Analytical Methods

Polyphenol extraction optimisation from Ceylon gooseberry (*Dovyalis hebecarpa*) pulp

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ABSTRACT

Originally from Asia, *Dovyalis hebecarpa* is a dark purple/red exotic berry now also produced in Brazil. However, no reports were found in the literature about phenolic extraction or characterisation of this berry. In this study we evaluate the extraction optimisation of anthocyanins and total phenolics in *D. hebecarpa* berries aiming at the development of a simple and mild analytical technique. Multivariate analysis was used to optimise the extraction variables (ethanol:water:acetone solvent proportions, times, and acid concentrations) at different levels. Acetone/water (20/80 v/v) gave the highest anthocyanin extraction yield, but pure water and different proportions of acetone/water or acetone/ethanol/water (with >50% of water) were also effective. Neither acid concentration nor time had a significant effect on extraction efficiency allowing to fix the recommended parameters at the lowest values tested (0.35% formic acid v/v, and 17.6 min). Under optimised conditions, extraction efficiencies were increased by 31.5% and 11% for anthocyanin and total phenolics, respectively as compared to traditional methods that use more solvent and time. Thus, the optimised methodology increased yields being less hazardous and time consuming than traditional methods. Finally, freeze-dried *D. hebecarpa* showed high content of target phytochemicals (319 mg/100 g and 1421 mg/100 g of total anthocyanin and total phenolic content, respectively).

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1. Introduction

Ceylon gooseberry or *Ketembilla* (*Dovyalis hebecarpa*, *Salicaceae* family), originally from Sri Lanka and introduced into USA (Florida) in 1920, is a small spherical fruit (0.5–1 inch) characterised by a deep purple-red colour, sour juice, and small seeds enclosed in the pulp. Fruits can be consumed as jams, juice, or fresh after skin removal. Despite an attractive appearance, skins are unpleasant in the mouth due to a velvety texture and could be considered co-product (Morton & Dowling, 1987). Recently, it is being cultivated as an exotic fruit in the southwest regions of Brazil. High production is obtained from March to May, but it is cultivated until August with satisfactory harvesting yields.

A hybrid of *D. hebecarpa* with *Dovyalis abyssinica* was reported as a source of vitamin C (120.3 mg/100 g of fresh fruit) and with good physical quality for market, showing an average of 75% pulp (Cavalcante & Martins, 2005). Phenolic composition of this hybrid revealed higher contents of anthocyanins in fruit peels and carotenoids in the pulp (De Rosso & Mercadante, 2007). Nevertheless, no reports were found about extraction and phytochemicals content in *D. hebecarpa* species.

Phenolic compounds are secondary metabolites in fruits and vegetables acting as a defense barrier against microorganism, insects, and UV radiation, or as attractants to promote pollination and seed dispersal. Some phytochemicals, as anthocyanins and phenolic acids, are very important for food acceptance acting as

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natural pigments or adding specific flavours, such as astringency and acidity (Shahidi & Naczk, 2003; Lattanzio, Kroon, Quideau, & Treutter, 2009).

Potential health benefits of phenolic compounds in the human diet have been extensively discussed in the literature and are still being investigated mainly linked to antioxidant activity and degenerative disease prevention (Zafra-Stone et al., 2007). The biological activity of berry phytochemicals is believed to be a result of multiple mechanisms that initially were believed to be mainly linked to antioxidant effects. However, nowadays the questionable polyphenols bioavailability has given rise to the discussion of different mechanisms by which it could act in the onset and in the development of degenerative diseases. Some of them are their anti-inflammatory activity, their enzyme inhibition capacity, or their potential modulation of the gut microbiota (Chiva-Blanch & Visioli, 2012).

Anthocyanins are glycosylated forms of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium, acylated or not, which are found as red to purple natural pigments in fruit and vegetables. Usually, extraction procedures are conducted using acidified solvents due to the higher anthocyanin stability at low pH values (Giusti & Jing, 2008). However, degradation of native chemical structure due to acid hydrolysis or oxidation can occur if the acid concentration is high or if the extraction time is long (Naczk & Shahidi, 2006; Revilla, Ryan, & Martín-Ortega, 1998). Thus, extraction conditions should be studied to ensure high extraction yields and minimal degradation in native chemical structure of these pigments. Finally, results obtained under optimised extraction conditions will better represent the real composition of the vegetable matrix being used for characterisation studies.

