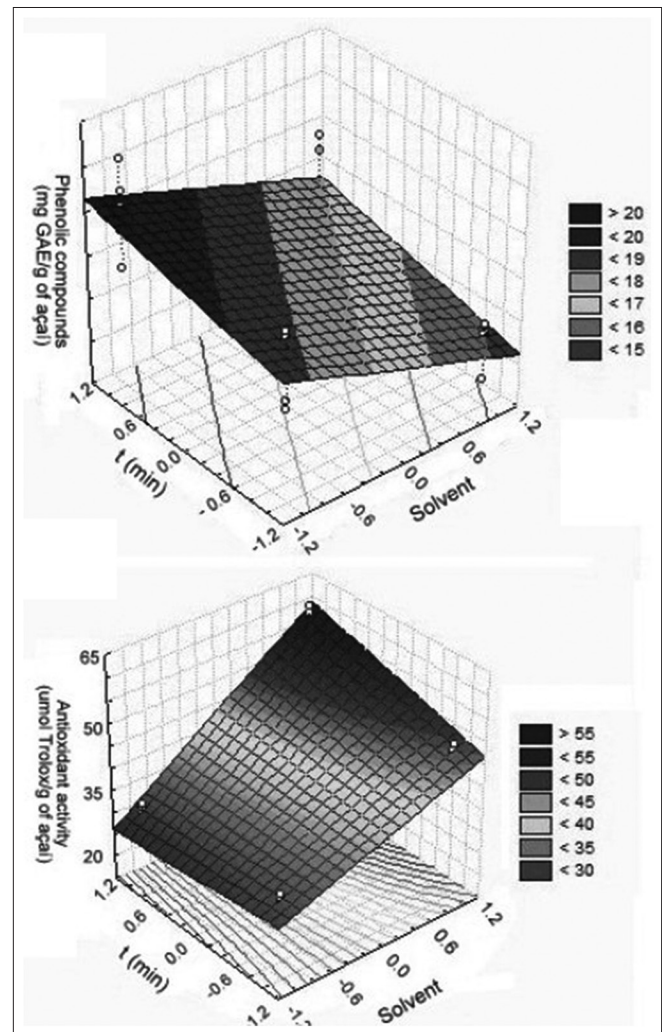
Table 3: Analysis of variance of global response in fruits subjected to treatments according to a Fractional Design

Source of variation	Dependent variable: global response				
	SS	DF	MS	F_{calc}	*p value*
Solvent (X1)	*10.7325*	*1*	*10.7325*	*659.9356*	*0.0000*
Time (X2)	*1.5724*	*1*	*1.5724*	*96.6864*	*0.0000*
Temperature (X3)	*1.5647*	*1*	*1.5647*	*96.2116*	*0.0000*
X1;X2	*0.2426*	*1*	*0.2426*	*14.9163*	*0.0012*
X1;X3	*0.1136*	*1*	*0.1136*	*6.9859*	*0.0170*
X2;X3	*0.3454*	*1*	*0.3454*	*21.2383*	*0.0002*
Residue	*0.2765*	*17*	*0.0163*		
Total	*14.8476*	*23*			

* Values in italic refer to significant differences, $F_{0.05;5;17}$=2.81

Fig 1. Reponse surface of the TPC (1a) in mg GAE/g of acai and AA (1b) in µmol Trolox/g of acai as a function of time and solvent nature

Table 4: Models generated for analysis of variance of the TPC and AA in açaí, blueberry and goji berry subjected to treatments, according to a Fractional Design (FD)

Equation number	Model generated	Correlation coefficient R²
2	*TPC açaí*=17.66−1.58χ_1+0.99χ_2+0.72χ_3+0.64χ1 χ_2	0.81
3	*AA açaí* =40.72+10.09χ_1+1.57χ_2+2.79χ_1 x_2+1.43χ_2 χ_3	0.94
5	*AA blueberry*=23.34 +13.12χ_1 + 2.61χ_2 + 2.96χ_3 + 2.17χ_1 χ_2 +1.77χ_1 χ_3 +2.06χ_2 χ_3	0.97
6	*TPC goji berry*= 15.84 − 1.47χ_1 + 1.47χ_{-2} + 1.32χ_3	0.85
7	*AA goji berry*= 13.172 + 3.198χ_1 + 0.830χ_2 + 1.160χ_3 + 0.312χ_1 χ_3	0.93
8	*GR*=3.84+0.67χ_1+0.25χ_2+0.25χ_3+0.10 χ_1 χ_2+0.07 χ_1 χ_3+0.12 χ_2 χ_3	0.98

