Anthocyanins and phenolic compounds in five ripening stages of *Byrsonima ligustrifolia* after extraction optimization

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Summary

Byrsonima ligustrifolia is an astringent fleshy berry fruit whose colour changes during the maturation process from green to purple-black at over-ripe. Like other berries, it can be a rich source of antioxidant phenolics. In the present study, the best combination between three extraction solvents (acetone, methanol, and water) was determined, using simplex-centroid design with axial points and two replicates at the center point. After that, phenolic compounds and anthocyanins from *B. ligustrifolia* fruit in five ripening stages were studied by colorimetric assays, high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS/MS), and HPLC equipped with diode array detector (DAD). Our results showed that acetone : methanol (20 : 80) worked better in extracting both compounds and, from the first to the last ripening stage, the content of total phenolic compounds decreased to 57% and total monomeric anthocyanins became 50 times more concentrated. HPLC-MS/MS allowed to identify cyanidin-3-glucoside, pelargonidin-3-glucoside, peonidin-3-glucoside, delphinidin-3-glucoside, gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, caffeic acid, *p*-coumaric acid, quercetin and catechin. Among these compounds, six were quantified by HPLC-DAD: cyanidin-3-glucoside, pelargonidin-3-glucoside, gallic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, and catechin.

Keywords

Byrsonima ligustrifolia; phenolic compounds; anthocyanins; simplex-centroid design; extraction optimization; high performance liquid chromatography

Murici-vermelho (Portuguese) or red-Murici (*Byrsonima ligustrifolia* A. Juss.) fruit belongs to the flowering plant family Malpighiaceae, in which *Malpighia emarginata* (acerola or Barbados cherry) is also included. The family comprises approximately 1300 species in 77 genera, and *Byrsonima* is one of the largest genera in this family, with nearly 150 species [1, 2].

B. ligustrifolia is a rounded and branched dense crown tree that can reach 4 m to 7 m in height. It has astringent fleshy fruits that can be consumed fresh or be pulped for use in juices and sweets, with greatly appreciated colour and flavour. The fruits colour changes during the maturation process from green to red, vinaceous, and then purple at over-ripe. It is a berry fruit and berries are exceptionally rich sources of antioxidant phenolics [3–5].

Phenolic compounds are the major group of phytochemicals in berry fruits including flavonoids, stilbenes, tannins, and phenolic acids. Among berry phenolics, anthocyanins (pigments that account for their attractive colours) are the most studied and have a wide range of bioactivities including antioxidant, anticancer and anti-inflammatory properties [6–8].

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Maturation of berries is accompanied by transformations, which include colour change, tissue softening, change of starch or acid into monosaccharide and become flavoursome, for example. It is the result of changes in gene expression, enzyme activities and shifting within the phenolic compound profile: contents of anthocyanins increases during successive ripening, and contents of other phenolic compounds can decrease or not change significantly. Thereby, due to a decrease in the contents of phenolics in some fruits, antioxidant capacity showed an apparent diminution during maturation [5, 9–13].

Extraction is the initial and the most important step in isolating different types of bioactive compounds from fruits [14]. Solvent concentration and liquid/solid ratio are two of the most important parameters affecting the extraction efficiency of polyphenols from plant sources [15, 16]. Different solvents have been used for extracting polyphenols from plant materials: water, either pure or diluted ethanol, methanol and acetone, or the mixtures of them are commonly used [17–19].

Optimization of antioxidants extraction may be achieved by either empirical or statistical methods. So, when the aim is to study the best combination of solvents, which allows extracting the principal compounds of interest, a multivariate statistical technique for optimization of mixtures is required due to the possibility of studying all solvent proportions (0–100%) by using a reduced number of experiments. Among the methods used to perform a qualitative and quantitative analysis of phenolics in plant extracts, high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS/MS), and HPLC equipped with diode array detector (DAD) were found to be powerful tools [20].

To our knowledge, information regarding *B. ligustrifolia* fruit is limited and there is no scientific literature about bioactive compounds from this fruit. In this study, the extraction of total monomeric anthocyanins and total phenolic compounds from *B. ligustrifolia* fruit in five ripening stages was optimized by applying the simplex-centroid design. After validating the optimized condition, the phytocompounds were quantified by colorimetric assays and its composition was identified by HPLC-MS/MS and quantified by HPLC-DAD.

MATERIALS AND METHODS

Plant materials

Samples of *Byrsonima ligustrifolia* A. Juss. (popularly known as Murici-vermelho) were obtained from Guaraqueçaba city (Paraná, Brazil), geographic coordinates 25°17'50"S 48°19'8"W, in March of 2014. Different ripening stages were collected at same time from the same trees. The species official identification was conducted by Municipal Botanical Museum of Curitiba city (Paraná, Brazil) and one specimen (exsiccate) was deposited in the Herbarium of this Museum, registered under number 388371. The fruits were selected, washed, sorted into five ripening stages (RS) according to external colour, weighed, whole freeze-dried, and stored at –20 °C. According to external colour, the fruits were classified in the following ripening stages: RS1 (green peel), RS2 (light-pink peel), RS3 (pink-red peel), RS4 (red-purple peel), RS5 (purple-black peel).

The extraction optimization was performed with the fruit in the intermediary ripening stage (RS3) once the amount of fruits in this stage was greater than the others. Since particle size is extremely relevant to extraction effectiveness, freeze-dried samples were ground until a fine and visually homogenous powder was obtained.