Aiming to develop efficient and fast new extraction techniques, sonication and subcritical fluids have been tested with promising results (Adil, Cetin, Yener, & Bayındırlı, 2007). However, these fluids increase the extraction costs and require equipment that is not typically present in most laboratory facilities. Moreover, in some cases, traditional methods still give better yields. Previous literature data revealed that solvent extraction showed higher results than an optimised subcritical fluid method (CO_2 plus ethanol) in apple and peach pomace for total phenolics and antioxidant activity (Adil et al., 2007). Nevertheless, the presence of even relatively safer solvents (such as alcohol) in the composition of natural colorants may limit its application in food. Thus, even that solvent extraction generates reliable results and satisfactory yields it should be optimised aiming the reduction or depletion of organic dissolvent agents. In addition, most extraction procedures were not optimised or validated and few details are reported about the selection of extraction variables. Considering it, anthocyanin and phenolic extraction could be one of the sources of high variation between reported values in a same plant material.

Furthermore, anthocyanins can be present in different compartments in the plant tissue, including vacuoles or in vacuolar inclusions. Thus, depending on the specific tissue characteristics, the optimum conditions for extraction to maximise yields may vary (Giusti & Jing, 2008).

Simplex lattice and central composite design are two multivariate experimental designs that allow investigating the effect of a mixture or a set of factors under one or more responses. The advantage of these techniques is that it allows the researcher to obtain knowledge of the whole system behaviour inside of the ranges, to evaluate simultaneously multiple variables, and to predict results using statically valid models (Ferreira et al., 2007). There is a wide range of applications of this statistical tool in the literature from bioprocesses in enzyme production (Ries & Alves Macedo, 2011) to extraction conditions for phenolics (Cacace & Mazza, 2002; Monrad, Srinivas, Howard, & King, 2012).

Due to the increasing need for efficient, simple, and mild extraction procedures for anthocyanins and other phenolics to allow its further evaluation and characterisation, this work was focused on optimising extraction methods for these types of compounds from *D. hebecarpa* pulp. Within this study, multivariate experimental designs were used to develop procedures investigating extraction variables to improve knowledge in this field.

2. Material and methods

2.1. Chemicals and equipment

The phenol reagent Folin–Ciocalteu and gallic acid were obtained from Sigma–Aldrich. A Büchi rotary evaporator and an UV-1600 spectrophotometer from Proanálise (São Paulo, Brazil) were used for concentration and quantification purposes. A food processor (Philips Mini Food Processor, model HR7625) Terroni Freeze-dryer, model LS-3000E (São Carlos, Brazil) and a Qhimis analytical grinder, model Q298A (São Paulo, Brazil) were used for sample preparation.

2.2. Sample preparation

Ripened samples (12.5 °Brix) were obtained from Bragança Paulista city (São Paulo, Brazil) in April of 2010 (see Supplementary material for fruit images). After harvesting, fruits were manually peeled while frozen (−20 °C) to minimise enzymatic degradation and decrease juice loss. Frozen pulps were crushed using a food processor and placed into trays to re-freeze. Materials were then freeze-dried until the pressure was reduced to stable values lower than 22 µHg. Since particle size is extremely relevant to extraction effectiveness, freeze-dried samples were grinded until a fine and visually homogenous powder was obtained.

2.3. Initial extraction procedure

A water/acetone (25:75 v/v) mixture, acidified with 2% of formic acid was the initial solvent tested (Mertz, Cheynier, Günata, & Brat, 2007). Freeze-dried powder samples (0.5 g) were mixed with the extraction solvent (15 ml) in a ratio of 1:30 w/v for 15 min, filtered and re-extracted under the same conditions. Both filtrates were combined, taken to a volume of 100 ml in a volumetric flask, and analysed for total phenolic and monomeric anthocyanins using the methods described below.

2.4. Extraction optimisation

2.4.1. Selection of the solid-to-liquid ratio

Solid–liquid ratios of 1:30; 1:60, 1:90, 1:120, 1:200 were studied in order to investigate the amount of solvent need to maximise the yield of anthocyanin and phenolic compounds extraction. Freeze-dried pulp powder (0.5 g) was extracted under agitation for 1 h with 75% aqueous acetone solution containing 2% formic acid in the different solid–liquid ratios chosen. Extraction media was filtered under vacuum, solids were discarded, and the filtrate was taken to an exact volume (100 mL) with the extraction solvent. The number of extractions was restricted to only one to avoid loses during the filtration process that could jumble the results. The solid–liquid ratio with highest extraction yield of anthocyanin and total phenolic compounds content was fixed for the next experimental step in the optimisation process. Three independent replications were performed and analysed in triplicate. ANOVA tests and means comparisons using Tukey test ($p \leqslant 0.05$) were performed in Statistica 7.0 (StatSoft, Inc.).