Response: TPC: Total phenolic compounds; AA: Antioxidant activity; GR: Global response - X1: solvent; X2: time; X3: temperature.

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The antioxidant activity in each fruit extract differed significantly (p <0.05) between analyzed methods (Table 5). Açaí extract showed superiority in antioxidant activity by the FRAP, ABTS and β-carotene method, while blueberry was superior through the DPPH test expressed as EC_{50}. Goji berry had the lowest antioxidant activity in all tests compared to açaí and to blueberry (Table 5).

Antioxidant activity of sodium erythorbate was used as positive control and for comparison with the samples with values of 0.05 ± 0.01 mg/mL, 2871.97 ± 26.97 μmol Fe^{2+}/g sample, 4777.93 ± 206.00 μmol Trolox/g sample and 77.27 % ± 0.07, through the EC_{50}, FRAP, ABTS and β-carotene method, respectively. These values were higher than those of the analyzed samples (Table 5), once sodium erythorbate - a chemical preservative much used in fruit products - is a pure substance when compared with fruits extract, which contain many other extracted compounds that can interfere with the chemical analysis.
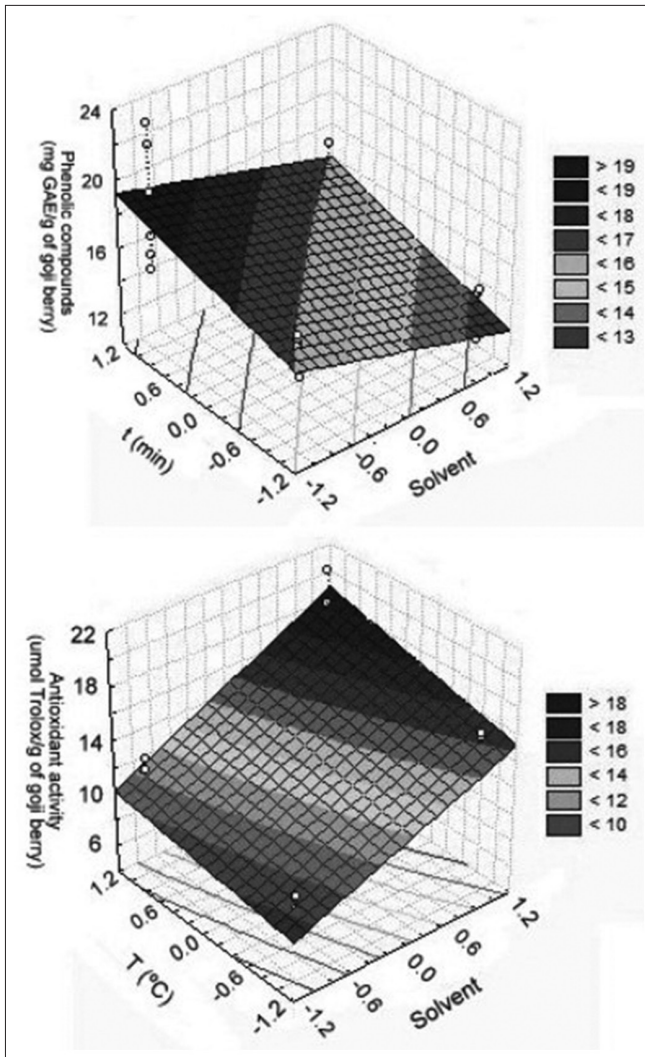