Evaluation of solvent effects by simplex-centroid design

Two different types of standard designs are commonly used for extraction experiments with mixtures: simplex-lattice design and simplex-centroid design. Both of them will evaluate the triangular response surface at the vertices (i.e., the corners of the triangle) and the centroids (sides of the triangle). In simplex-lattice designs, $m+1$ equally spaced proportions are tested for each factor or component in the model ($x_i = 0, 1/m, 2/m, ..., 1$; $i = 1, 2, ..., q$) and all combinations of factor levels are tested. The resulting design is called a $\{q,m\}$ simplex lattice design [21]. An alternative arrangement of settings introduced by Scheffé [22] is the so-called simplex-centroid design. Here, the different conditions tested form a triangle, with pure components in the vertex, representing 100% of one of each solvent. Middle points on each side representing permutations of the binary blends (1/2 : 1/2 : 0; 1/2 : 0 : 1/2; 0 : 1/2 : 1/2), and, the center point as a ternary mixture (1 : 1 : 1). This design is sometimes augmented with interior points (axial points) representing 2/3 of one of the solvents and 1/6 for the others (Fig. 1). With a reduced number of experiments, this multivariate statistical technique for optimization of mixtures allows to study all solvent proportions (0–100%) and determine possible interaction effect among variables [21]. The simplex-centroid design with axial points and two replicates at the center point was chosen to determine the solvent combination (of acetone,

	β_1	β_2	β_3
●	1	0	0
●	0	1	0
●	0	0	1
●	1/2	1/2	0
●	1/2	0	1/2
●	0	1/2	1/2
○	1/3	1/3	1/3
●	2/3	1/6	1/6
●	1/6	2/3	1/6
●	1/6	1/6	2/3

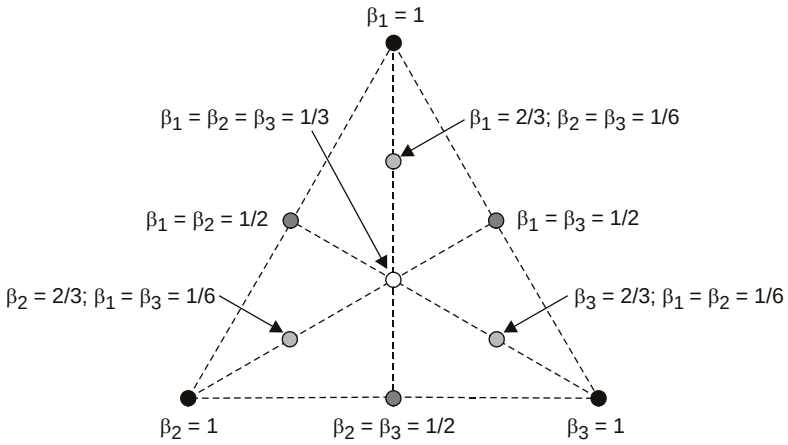

Fig. 1. Simplex-centroid design for 3 factors with axial points.

methanol and water; v/v/v) that generates the greater efficiency in the extraction of total monomeric anthocyanins content (TMA) and total phenolic compounds content (TPC), expressed as grams per kilogram of the freeze-dried sample. Tab. 1 presents all conditions tested. This design permitted the evaluation of linear (β_1, β_2, and β_3), quadratic (β_{12}, β_{13}, and β_{23}) and special cubic models (β_{123}) for the response under study.

Extraction procedure and sample preparation

Freeze-dried pulp powder (0.5 g) was extracted with 15 ml of solvents mixture, acidified with 2% of acetic acid, under agitation, for 40 min. After that, the extracts were centrifuged and the supernatants were transferred to a volumetric flask and the solutions were taken to an exact volume (25 ml) with the extraction solvent. For HPLC-MS/MS analysis, the extracts were evaporated to

Tab. 1. Simplex centroid design of total monomeric anthocyanins and total phenolic compounds extraction optimization.

Run number	Independent variable			Response	
	Acetone (β_1)	Methanol (β_2)	Water (β_3)	TMA [g·kg^{-1}]	TPC [g·kg^{-1}]
14	1	0	0	0.30	41.94
2	0	1	0	1.87	126.02
18	0	0	1	1.13	64.62
9	1/2	1/2	0	1.93	111.16
8	1/2	0	1/2	1.50	93.61
1	0	1/2	1/2	1.70	98.03
16	1/3	1/3	1/3	1.88	108.63
13	2/3	1/6	1/6	1.48	103.20
4	1/6	2/3	1/6	1.80	112.76
10	1/6	1/6	2/3	1.42	85.89
12	1	0	0	0.39	36.96
15	0	1	0	1.93	108.90
6	0	0	1	1.27	73.50
11	1/2	1/2	0	1.65	112.37
5	1/2	0	1/2	1.42	105.94
17	0	1/2	1/2	1.45	88.41
7	1/3	1/3	1/3	1.75	110.20
3	1/3	1/3	1/3	1.66	105.63

TMA – total monomeric anthocyanins expressed as grams of cyanidin-3-glucoside equivalent per kilogram of freeze-dried sample, TPC – total phenolic compounds expressed as grams of gallic acid equivalent per kilogram of freeze-dried sample.

dryness in vacuum at 30 °C, and the residue was dissolved in methanol, HPLC grade, membrane-filtered (0.45 μm) and mixed (1:1) with the mobile phase A of the respective system.

Validation of the models

Intending to validate the models obtained for total monomeric anthocyanins content and total phenolics content, five new extractions, with the same procedure showed for the step above, were performed with different proportions between solvents and at the best conditions achieved.

Selection of the solid-to-liquid proportion

The solvent volume needed to maximize the yield of anthocyanins and phenolic compounds at extraction was investigated. Freeze-dried pulp powder (0.5 g) was extracted under agitation for 40 min with the combination of solvents that gave the highest response in the previous step, in proportions of 1:10, 1:20, 1:30, 1:60, 1:90, 1:120 and 1:150 (gram of sample per millilitre of solvent). Extracts were centrifuged and the supernatants were transferred to a volumetric flask and the solutions were taken to an exact volume with the extraction solvent.

Total monomeric anthocyanins and total phenolic compounds content

Total monomeric anthocyanins (TMA) was determined by using a spectrophotometric pH differential method [23] and the results were expressed as grams of cyanidin-3-glucoside equivalents per kilogram of freeze-dried pulp.

Total phenolic compounds (TPC) was measured using a colorimetric Folin-Ciocalteu method [24]. Gallic acid was used as a standard and results were expressed as grams of gallic acid equivalents per kilogram of freeze-dried pulp.

After determination of the best condition of extraction, the content of anthocyanins and phenolic compounds in five ripening stages was investigated. The steps of extraction were the same as in previous experiments.