2.4.2. Evaluation of solvent effects by simplex lattice design (SLD)

Using the best solid–liquid ratio determined in the previous experiment, extraction efficiency with different proportions of acetone, water, and ethanol were studied aiming to determine the solvent combination to achieve higher extraction yields with lower percentage of organic solvents. A multivariate statistical technique for optimisation of mixtures, the simplex centroid design with axial points, was chosen due to the possibility of study all solvent proportions (0–100%) by using a reduced number of experiments. Moreover, this design allowed us to examine possible interaction effect among variables.

All conditions tested are presented in Table 1. In this design, the different conditions tested form a triangle, with pure components in the vertex, representing 100% of one of each solvent. Middle points in each side representing a binary mixture (1:1), the center point as a ternary mixture (1:1:1), and axial points representing 2/3 of one of the solvents and 1/6 for the others. This design permits 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}) (Eq. (1)) for the response under study.

$$Y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 \tag{1}$$

Total monomeric anthocyanin content (TMA, Y_1, mg/g freeze-dried sample) and total phenolic compounds content (TPC, Y_2, mg/g freeze-dried sample) were the two responses evaluated as described in Sections 2.5 and 2.6. The proportion of solvents that gave the highest response with the lowest concentration of organic solvent was fixed for the following experiments of time and acid concentration optimisation.

2.4.3. Time and acid concentration optimisation using response surface methodology (RSM)

Time and acid concentration were the two last variables optimised using the response surface methodology. The best solid–liquid ratio and mixture of solvents to obtain the highest concentration of anthocyanin and phenolic compounds were fixed based on results obtained from the previous experiments. At these conditions, a five levels ($-\alpha$, -1, 0, $+1$, $+\alpha$) central composite rotational design was used to optimise extraction time (X_1, from 17.6 to 102.43 min) and formic acid concentration (X_2, from 0.35% to

2.05%, see Supplementary material) variables. Eleven extraction conditions were tested which included 2^2 complete factorial, 4 axial points, and 3 repetitions of central conditions. Total monomeric anthocyanin content (TMA, Y_1, mg/g freeze-dried sample) and total phenolic compounds content (TPC, Y_2, mg/g freeze-dried sample) were the two responses studied evaluated as described in Sections 2.5 and 2.6.

Results were used to build a regression model with linear, quadratic, and interactive components (Eq. (2)).

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x^1 x^2 + \varepsilon \tag{2}$$

All experiments in the SLD and RSM were performed in random order to minimise the effects of unexplained variability in the observed responses due to systematic errors. ANOVA, regression results, and surfaces from fitted models were obtained using *Statistica* 7.0 (StatSoft). The final extraction procedure, using optimal conditions, was validated based on predictions of the developed model.

2.4.4. Comparison among extraction conditions and extraction efficiency with different berry matrices

Optimised conditions determined after the series of experiments were compared to another extraction methodology reported in the literature (Rodriguez-Saona & Wrolstad, 2001). Freeze-dried fine powders were exhaustively extracted with a mixture of 70/30 (v/v) of acetone in acidified water (0.01% HCl) under maceration. The resulting filtrates were combined, shaken, placed in a separatory funnel with chloroform (1:2 acetone: chloroform v/v), and stored overnight at 5 °C. The aqueous portion (top portion) was collected and placed on a rotary evaporator at 38 ± 2 °C until all residual acetone was evaporated. The aqueous extract was made up to a known volume with acidified distilled water (0.01% HCl). For the optimised procedure, extracts were placed in a rotatory evaporator to remove organic solvent and made up to a known volume using diluted formic acid solution (0.35%, pH 2.52 ± 0.01). Freeze-dried powder of Ceylon gooseberry pulp samples and entire berries of blueberry (*Vaccinium myrtillus*), blackberry (*Rubus ap.*, Guarani cultivar), Pitanga Brazilian cherry (*Eugenia uniflora* L.), and grape (*Vitis labrusca*, Isabel cultivar) were used in this experiment.

Table 1
Solvent optimisation experiments: simplex lattice design and observed responses.