Fig 2. Reponse surface of the TPC (2a) in mg GAE/g of goji berry as a function of time and solvent nature and AA (2b) in μmol Trolox/g of goji berry as a function of temperature and solvent nature
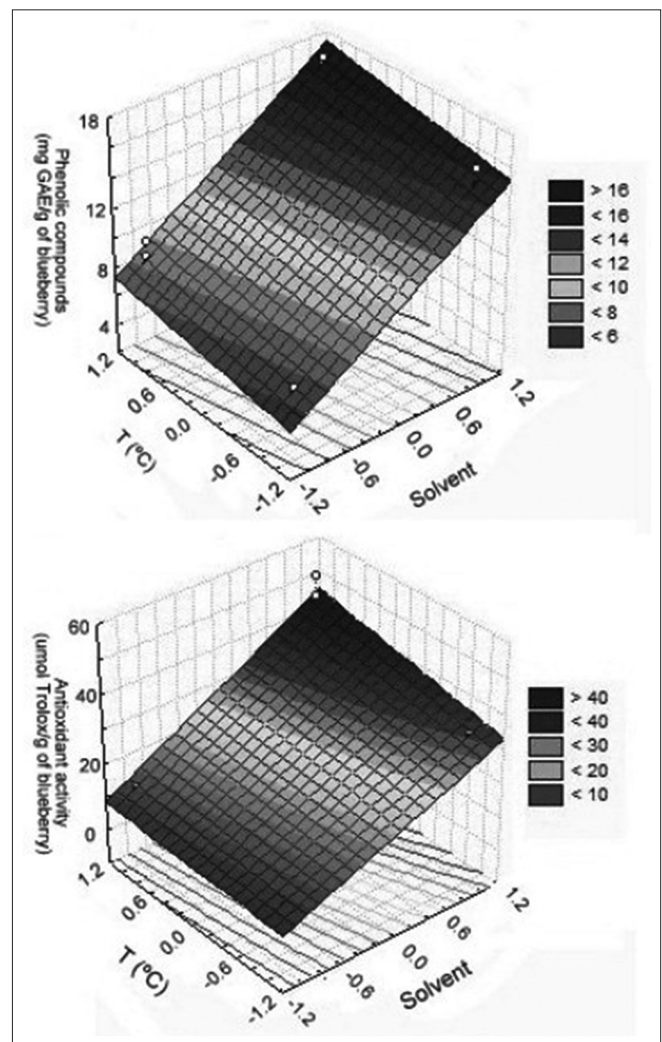

Fig 3. Reponse surface of the TPC (3a) in mg GAE/g of blueberry and (3b) in μmol Trolox/g of blueberry as a function of temperature and solvent nature

Table 5: Total anthocyanin and antioxidant activity by different methods for açaí, blueberry and goji berry fruits in the best extraction condition

Fruits	Total anthocyanin (mg/100 g)	EC_{50} (mg/mL)	FRAP (μmol de Fe^{2+}/g)	ABTS (μmol Trolox/g)	β-caroteno (%)
Açaí	410.24±1.18[b]	0.62±0.01[b]	106.35±0.61[a]	15.28±0.16[a]	74.66±0.63[a*]
Blueberry	874.17±3.61[a]	0.50±0.03[a]	99.38±0.76[b]	13.11±0.04[b]	66.28±0.29[b*]
Goji berry	4.17±0.83[c]	2.48±0.02[c]	47.54±0.55[c]	10.67±0.03[c]	47.14±0.46[c**]
Pooled SD	351.28	0.90	26.32	1.98	11.26

EC_{50}: Equivalent concentration; *concentration: 0.01 g/mL: **0.03 g/mL

Table 6: Phenolic profile determined by HPLC in açaí, blueberry and goji berry extracts