HPLC-MS/MS instrumentation and conditions

HPLC-MS/MS analyses were performed using an Agilent 1200 HPLC System (Wilmington, Delaware, USA) consisting of a G1312B binary pump, a G1379B degasser, and a G1316B column oven, connected to a CTC Sample Manager (Model 2777; Waters, Milford, Massachusetts, USA). The HPLC system was coupled to a MDS Sciex API 3200 Triple Quadrupole Mass Spectrometer (Applied Biosystems, Toronto, Canada) equipped with a Harvard 22 Dual Model syringe pump (Harvard Apparatus, South Natick, Massachusetts, USA) and an electrospray ionization (ESI) source.

The ESI source was operated in the positive ion mode for monitoring anthocyanins and in the negative ion mode for monitoring phenolic acids, anthoxanthins and stilbenes.

For the positive ion mode, the mobile phase consisted of a gradient of water:formic acid:acetonitrile (A) (95:2:3, v/v/v) and water:formic acid:acetonitrile (B) (48:2:50, v/v/v). The injection volume was 25 μl, the flow rate was maintained at 800 μl·min^{-1}, and the gradient profile was as follows: A = 90% (0 min); A = 75% (10 min); A = 69% (5 min); A = 60% (5 min); A = 54% (5 min); A = 10% (0.10 min); A = 10% (2.9 min); A = 90% (3 min).

For the negative ion mode, the mobile phase consisted of a gradient of water:acetic acid (98:2, v/v) (A) and water:acetonitrile (B) (50:50, v/v) containing 0.5% of acetic acid. The injection volume was 20 μl, the flow rate was maintained at 1 ml·min^{-1}, and the gradient profile was as follows: A = 90% (0 min); A = 85% (10 min); A = 75% (10 min); A = 65% (10 min); A = 0% (2 min); A = 0% (5 min); A = 90% (4 min).

For both methods, the analyte separations were achieved on an XBridge C18 150 × 4.6 mm (5 mm particle size) column (Waters) whose temperature was maintained at 25 °C.

The analysis was performed with the MS Workstation using Analyst 1.4 software (Sciex, Framingham, Massachusetts, USA), in multiple reaction monitoring mode, maintaining the dwell time at 300 ms for both positive and negative ion modes. The ion transitions, the individual compound parameters, including the declustering potential, entrance potential, collision cell entrance potential, collision energy and cell exit potential, as well as the ion-source parameters for ESI-positive and negative modes are summarized in Tab. 2. The high-purity nitrogen and zero-grade air, which were used as the curtain gas, collision gas, nebulizer gas and turbo gas, were produced by a high-purity nitrogen generator from PEAK Scientific Instruments (Chicago, Illinois, USA).

Liquid chromatography analysis of phenolic compounds

Analyses were performed with the use of Agilent 1200 Series HPLC system (Wilmington, Delaware, USA) equipped with diode array detector (DAD), controlled by Software EZChrom Elite (Agilent), with automatic liquid sampler (ALS) and quaternary pump. Separations were made using a Zorbax Eclipse XDB-C18 column

Tab. 2. Compound-dependent parameters and ion transitions of the analytes used for monitoring anthocyanins, phenolic acids, anthoxanthins and stilbenes.

Ionization mode	Compound	Molecular ion (*m*/*z*)	Ion transition (*m*/*z*)	CE [eV]	CXP [V]	CEP [V]	DP [V]	EP [V]
Positive $[M+H]^+$	Cyanidin-3-glucoside	449	449→287	25	3.8	20	40	10
	Pelargonidin-3-glucoside	433	433→271	25	3.8	21	40	10
	Peonidin-3-glucoside	463	463→301	25	3.8	20	40	10
	Cyanidin-3-rhamnoside	433	433→287	25	3.8	21	40	10
	Delphinidin-3-glucoside	465	465→303	25	3.8	20	40	10
	Pelargonidin-3-rhamnoside	417	417→271	25	3.8	21	40	10
	Petunidin-3-glucoside	479	479→317	25	3.8	20	40	10
	Malvidin-3-glucoside	493	493→331	25	3.8	20	40	10
	Peonidin-3-acetylglucoside	505	505→301	25	3.8	20	40	10
	Delphinidin-3-acetylglucoside	507	507→303	25	3.8	20	40	10
	Petunidin-3-acetylglucoside	521	521→317	25	3.8	20	40	10
	Malvidin-3-acetylglucoside	535	535→331	25	3.8	20	40	10
	Cyanidin-3-rutinoside	595	595→449 595→287	25	3.8	27	40	10
	Peonidin-3-rutinoside	609	609→463 609→301	25	3.8	27	40	10
	Petunidin-3-*p*-coumaroylglucoside	625	625→317	25	3.8	20	40	10
	Malvidin-3-*p*-coumaroylglucoside	639	639→331	25	3.8	20	40	10
Negative $[M-H]^-$	Protocatechuic acid	153	153→109	-25	-2.5	-12.0	-40	-10
	p-hydroxybenzoic acid	137	137→ 93	-25	-2.5	-9.5	-40	-10
	Procyanidin	577	577→425 577→407	-25	-2.5	-26.5	-40	-10
	Fertaric acid	325	325→193	-25	-2.5	-16.8	-40	-10
	Catechin	289	289→245	-25	-2.5	-15.4	-40	-10
	Caffeic acid	179	179→135	-25	-2.5	-11.2	-40	-10
	Gallic acid	169	169→125	-25	-2.5	-11.0	-40	-10
	p-coumaric acid	163	163→119	-25	-2.5	-10.5	-40	-10
	Quercertin 3-glucoside	463	463→301	-25	-2.5	-22.1	-40	-10
	Quercertin 3-rhamnoside	447	447→301	-25	-2.5	-21.5	-40	-10
	Rutin	609	609→301	-25	-2.5	-27.8	-40	-10
	Quercertin	301	301→179 301→151	-25	-2.5	-15.9	-40	-10
	Ferulic acid	193	193→134	-25	-2.5	-11.7	-40	-10
	Syringic acid	197	197→182 197→153	-25	-2.5	-11.8	-40	-10
	Sinapic acid	223	223→208 223→164	-25	-2.5	-12.8	-40	-10
	Coutaric acid	295	295→163	-25	-2.5	-15.6	-40	-10
	Caftaric acid	311	311→179	-25	-2.5	-16.3	-40	-10
	Resveratrol derivative	535	535→227	-25	-2.5	-24.9	-40	-10

CE – collision energy, CXP – cell exit potential, CEP – collision cell entrance potential, DP – declustering potential, EP – entrance potential.