Run number	Independent variables						Responses	
	Acetone (X_1)		Ethanol (X_2)		Water (X_3)		TPC[a]	TMA[a]
	Coded value	Real value (%)	Coded value	Real value (%)	Coded value	Real value (%)		
1	1	100	0	0	0	0	5.53	0.72
3	0	0	1	100	0	0	8.88	2.60
5	0	0	0	0	1	100	9.53	2.84
7	1/2	50	½	50	0	0	11.02	2.76
11	1/2	50	0	0	1/2	50	10.91	2.64
9	0	0	½	50	1/2	50	10.02	3.00
13	2/3	66.7	1/6	16.7	1/6	16.7	10.8	2.88
15	1/6	16.7	2/3	66.7	1/6	16.7	14.32	2.79
16	1/6	16.7	1/6	16.7	2/3	66.7	12.98	3.18
17	1/3	33.3	1/3	33.3	1/3	33.3	13.3	3.11
2	1	100	0	0	0	0	5.31	0.25
4	0	0	1	100	0	0	7.8	1.95
6	0	0	0	0	1	100	9.82	2.70
8	1/2	50	1/2	50	0	0	10.23	2.29
12	1/2	50	0	0	1/2	50	10.27	2.61
10	0	0	1/2	50	1/2	50	10.4	2.88
14	1/3	33.3	1/3	33.3	1/3	33.3	10.73	2.87

[a] Results were expressed as mg of cyanidin-3-glucoside equivalent/g of freeze-dried sample for total monomeric anthocyanins (TMA) and mg of gallic acid equivalent/g of freeze-dried sample for total phenolic compounds (TPC).

2.5. Total monomeric anthocyanin content (TMA)

Monomeric anthocyanin content was determined by using the pH-differential method described by Giusti and Wrolstad (2001). This method is based on the anthocyanin equilibrium forms obtained under acid (pH 1.0) and in a higher pH (pH 4.5) conditions. The coloured flavylium cation forms in acid conditions are measured against a correction in basic conditions, when colourless hemiketal forms of anthocyanins are present.

Readings were performed at 520 and 700 nm and the concentration of total monomeric anthocyanin content was calculated according to the Eq. (3). Results were expressed as mg of cyanidin-3-glucoside equivalents (CGE) per gram of freeze-dried pulp and, using values obtained for water loss after freeze-drying process, results were converted to mg per 100 grams of pulp fresh weight. Values of 449.2 and 26.900 were considered as molecular weight (MW) and molar absorptivity (ε) of cyanidin-3-glucoside, respectively (Eq. (4)).

$$A = (A_{520nm} - A_{700nm})_{pH1.0} - (A_{520nm} - A_{700nm})_{pH4.5} \qquad (3)$$

Monomeric anthocyanin pigment (mg/litre)

$$= (A \times MW \times DF \times 1000)/(\varepsilon \times 1) \qquad (4)$$

MW: molecular weight; DF: dilution factor. Since it is a response used for evaluation of extraction variables in the optimisation experiments, quality assurance was checked using the relative standard variation (RSD) for results obtained from extractions ($n = 3$) over 3 days of analysis.

2.6. Total phenolic compounds content (TPC)

Total phenolic content was measured using the Folin–Ciocalteu method as described by Singleton and Orthofer (1999) and modified by Scherer and Godoy (2014). This method is based on the reaction between phenolic compounds and sodium carbonate to form phenolates, which are able to reduce the reagent mixture Folin–Ciocalteu. Color in the reaction mixture changes from yellow to blue and is monitored at 760 nm. The extraction solvent was used for the blank. In the experiment with different solvent (mixture design), one curve was performed for each mixture of solvents. All results were presented as mg of gallic acid equivalents (GAE) per gram of freeze-dried sample and, using values obtained for water loss after freeze-drying process, results were converted to mg per 100 grams of pulp fresh weight. Quality assurance was checked using results obtained for the calibration curve over different days of analysis ($n = 3$).

3. Results and discussion

The total content of monomeric anthocyanins and phenolic compounds were the main responses used during optimisation studies and the relative standard deviation (RSD) values were checked for quality assurance. Thus, for the average value of 3.01 ± 0.22 mg of cyaniding-3-glucoside equivalents/g of freeze dried sample, RSD values in different days of analysis ranged from 3.2% to 4.3%. For the total content of phenolic compounds, a linear range was obtained from 10 to 65 μg/ml and the regression coefficient (r^2) was equal to 0.9993 ± 0.0008, using a seven-point gallic acid calibration curve. Relative standard deviation (RSD) at the lowest, the intermediate, and the highest concentration tested for calibration curves were 4.66%, 3.67%, and 2.83%, respectively. Second Horwitz & Albert, 1995, all values observed for RSD in both methods are acceptable and indicate satisfactory method precision.
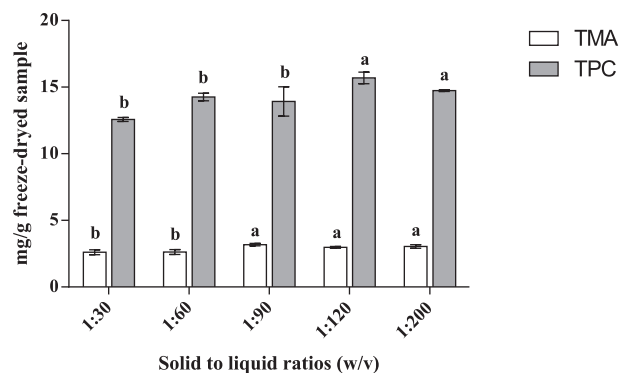
3.1. Solid–liquid ratio effect