Compounds	TR (min)	RE (Correlation coefficient)	Açaí (mg/g)	Blueberry (mg/g)	Goji berry (mg/g)	Pooled SD
Catequin	11.64	y=0.064x+0.129 (R^2 = 0.988)	0.023±0.001	0.056±0.001	0.050±0.002	0.014
Cafeic acid	15.68	y=0.071x+0.207 (R^2 = 0.988)	n.d.	0.017±0.001	0.151±0.002	0.067
Epicatequin	17.77	y=0.065x+0.137 (R^2 = 0.988)	0.030±0,001	0.008±0.001	n.d.	0.011
Cumaric acid	22.07	y=0.349x+1.095 (R^2 = 0.988)	n.d.	n.d.	0.123±0.005	0.003
Ferulic acid	23.71	y=0.061x+0.196 (R^2 = 0.989)	n.d.	0.119±0.002	0.069±0.001	0.025
Rutin	24.89	y=0.145x+0.428 (R^2 = 0.988)	0.001±0.000	0.091±0.000	0.326±0.001	0.137
Myricetin	27.95	y=0.066x - 0.158 (R^2 = 0.989)	0.054±0.001	n.d.	n.d.	0.001

TR: Retention time; RE: Regression equation; Detection and quantification limits were defined as the concentration of 0.12 μg/mL and 0.35 μg/mL, respectively
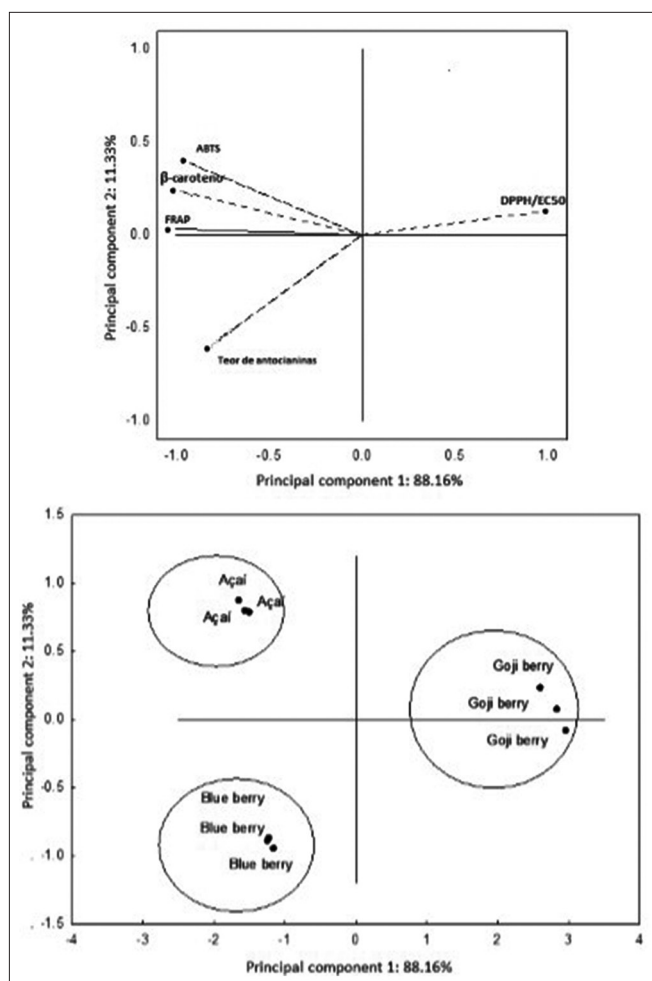

Fig 4. PCA plot Antioxidant activity and total anthocyanin on the acai, blueberry and goji berry a) scores; b) loding plot for the antioxidant actitivty and total anthocyanin on prinicipal 1 and 2

The highest antioxidant activity performed through DPPH method and expressed as EC_{50} (concentration of extract necessary to decrease the initial concentration of DPPH by 50%) was found in blueberry (0.50 mg/mL), and calculated through the line equation y = 79.141x + 10.647 (R^2 = 0.990). Goji berry showed the lowest antioxidant activity with EC_{50} of 2.48 mg/mL (y = 20.303x - 0.7711, R^2 = 0.996) (Table 5).

ABTS radical scavenging performed using the line equation y = -11.244x + 0.689 (R^2 = 0.997) ranged from 10.675 μmol Trolox/g (goji berry) to 15.285 μmol Trolox/g (açaí) (Table 5). These results were similar to those reported by Santos et al. (2008) who obtained values between 10.21 and 52.47 μmol of Trolox/g sample in açaí Brazillian commercial pulps, still Pertuzatti et al. (2014) found values from 40.30 to 260.80 μmol Trolox/g in blueberry Brazilian varieties.