Ion-source parameters for ESI-positive mode: curtain gas 69 kPa; collision gas 34 kPa; ion spray voltage 5500 V; nebulizer gas 345 kPa; turbo gas 345 kPa and temperature 500 °C.

Ion-source parameters for ESI-negative mode: curtain gas 69 kPa; collision gas 21 kPa; ion spray voltage –4500 V; nebulizer gas 345 kPa; turbo gas 345 kPa and temperature, 600 °C.

(4.6 × 150 mm, 5 μm particle size; Agilent) connected to a guard-column Zorbax Eclipse XDB-C18 (4.6 × 12.5 mm). The separation of anthocyanins, phenolic acids and anthoxanthins was done under different conditions. Thus, there were three systems with different mobile phase compositions, gradient program, total run time, injection volume of samples, wavelengths monitored and flow rate.

System I (anthocyanins)

The mobile phase consisted of water : formic acid : acetonitrile (95 : 2 : 3, v/v/v; eluent A) and water : formic acid : acetonitrile (48 : 2 : 50, v/v/v; eluent B) using a gradient program as follows: from 10% to 25% B (10 min), from 25% to 31% B (5 min), from 31% to 40% B (5 min), from 40% to 46% B (5 min), from 46% to 90% B (1 min), from 90% to 10% B (2 min). Total run time was 30 min. The injection volume for all samples was 25 μl. Monitoring was performed at 520 nm at a flow rate of 0.8 ml·min^{-1}.

System II (phenolic acids)

The mobile phase consisted of 2% (v/v) acetic acid in water (eluent A) and 0.5% acetic acid in water and acetonitrile (50 : 50, v/v; eluent B) using a gradient program as follows: from 10% to 15% B (10 min), 15% B isocratic (3 min), from 15% to 55% B (12 min), from 55% to 100% B (5 min), 100% B isocratic (4 min), from 100% to 10% B (3 min). Total run time was 40 min. The injection volume for all samples was 15 μl. Simultaneous monitoring was performed at 280 nm (hydroxybenzoic acids) and at 320 nm (hydroxycinnamic acids) at a flow rate of 1.0 ml·min^{-1}.

System III (anthoxanthins)

The mobile phase consisted of the same solvents as described for system II using a gradient program as follows: from 10% to 24% B (20 min), from 24% to 30% B (20 min), from 30% to 55% B (20 min), from 55% to 100% B (15 min), 100% B isocratic (8 min), from 100% to 10% B (2 min). Total run time was 86 min. The injection volume for all samples was 15 μl. Simultaneous monitoring was performed at 280 nm (flavanols) and at 370 nm (flavonols) at a flow rate of 1.0 ml·min^{-1}.

The separated compounds were identified on the basis of the following combined information: elution order on the reversed phase column, co-chromatography with standards, UV-Vis spectra (λ_{max}), and calibration curves for each standard were prepared for quantification. The following individual phenolic standards were purchased from Sigma-Aldrich (St. Louis, Missouri, USA): cyanidin-3-glucoside, gallic acid, caffeic acid, *p*-coumaric acid, protocatechuic acid, *p*-hydroxybenzoic acid, quercetin and catechin.

Statistical analysis

All the experiments for models validation, selection of the solid-to-liquid proportion, as well as total monomeric anthocyanins content and total phenolic compounds content in five ripening stages were performed in triplicate, and the results were reported as mean ± standard deviation. One-way analysis of variance for comparison of means and significant differences according to Tukey's test at the 5% level were performed by Statistica 8.0 (StatSoft, Tulsa, Oklahoma, USA).

RESULTS AND DISCUSSION

Solvent effects by simplex-centroid design

The total content of phenolic compounds was obtained using a seven-point gallic acid calibration curve from 10 μg·ml^{-1} to 70 μg·ml^{-1}, with a regression coefficient (R^2) equal to 0.9992. The extraction with pure acetone showed the lowest values for anthocyanins (0.35 ± 0.06 g·kg^{-1} of freeze-dried sample) and total phenolics content (39.45 ± 3.52 g·kg^{-1}) and the best results were obtained with pure methanol (1.90 ± 0.04 g·kg^{-1} and 117.46 ± 12.10 g·kg^{-1} to TMA and TPC, respectively). However, the experiments with the same solvents proportion (1 : 1 : 1) resulted in a high value both for anthocyanins (1.76 ± 0.11 g·kg^{-1}) and for total phenolic compounds (108.16 ± 2.32 g·kg^{-1}). Nevertheless, the extractions with acetone and methanol (1 : 1) and one of axial points (1 : 4 : 1, acetone : methanol : water) also produced good results.

Tab. 3 presents results of ANOVA testing of regression models. Fitness of purpose for quadratic models is shown in Tab. 4. Significant quadratic models were obtained for both responses and both models had no lack of fit and could be used to make predictions. Significant coefficients were obtained for all variables studied (β_1, β_2, and β_3) and the interaction between acetone and methanol (β_{12}) and between acetone and water (β_{13}) showed an important synergistic effect. However, the interaction coefficient between methanol and water (β_{23}) was not significant for the studied response in both models. Therefore, this coefficient was removed from the model before predictions.

Thereby, fitness of purpose for TMA and TPC quadratic models without the coefficient β_{23} is shown in Tab. 5. The results displayed on the

Tab. 3. ANOVA results of regression models estimated from simplex-centroid design for solvent optimization.