Considering that solvents will extract target compounds until saturation or until reaching to equilibrium with the sample, the ratio between sample solids and solvent is one of the major variables that could affect the extraction efficiency. Aiming to ensure that all variables were efficiently studied, solid–liquid ratios were evaluated separately and fixed using the best condition before continuing with the optimisation experiments. Results of solvent (75% aqueous acetone containing 2% formic acid) to solid (freeze-dried powder) proportion experiments are illustrated in Fig. 1. A significant increase in phytochemical extraction yields was observed when the method was performed using 1:90 solid–liquid ratio for total anthocyanin content response, and 1:120 for total phenolic compounds. Thus, solid–liquid ratio was fixed in 1:120 for the next experiments, since no changes were observed with proportions higher than it and this value was suitable for both responses.

3.2. Solvent effects

Acidified methanol, ethanol, and acetone are often used as organic solvents for anthocyanin extraction (Giusti & Jing, 2008). Nevertheless, extraction with organic solvents has economic and environmental disadvantages, and the concept of "green chemistry" encourages the development and the use of less hazardous processes and materials without reduction in efficiency (Chemat, Vian, & Cravotto, 2012). Accordingly, solvent extraction of anthocyanin should be optimised for the maximum response using less organic solvents. Thus, water was chosen to be studied in combination with ethanol and acetone since native chemical structures of anthocyanins are usually linked to sugar moieties having increased water solubility (Giusti & Jing, 2008). Ethanol was chosen instead of methanol as extraction solvent due to the high human toxicity of methanol (EPA, 2013). Moreover, low ethanol concentrations could be used in some food industries processes, as it has already been classified as GRAS (Generally Recognized as Safe) by the FDA (Food and Drug Administration, USA) for some food additive preparations (FDA, 2012).

Results from complete SLD obtained in the solvent optimisation experiments are shown in Table 1. The lowest values for anthocyanin and total phenolic content were obtained in the *experiment number 1* which corresponds to extraction with pure acetone. Extraction with pure ethanol (*experiment 3*) and pure water (*experiment 5*) showed better extraction efficiency than with pure acetone (*experiment 1*). However, the best results were obtained

Fig. 1. Total monomeric anthocyanin (TMA) and total phenolic content (TPC) of Ceylon gooseberry freeze-dried sample extracted with different solid/liquid ratio w/v. Extraction conditions: 1 h under agitation with 2% of formic acid in 75% acetone in water. Results are mean ± standard deviation. Different letters in the same group represent significant differences (Tukey test; $p \leqslant 0.05$).

Table 2
ANOVA results of regression models estimated from SLD[*] for solvent optimisation and fitness of quadratic model.

ANOVA Source	SS	DF	*F*-value	*p*-Value	R^2
TMA[a]					
Linear	5.37	2	7.44	0.006	0.52
Quadratic	4.15	3	16.74	0.0002	0.91
Cubic	0.003	1	0.03	0.88	0.91
TPC[b]					
Linear	10.7	2	0.94	0.41	0.12
Quadratic	50.9	3	6.58	0.008	0.68
Cubic	0.6	1	0.23	0.64	0.69

Fitness of purpose for total monomeric anthocyanin's quadratic model

Source	SS	DF	*F*-value	*p*-value
TMA[a]				
Model	9.52	5	23.05	0.000017
Total Error	0.91	11		
Lack of fit	0.46	4	1.79	0.24
Pure error	0.45	7		
TPC[b]				
Model	61.56	5	4.78	0.01
Total error	28.32	11		
Lack of fit	27.08	4	38.19	0.00008
Pure error	1.24	7		

[*] Simplex lattice design.
[a] Total monomeric anthocyanins.
[b] Total phenolic compounds.

with a mixture of solvents (*experiments 15 and 16*). Thus, for total phenolic content the highest yield (14.32 mg of GAE/g freeze-dried sample, *experiment 15*) was obtained with ethanol as the main solvent in the mixture composed of 1/6, 2/3, and 1/6 fractions of acetone, ethanol, and water in the whole solvent volume, respectively. A similar behaviour was observed for total monomeric anthocyanin where the highest concentration was obtained in *experiment 16*, a mixture of all solvents with higher amount of water than acetone and ethanol (1/6, 1/6, and 2/3 fractions of acetone, ethanol, and water in the whole solvent mixture, respectively).