The reducing activity power of Fe^{3+} to Fe^{2+} (FRAP) of fruit extracts was calculated using the line equation y = 32.991x − 0.050 with R^2 of 0.999 and ranged from 47.54 to 106.35 μmol Fe^{2+}/g sample (Table 5). Açaí and goji berry showed the highest and lowest values, respectively (Table 5) and differed statistically ($p<0.05$). The antioxidant activity by coupled β-carotene/linoleic acid method at a concentration of 0.01 g/mL for blueberry and açaí samples and at 0.025 g/ml for goji berry, ranged from 74.66 to 47.14%. The highest values was found in açaí extract and smaller values in goji berry extract (Table 5).

Donno et al. (2015) found 20.89 μmol Fe^{2+}/g in the goji berry from Italy and was lower than those found in this study (Table 5). Pertuzatti et al. (2014) achieved antioxidant activity of 60.9% by the β-carotene/linoleic acid method in the blueberry extracts (0.46 g/mL concentration) with similar results of this study (Table 5).

The quantification of the phenolic compounds by high-performance liquid chromatography in the açaí, blueberry and goji berry extracts was performed in different wavelengths from 277 to 371 nm. Retention times, wavelengths and regression equation data of compounds are given in Table 6. It was possible to visualize the presence of catechin and rutin flavonoids in the three studied fruit extracts (Table 6). Catechin, epicatechin, rutin and myricetin were found in açaí, among them myricetin (0.054 mg/g) and rutin (0.001 mg/g) were present in higher and lower concentration, respectively. No phenolic acids were found in the açaí extracts (Table 6).

Catechin, epicatechin, caffeic acid, ferulic acid and rutin were found in blueberry extract. In this extract, epicatechin (0.008 mg/g) and ferulic acid (0.119 mg/L) were the phenolic compounds found in lower and higher concentrations, respectively (Table 6).

Three phenolic acids (caffeic acid, coumaric acid, ferulic acid) and two flavonoids (catechin, rutin) were found in goji berry extract in concentrations ranging from 0.050 mg/L (catechin) to 0.326 mg/L (rutin) (Table 6).

Studies for identification and quantification of phenolic compounds from fruits are common (Castrejón et al., 2008; Yuyama et al., 2011; Borges et al., 2016), however, the method used in extraction, equipment, identification and separation conditions are different. Furthermore, the wide species diversity and crops types make it relatively difficult to compare these studies (Rodrigues et al., 2011; Melo et al., 2015). Donno et al. (2015) found caffeic acid, coumaric acid, ferulic acid and epicatechin in goji berry from Italy, while Wang et al. (2010) in their studies found another composition in the same fruit (rutin, quercetin and caffeic acid). The phenolic compound contents found by these authors were higher than those found in this study (Table 6). Gordon et al. (2012) in their studies with açaí had previously identified, compounds like gallic acid, caffeic acid, and vanillic acid.

CONCLUSION

Experimental design and RSM could be used to optimize extraction of phenolic compounds from açaí, blueberry and gojy berry for maximizing the antioxidant capacity. Further, the use of global response was very useful for simplifying and improving the phenolic compounds extraction performance with high antioxidant activity. In the best extraction condition it was possible to extract phenolic compounds such as phenolic acids and flavonoids with high antioxidant activity. The açaí, blueberry and gojy berry extracts obtained in this study can be a potential source of the phenolic compounds for food technology application, as natural antioxidant alternatives.

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Author's contributions

C. M.: Designed the study and colected of the samples, did the analysis; A. S. R. and L. D. S.: Participate on carrying out experiments as antioxidant activity; V. A. L.: Was involved in designing the study and statistical analysis; T. L. C. O.: Was involved in HPLC analysis; C. P.: Was involved in literature collection; S. T. C.: Was involved in manuscript preparation and supervised the research project.

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