Source	SS effect	DF effect	*F*-value	*p*-value	R^2
Total monomeric anthocyanins					
Linear model	2.096	2	9.463	0.002	0.558
Quadratic model	1.504	3	38.161	2.0×10^{-6}	0.958
Special cubic model	1.07×10^{-4}	1	0.007	0.933	0.958
Total phenolic compounds					
Linear model	4818.88	2	6.441	9.6×10^{-3}	0.462
Quadratic model	5064.30	3	37.062	0.2×10^{-5}	0.948
Special cubic model	7.46	1	0.152	0.704	0.948

Tab. 4. Fitness of the quadratic model.

Source	SS effect	DF effect	*F*-value	*p*-value
Total monomeric anthocyanins				
Model	3.599	5	54.813	0.1×10^{-6}
Total error	0.158	12		
Lack of fit	0.044	4	0.771	0.574
Pure error	0.114	8		
Total phenolic compounds				
Model	9883.17	5	43.397	0.1×10^{-6}
Total error	546.57	12		
Lack of fit	214.40	4	1.291	0.350
Pure error	332.18	8		

Tab. 5. Fitness of total monomeric anthocyanins and total phenolic compounds quadratic model without the coefficient β_{23}.

Source	SS effect	DF effect	*F*-value	*p*-value
Total monomeric anthocyanins				
Model	3.599	4	74.199	0.1×10^{-6}
Total error	0.158	13		
Lack of fit	0.044	5	0.617	0.691
Pure error	0.114	8		
Total phenolic compounds				
Model	9877.33	4	58.110	0.1×10^{-6}
Total error	552.42	13		
Lack of fit	220.24	5	1.061	0.447
Pure error	332.18	8		

compound response profile graph, using the optimal values of the independent variables as their current values, and 20 steps from the observed minimum to the observed maximum to define the 21 grid points for each factor, show that desirability is improved by setting the factors at levels other than their means. The overall desirability value for TMA was 1.93 with the proportion of acetone : methanol : water at 30 : 60.9 : 9.1 and, for TPC, was 122.66 with 20% of acetone and 80% of methanol.

The quadratic model, without the interaction β_{23} for TMA with a value of $R^2 = 0.958$, is given by Eq. 1 and is shown in Fig. 2A.

$$TMA = 0.368\beta_1 + 1.890\beta_2 + 1.185\beta_3 + + 2.676\beta_{12} + 2.745\beta_{13} \quad (1)$$

The quadratic model, without the interaction β_{23} for TPC with a value of $R^2 = 0.947$, is given by Eq. 2 and is shown in Fig. 2B:

$$TPC = 41.48\beta_1 + 116.54\beta_2 + 67.13\beta_3 + + 132.07\beta_{12} + 178.88\beta_{13} \quad (2)$$

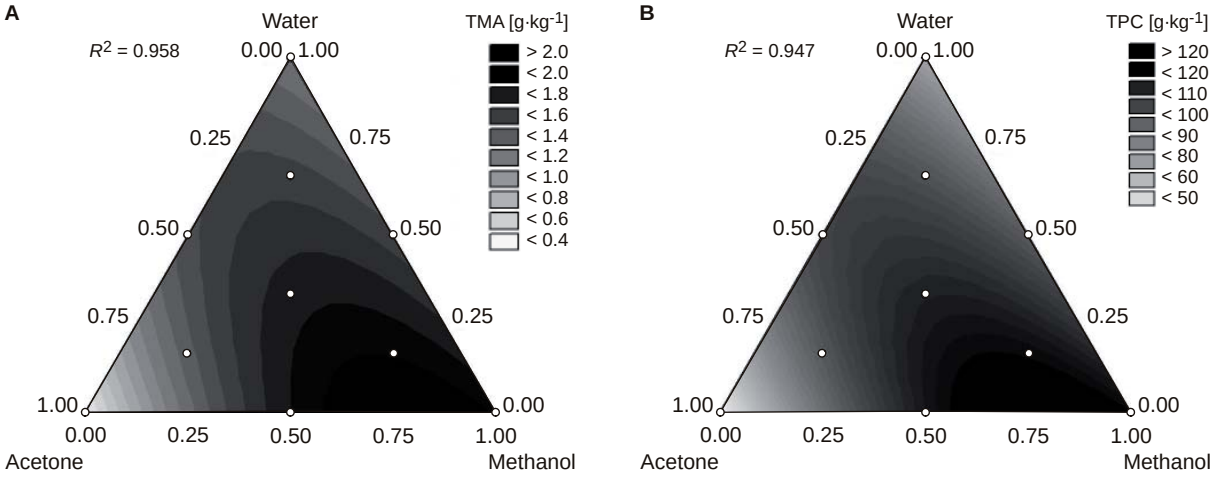

Fig. 2. Response-surface plots of the valid models.

A – Total monomeric anthocyanins content; B – Total phenolic compounds content.

Models validation

The seven new extractions performed to validate the models obtained for total monomeric anthocyanins content and total phenolics content are shown in Tab. 6. Note that experiment A presents the best solvents combination to extract anthocyanins, and experiment B used the solvents combination that displayed the greater phenolic compounds content extraction. The following experiments (D to G) were carried out to show if the models were effective.

All the results obtained for total monomeric anthocyanins content were in agreement with the model predictions and within the confidence interval (95%). Almost all results obtained for phenolics content were in agreement with the model predictions and within the confidence interval (95%), except the result in condition E that was higher than the predicted value.

Since the results of experiments A and B were not statistically different both regarding TMA and TPC contents, and because the predicted values and the experimental values from TMA and also from TPC in the solvents blend B were greater numerically than these values in experiment A, we fixed the condition B to simplify the following experiments.

Solid-to-liquid ratio effect

Since the ratio between sample solids and the solvent could affect the extraction efficiency, the optimization of this parameter was realized; the

Tab. 6. Validation results for quadratic model obtained from simplex-centroid design in solvent optimization experiments.