ANOVA from regression models and fitness of purpose for quadratic models are shown in Table 2. Significant quadratic models were obtained for both responses; however, just the model obtained for anthocyanin content has no lack of fit and could be used for make predictions. Significant coefficients were obtained for all variables studied (0.55 ± 0.2 for β_1, 2.29 ± 0.2 for β_2, and 2.75 ± 0.2 for β_3 with *p*-values of 0.018, 0.1×10^{-6}, and 0.1×10^{-6}, respectively) and almost all interactions showed an important synergistic effect between acetone and ethanol (4.82 ± 0.91 for β_{12}), and between acetone and water (4.15 ± 0.91 for β_{13}). However, the interaction coefficient between ethanol and water (β_{23}) was no significant for the studied response. Because of it, this coefficient was removed from the model before predictions (see estimated regression coefficients, standard error and *p*-values at Supplementary material). The model prediction

with the highest desirability revealed that the best proportion of solvents was 20% of acetone with 80% of water. The same proportion was obtained with all coefficients in the model, but adjusting for the highest desirability in a proportion with the lowest amount of organic solvents (ethanol and acetone).

Aiming to validate the obtained fitted model for anthocyanin content new experiments were performed with different proportions between solvents and in the best condition achieved (Table 3). Three independent experiments were performed in each condition and analysed. All results obtained for anthocyanin content are in agreement with the model predictions and within the confidence interval (95%). Notwithstanding the model obtained for total phenolic had significant lack of fitness (*p*-value of 0.00008) being not useful for predictions, solvent mixtures used for the anthocyanin model validation were also monitored for this response. Results in conditions B and D (Table 3) were inside of the interval of confidence. However, as expected for a non-fitted model, conditions A and C were higher than predicted values. Condition D showed the highest content of anthocyanin and total phenolic compounds which is in agreement with the optimum condition predicted by the anthocyanin quadratic model.

Fig. 2 shows the surface obtained with the valid model for total monomeric anthocyanin content response, only with significant coefficients ($Y = +0.533 * X_1 + 2.456 * X_2 + 2.911 * X_3 + 4.780 * X_1 * X_2 + 4.110 * X_1 * X_3 + 0.100$). Even though the best condition was the mixture of 20% acetone, 80% water, it is possible to determine that extraction with pure water will also produce acceptable results. In agreement with this observation, some recent works (Li, Fabiano-Tixier, Vian, & Chemat, 2013; Li et al., 2012; Petersson, Liu, Sjöberg, Danielsson, & Turner, 2010; Puértolas, Cregenzán, Luengo, Álvarez, & Raso, 2012) have proposed water as environmental-friendly solvent for extraction of phenolic compounds and anthocyanins, in some cases with similar yield to organic solvents commonly used. However, in previous experiments, pure water was applied in combination with pressure and high temperatures (Petersson et al., 2010), micro-wave-assisted techniques (Li et al., 2012, 2013), and pulsed-eletric-field-assisted (Puértolas et al., 2012) extraction with results that were similar to values reported when using organic solvents. In our experiments, we obtained high extraction yields without assistance of additional extraction technologies other than simple homogenisation.

3.3. Time and acid concentration effect

For the studies of time and acid effects the extracting solvent was fixed according to the results of previous experiments at 20% of acetone in water as the optimum for anthocyanin extraction. Using this proportion, time and acid concentration were optimised in a central composite design. There was almost no difference in the anthocyanin and total phenolic content obtained from all different experiments (see complete experimental design and results in

Table 3
Validation results for quadratic model obtained from SCD[*] in solvent optimisation experiments.

Condition	Acetone	Ethanol	Water	TMA[a] (mg/g freeze-dried sample)				TPC[b] (mg/g freeze-dried sample)			
				Predicted Values	Interval of confidence		Experimental Values	Predicted Values	Interval of confidence		Experimental Values
	X_1	X_2	X_3		−95%	+95%			-95%	+95%	
A	0.37	0	0.63	2.99	2.59	3.4	2.73 + 0.02	11.89	9.82	13.96	13.08 + 0.48
B	0.25	0.32	0.43	2.99	2.77	3.21	3.07 + 0.04	11.6	10.46	12.73	13.28 + 0.13
C	0.25	0.75	0	2.87	2.52	3.22	2.78 + 0.02	11.34	9.53	13.15	12.84 + 0.14
D	0.2	0	0.8	3.1	2.76	3.42	3.19 + 0.05	11.64	9.92	13.35	14.21 + 0.06

[*] Simplex lattice design.
[a] Total monomeric anthocyanins expressed as cyanidin-3-glucoside equivalent.
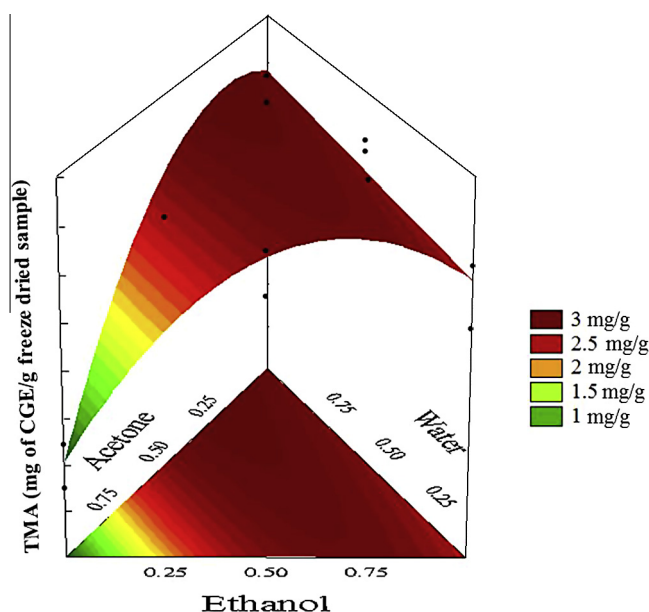[b] Total phenolic compounds expressed as gallic acid equivalent.

Fig. 2. Total monomeric anthocyanin (TMA) content surface obtained with quadratic model for solvent mixture optimisation using simplex centroid design (SCD). Results are expressed as mg of cyanidin-3-glucoside/g of freeze-dried sample.

Supplementary material). ANOVA from regression analyses showed that linear and quadratic models are not significant to explain the oscillation among the results. In other words, in the range studied, acid and time had no effect on the total phenolic compounds and total monomeric anthocyanin content yields obtained. Results ranged from 3.32 to 3.65 mg of CGE/g and from 12.55 to 14.21 mg of GAE/g of freeze-dried pulp sample for total monomeric anthocyanin content and total polyphenol content, respectively.

Considering that short extraction times are desirable and lower acid concentration are desirable to avoid hydrolysis and degradation of target compounds, 0.35% of formic acid and 17.6 min were the conditions chose as the optimum for extraction of anthocyanins and phenolic compounds of Ceylon gooseberry. Larger ranges could be tested for extraction time and acid concentration aiming to find a valid model. Nevertheless, the obtained results probably are very close to the real amount in the pulp and extreme conditions could result in less stable extracts.

3.4. Comparison between extraction conditions method efficiency with different berry matrices

There is a diversity of extraction methods for anthocyanins and phenolic compounds which are recognised as able to be applied from berries to more complex samples. Rodriguez-Saona and Wrolstad (2001) have published a rather universal protocol that allows for the extraction of anthocyanin from a range of different materials (Duan, Wu, Strik, & Zhao, 2011; Lima, Melo, Pinheiro, & Guerra, 2011) with a subsequent partition in chloroform that partially purifies the pigments. Extraction is exhaustively performed with 70% of acetone in water with hydrochloric acid (0.01%). This procedure was reproduced using freeze-dried powder of Ceylon gooseberry and compared to the extraction in the optimised and initial conditions aiming to evaluate the efficiency of final extraction method. Moreover, it was evaluated the applicability of the optimised extraction in other berry sample matrices (entire grapes, Pitanga cherry, blackberry, and blueberry). All results obtained with our optimised protocol were compared to the results obtained with exhaustive extraction described above (Table 4).

For Ceylon gooseberry samples, optimised conditions showed higher ($p \leqslant 0.05$) values for TMA (Table 4) and TPC (13.96 ± 0.06 mg GAE/g of freeze-dried sample) when compared to the initial procedure (12.57 ± 0.05 mg GAE per gram of freeze-dried pulp sample, respectively) representing an increase of 31.54% and 11.06%, respectively. RSD using our optimised conditions was lower than the values obtained with the traditional extraction conditions showing an improvement in method repeatability.