Experiment	Acetone (β_1) [%]	Methanol (β_2) [%]	Water (β_3) [%]	TMA [g·kg^{-1}]				TPC [g·kg^{-1}]			
				Predicted values	Interval of confidence		Experimental values	Predicted values	Interval of confidence		Experimental values
					–95%	+95%			–95%	+95%	
A*	30	60.9	9.1	1.93	1.83	2.04	1.85 ± 0.07^{ab}	118.53	112.28	124.78	116.79 ± 4.21^{a}
B**	20	80	0	2.01	1.90	2.13	2.03 ± 0.09^{a}	122.66	116.00	129.32	122.31 ± 2.70^{a}
C	0	45	55	1.50	1.41	1.60	1.42 ± 0.02^{c}	89.37	83.84	94.89	86.79 ± 2.94^{b}
D	35	65	0	1.97	1.83	2.10	1.84 ± 0.04^{ab}	120.31	112.46	128.17	117.63 ± 0.60^{a}
E	35	40	25	1.80	1.71	1.88	1.77 ± 0.20^{b}	112.06	107.17	116.95	120.26 ± 3.98^{a}
F	10	90	0	1.98	1.86	2.09	1.86 ± 0.09^{ab}	120.92	114.13	127.72	115.58 ± 0.82^{a}
G	15	80	5	1.97	1.87	2.07	1.98 ± 0.15^{ab}	120.00	113.94	126.06	116.95 ± 1.52^{a}

Same superscript letter in the column means no significant difference at 95% of confidence ($p \leq 0.05$) by Tukey's test.
TMA – total monomeric anthocyanins, expressed as grams of cyanidin-3-glucoside equivalent per kilogram of freeze-dried sample; TPC – total phenolic compounds, expressed as grams of gallic acid equivalent per kilogram of freeze-dried sample.
* – best condition for TMA, ** – best condition for TPC.

Tab. 7. Total monomeric anthocyanins and total phenolic compounds in *Byrsonima ligustrifolia* freeze-dried pulp extracted with different proportions of solvent.

Responses	Solid-to-liquid proportion [g : ml]						
	1 : 10	1 : 20	1 : 30	1 : 60	1 : 90	1 : 120	1 : 150
TMA [g·kg^{-1}]	1.10 ± 0.03^{c}	1.69 ± 0.03^{b}	1.97 ± 0.04^{a}	2.00 ± 0.05^{a}	1.94 ± 0.03^{a}	1.91 ± 0.03^{a}	1.95 ± 0.08^{a}
TPC [g·kg^{-1}]	104.77 ± 5.06^{b}	116.72 ± 2.48^{a}	121.56 ± 1.69^{a}	123.32 ± 2.49^{a}	122.83 ± 1.76^{a}	119.72 ± 1.88^{a}	122.05 ± 2.93^{a}

Solid-to-liquid proportion is expressed as gram of sample per millilitre of solvent. Results are mean values ± standard deviation (n = 3). Same superscript letter in the line means no significant differences at 95% of confidence ($p \leq 0.05$) by Tukey's test.
TMA – total monomeric anthocyanins expressed as grams of cyanidin-3-glucoside equivalent per kilogram of freeze-dried sample, TPC – total phenolic compounds expressed as grams of gallic acid equivalent per kilogram of freeze-dried sample.

results are presented in Tab. 7. The ratio between sample and solvent (in grams per millilitre) in the range from 1 : 30 to 1 : 150 had no significant effect an the amounts of extracted TMA and TPC, at 95% confidence ($p \leq 0.05$) by Tukey's test. Based on these results, 30 ml of solvent for one gram of sample (1 : 30) was fixed for the following experiments.

Total monomeric anthocyanins and total phenolic compounds content in five ripening stages

TMA and TPC of *B. ligustrifolia* in five ripening stages are presented in Tab. 8. The results demonstrate a significant decrease of TPC during the ripening of red-murici. At the first ripening stage (RS1), TPC was 160.8 ± 3.5 g·kg^{-1}, and, at the last one (RS5), the content of TPC was 57% of this value. Celli et al. [25] described the changes in the total phenolics content throughout the developmental stages of fruits from two different varieties of Brazilian cherry (*Eugenia uniflora*). They concluded that ripening reduced TPC in these fruits, similarly to what has been reported for other berries, such as red raspberry [26], blackberry, strawberry [27] and acerola [11]. This gradual decrease in the total fruit phenolic contents can be connected with an increased polyphenol oxidase activity, transformations (polymerization, oxidation and conjugation reactions) of phenolic acids, and the reduction of primary metabolism in the over-ripe fruit, resulting in a lack of substrates necessary for the biosynthesis of phenolic compounds [9]. TPC of *Byrsonima crassifolia* fruit extracts were analysed by Almeida et al. [28], De Souza et al. [29], and Ribeiro et al. [30] using Folin Ciocalteu reagent. These authors found values 1.599 ± 0.560 g·kg^{-1} (expressed as grams of gallic acid per kilogram of fruit fresh weight, FW), 3.343 ± 0.907 g·kg^{-1} and 2.434 ± 0.279 g·kg^{-1}, respectively. So, considering the moisture of *B. ligustrifolia* (in this study: 88.5 ± 1.0% for fresh fruit, and 15.3 ± 1.0% for lyophilized fruit), TPC ranged from 220.195 g·kg^{-1} (RS1) to 142.337 g·kg^{-1} (RS5), that is much higher than for *B. crassifolia*.

The TPC determination by Folin-Ciocalteu reagent is based on reduction of phosphomolybdic-phosphotungstic acid reagent to a blue-coloured complex in an alkaline solution, which occurs in the presence of phenolic compounds. Nevertheless, considering the heterogenity of natural phenols and the possibility of interference from other readily oxidized substances, this method is being progressively considered as a reducing power assay [9, 31]. So, our results indicate that *B. ligustrifolia* has high levels of antioxidant activity, based on the Folin-Ciocalteu assay. This is greater than in other berries, such as *B. crassifolia* [28–30], blackberry [27, 32], raspberry [26, 27, 32], strawberry [27], red currant, gooseberry, Cornelian cherry [32], açaí [33–35], and Chilean myrtle

Tab. 8. Extraction results for total monomeric anthocyanins and total phenolic compounds in five ripening stages.

Responses	Ripening stages				
	RS1	RS2	RS3	RS4	RS5
TMA [g·kg^{-1}]	0.14 ± 0.01^{e}	0.45 ± 0.01^{d}	1.95 ± 0.05^{c}	3.91 ± 0.09^{b}	7.05 ± 0.09^{a}
TPC [g·kg^{-1}]	160.78 ± 3.51^{a}	140.82 ± 3.12^{b}	123.07 ± 1.77^{c}	98.34 ± 1.22^{d}	91.68 ± 1.96^{e}

Results are mean values ± standard deviation (n = 3). Same superscript letter in the line means no significant differences at 95% of confidence ($p \leq 0.05$) by Tukey's test.
TMA – total monomeric anthocyanins expressed as grams of cyanidin-3-glucoside equivalent per kilogram of freeze-dried sample, TPC – total phenolic compounds expressed as grams of gallic acid equivalent per kilogram of freeze-dried sample.

[36], and comparable to acerola [11, 33, 35, 37, 38].

Opposite to what happened with TPC, there was a significant increase of total monomeric anthocyanins during the ripening. Since the first to the last stage, TMA became 50 times more concentrated. TMA at five ripening stages differed significantly showing that, during ripening, anthocyanins biosynthesis in plant tissues was increased resulting in higher pigment content, a modified anthocyanins pattern or a pH change along with a change in appearance. This was previously exemplified in ripening fruits from bilberry, blackberry, strawberry, red currant and raspberry [27, 39].

Despite being from the same genus, *B. ligustrifolia* is a species very different from *B. crassifolia*, a yellow fruit on over-ripe with low TMA (0.102 $g \cdot kg^{-1}$ FW) [28]. Our fruit presents 10.945 $g \cdot kg^{-1}$ FW at RS5 (purple-black peel). According to Lima et al. [40], acerola fruits may contain 0.379–5.974 $g \cdot kg^{-1}$ FW total anthocyanins, depending on the varieties which show different colour (from yellow to red). Besides that, studying the same fruit, Mezadri et al. [37], Rufino et al. [33] and Silva et al. [38] values of total anthocyanins of 0.498 ± 0.022 $g \cdot kg^{-1}$ FW, 1.89 ± 0.09 $g \cdot kg^{-1}$ FW, and 14.427 ± 0.653 $g \cdot kg^{-1}$ DW (dried weight), respectively, being also less than our results for *B. ligustrifolia* (10.945 $g \cdot kg^{-1}$ FW or 83.235 $g \cdot kg^{-1}$ DW). Our results can be compared to blackberry [27, 32, 41], raspberry [27] and açaí [33].

Due to its considerable contents of phenolics and anthocyanins, *B. ligustrifolia* fruit is a promising source of natural antioxidant compounds, insofar as phenolic compounds are considered the major determinants for the antioxidant capacity of plants.

Tab. 9. Retention times of metabolites detected in extracts.

No.	Compound	Retention time [min]
Anthocyanins		
1	Cyanidin-3-glucoside	8.00
2	Pelargonidin-3-glucoside	9.72
3	Peonidin-3-glucoside	10.64
4	Delphinidin-3-glucoside	15.15
Phenolic acids and other flavonoids		
5	Protocatechuic acid	3.00
6	*p*-hydroxybenzoic acid	4.59
7	Catechin	8.51
8	Caffeic acid	9.17
9	Gallic acid	16.40
10	*p*-coumaric acid	24.83
11	Quercetin 3-glucoside	28.05
12	Quercetin	33.44

Identification of phenolic compounds by HPLC–MS/MS

The identification of major compounds present in extracts of *B. ligustrifolia* was assessed via HPLC-MS/MS analysis, and these data were compared to those described in the literature [42–45]. Of the thirty-four monitored metabolites, twelve were detected in the analysed extracts: four were classified as anthocyanins and eight as phenolic acids and other flavonoids. The retention time found for each of the metabolites is shown in Tab. 9.

Compound 1 was identified as cyanidin-3-glucoside, taking in consideration the molecular ion at *m/z* 449 and a fragment ion at *m/z* 287 ($[M-162]^+$) corresponding to the aglycon cyanidin, by the loss of one molecule of glucoside (M = 162). The molecular ions of compounds 2, 3, and 4 were at *m/z* 433, 463, and 465, respectively, and the spectra contained fragments with *m/z* of 271, 301, and 303, which correspond to the loss of glucoside. Therefore, these compunds can be identified as pelargonidin-3-glucoside, peonidin-3-glucoside and delphinidin-3-glucoside, respectively. According to the signal intensity of molecular ion and a fragment ion, probably cyanidin-3-glucoside is the major anthocyanin in this fruit. The increase of signal intensity of molecular and fragment ions corresponding to cyanidin-3-glucoside, pelargonidin-3-glucoside and peonidin-3-glucoside throughout the ripening shows that these compounds are possibly synthesized during maturation. Our results are in agreement with Wu and Prior [43]. They identified and characterized anthocyanins in berries (black raspberry, blackberry, blueberry, Concord grape, cranberry, marionberry, raspberry, red grape, strawberry and sweet cherry) by HPLC-MS/MS and concluded that, among all six widely distributed anthocyanidins (delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin), cyanidin was found in all samples and was a major anthocyanidin in all ftuits except strawberry. Glucose was the dominant monosaccharide that was linked to aglycons (anthocyanidins) to form anthocyanins. Studying acerola (*Malpighia emarginata*), a fruit from Malpighiaceae family, De Rosso et al. [45] and Hanamura et al. [46] identified cyanidin 3-rhamnoside and pelargonidin 3-rhamnoside as the major anthocyanins. Malta et al. [47] analysed *Byrsonima verbascifolia* extracts by HPLC–MS and identified resveratrol and ferulic acid as main phe-

nolic constituents. In accordance with the molecular ion and a fragment ion corresponding to ferulic acid and resveratrol derivative, the present study did not identify these compounds in *B. ligustrifolia*. HPLC-MS showed that the main phenolic acid in *B. ligustrifolia* is gallic acid, followed by protocatechuic acid, *p*-hydroxybenzoic acid, caffeic acid and *p*-coumaric acid. Among the other flavonoids, quercetin and catechin were identified, taking in consideration the molecular and a fragment ion. We know that molecular weight information can identify predicted compounds with certainty and identify true unknowns by obtaining a mass spectrum or MS/MS spectrum. However, mass spectra alone are not 100% effective because MS cannot provide complete structural information. For different compounds with the same mass spectra, additional information has to be utilized to identify the peak [43].

Tab. 10. Anthocyanins in *B. ligustrifolia* fruit at five ripening stages.

	Cyanidin-3-*O*-glucoside [g·kg^{-1}]	Pelargonidin-3-*O*-glucoside [g·kg^{-1}]
Retention time [min]	9.27 ± 0.20	11.04 ± 0.05
RS1	1.70 ± 0.09^{e}	ND
RS2	10.65 ± 0.60^{d}	ND
RS3	34.68 ± 1.47^{c}	ND
RS4	69.97 ± 1.07^{b}	0.96 ± 0.02^{b}
RS5	167.89 ± 1.38^{a}	4.04 ± 0.01^{a}

Results are mean values ± standard deviation (n = 3), expressed as grams per kilogram of fruit pulp on a dry weight basis. Same superscript letter in the column means no significant differences at 95% of confidence ($p < 0.05$) by Tukey's test. ND – not detected.

Individual quantification of phenolic compounds

Bioactive compounds from *B. ligustrifolia* identified by LC-MS analysis were individually quantified and confirmed through the following combined information: elution order on the reversed phase column, co-chromatography with standards and UV–Vis spectra (λ_{max}).

The major anthocyanin was cyanidin-3-*O*-glucoside, which was identified in all five ripening stages of the fruit, with a significant increase during the maturity stages that ranged from 1.698 g·kg^{-1} DW to 167.892 g·kg^{-1} DW, raising nearly 100 times its content. Pelargonidin-3-*O*-glucoside was detected only in stages four and five (Tab. 10). In a large number of studies, individual anthocyanins were quantified in berries, but it is difficult to find a fruit that presents values so high for a single anthocyanin like *B. ligustrifolia*. For example, VEBERIC et al. [48] determined the anthocyanins composition of 24 berry species and only one species (Eastern shadbush) presented the content of cyanidin-3-glucoside (21.9 g·kg^{-1} FW) similar to our fruit at RS5 (22.1 g·kg^{-1} FW). Another berry (Cultivated elderberry) had this value greater than *B. ligustrifolia*. Chokeberry presented 24.8 g·kg^{-1} FW of cyanidin-3-galactoside and the other 21 berries presented values of individual anthocyanins much lower than the value of cyanidin-3-glucoside in our study. Besides that, raspberry presented values of pelargonidin-3-glucoside (0.3 g·kg^{-1} FW) similar to our fruit at RS5 (0.53 g·kg^{-1} FW).

In this study, three phenolic acids and one anthoxanthin were quantified in freeze-dried pulp of *B. ligustrifolia* fruit (Tab. 11). Even when contents decreased with maturation, gallic acid was the most abundant phenolic acid in the five ripening stages, followed by *p*-coumaric and *p*-hydroxybenzoic acids. This gradual decrease can be connected with an increased polyphenol oxidase activity, transformations (polymerization, oxidation and conjugation reactions) of phenolic acids, and the

Tab. 11. Gallic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid and catechin in *B. ligustrifolia* fruit at five ripening stages.

	Gallic acid	*p*-Hydroxybenzoic acid	*p*-Coumaric acid	Catechin
Retention time [min]	2.86 ± 0.02	9.54 ± 0.05	20.46 ± 0.10	10.53 ± 0.50
RS1	306.03 ± 5.88^{a}	28.24 ± 0.36^{a}	52.65 ± 1.67^{a}	29.38 ± 1.48^{a}
RS2	272.21 ± 4.57^{b}	20.59 ± 0.68^{b}	45.02 ± 1.63^{bc}	23.38 ± 0.74^{b}
RS3	258.90 ± 3.01^{b}	20.08 ± 0.46^{b}	42.19 ± 1.68^{cd}	23.27 ± 0.51^{b}
RS4	163.07 ± 1.60^{c}	12.73 ± 0.20^{c}	38.60 ± 0.96^{d}	22.92 ± 0.96^{b}
RS5	141.85 ± 1.70^{d}	10.09 ± 0.27^{d}	28.98 ± 1.04^{e}	16.31 ± 0.42^{c}

Results are mean values ± standard deviation (n = 3), expressed as grams per kilogram of fruit pulp on a dry weight basis. Same superscript letter in the column means no significant differences at 95% of confidence ($p < 0.05$) by Tukey's test.

reduction of primary metabolism in the over-ripe fruit, resulting in a lack of substrates necessary for the biosynthesis of phenolic compounds [9]. Studying the phenolic acids profiles of six berry fruits, ZADERNOWSKI et al. [49] found values of gallic acid, *p*-hydroxybenzoic acid and *p*-coumaric acid much lower than our results for *B. ligustrifolia* at RS5. TSANOVA-SAVOVA et al. [50] analysed the presence of catechin in 15 fruits, among these eight being berries, and all their results were much lower than the catechin values detected in our fruit in this study.

CONCLUSIONS

Since accurate quantification of bioactive substances in plant tissues depends largely on the extraction conditions, their optimization is fundamental to get the better contents of TMA and TPC. Our results showed that 20% acetone and 80% methanol worked best at extracting TMA and TPC from *B. ligustrifolia* fruits. Besides that, no changes were observed with proportions higher than 30 ml of solvent per 1 g of sample, and this value was suitable for both responses. During ripening, *B. ligustrifolia* fruit presented a significant decrease of TPC and a significant increase of TMA. From the first to the last ripening stage, TPC decreased to 57% and TMA became 50 times more concentrated. The anthocyanins identified and quantified were cyanidin-3-glucoside and pelargonidin-3-glucoside. The other phenolic compounds identified and quantified were gallic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid and catechin. This information suggests that the fruit is suitable for industrial utilization as a source of compounds with antioxidant activities. This is in line with the demand of the food industry for new food ingredients for developing commercial foods. The fruits can be included as functional ingredients in different foods, supplements or medicinal products.

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