Moreover, for all matrices tested, ANOVA revealed no significant differences between our optimised conditions determined in this work and the exhaustive traditional extraction procedure. Thus, our optimised extraction conditions were applied to the extraction of phenolics and anthocyanins from Ceylon gooseberry pulp samples and in all tested matrices with similar extraction yields than those obtained with the exhaustive extraction procedure with 70% acetone. For sources other than berry fruits and with a different cellular structure, the optimum extraction conditions for maximised compound recovery should be checked prior to their chemical analyses. Both extraction methods showed satisfactory repeatability, with RSD (from 1.9% to 5.4%, Table 4) inside of the limits described by Horwitz and Albert (1995) in the range of concentrations found in fruit matrices.

ANOVA showed significant differences among mean values of total anthocyanin content determined in berry samples. Ceylon

Table 4
Extraction comparison results for total monomeric anthocyanin content in Ceylon gooseberry freeze-dried pulp and other berry fruit matrices.

Sample	Extraction condition	TMA content (mg/g freeze-dried sample)	RSD (%)
Ceylon gooseberry pulp (*Dovyalis hebecarpa*)	A	2.79 ± 0.19	6.73
	B	3.67 ± 0.09	3.67
	C	3.29 ± 0.22	3.29
	Mean value[*]	3.48[b]	
Entire blueberry (*Vaccinium myrtillus*)	B	5.25 ± 0.39	5.24
	C	5.32 ± 0.27	5.29
	Mean value	5.28[a]	
Entire blackberry (*Rubus ap.*, Guarani cultivar)	B	5.75 ± 0.70	5.36
	C	4.66 ± 1.21	4.78
	Mean value	5.20[a]	
Entire Pitanga Brazilian cherry (*Eugenia uniflora* L.)	B	2.40 ± 0.31	2.49
	C	1.91 ± 0.17	1.90
	Mean value	2.15[c]	
Entire grape (*Vitis labrusca*, Isabel cultivar)	B	2.66 ± 0.25	2.56
	C	2.32 ± 0.19	2.71
	Mean value	2.49[c]	

RSD: relative standard deviation. Results are mean values (n = 3) plus standard deviations. Same lower case letter in the column means no significant differences at 95% of confidence ($p \leqslant 0.05$) by Tukey test. A: Extraction at initial conditions which were 2% of formic acid in 75% of acetone in water under one hour of mixing. B: Final optimised conditions which were 0.35% of formic acid in 20% of acetone in water under 18 min of mixing. C: Exhaustive extraction with 0.01% of HCl in 70% of acetone in water as reported by Rodriguez-Saona and Wrolstad (2001). [*] Mean value of results obtained in conditions B and C.

gooseberry pulp sample showed higher anthocyanin levels than entire Pitanga cherry and grape berries (Isabel cultivar) and lower than blueberries and blackberries. Since, Ceylon gooseberry peels have a velvety texture and it is usually removed before consumption, only pulp fruit part was considered as edible part and used for analysis. As previously reported (Cevallos-Casals, Byrne, Okie, & Cisneros-Zevallos, 2006; Lee & Wrolstad, 2006), higher concentration of polyphenols are normally found in the exocarp than in the mesocarp fruit part. Thus, results for whole Ceylon gooseberry fruits are expected to be higher and possibly closer to blackberry and blueberry anthocyanin levels. Finally, Ceylon gooseberry pulp can be considered a source of anthocyanin and other phenolic compounds with concentration values converted to fresh weight (49.04 ± 0.08 mg CGE/100 g and 187.06 ± 0.08 mg of GAE/100 g of FW) similar to other gooseberry species (2.4–43.3 mg of CGE/100 g FW) and raspberries (1.3–88.4 mg of cyanidin-3-gluoside equivalents/100 g FW 129–184.6 mg GAE/100 g) (Dobson, Grahan, Stewart, & Brennan Hackett, 2012; Pantelidis, Vasilakakis, Manganaris, & Diamantidis, 2007).

4. Conclusion

Extraction conditions were optimised and maximum anthocyanin yields were obtained in experiments with high proportions of water (from 50% to pure water, with the optimum conditions using 80% of water with 20% acetone). Since pH values in final solvent media did not reach to values above 2.0, acid concentration had no effect in anthocyanin and phenolic extraction and neither did the extraction time in the ranges tested. Consequently, both variables were fixed in the minimum values studied. The optimised condition, using less organic solvent in a shorter time, produced phytochemical yields comparable to those obtained with other traditional methods using harsh chemicals. Moreover, Ceylon gooseberry is a source of anthocyanin and other phytochemical with great potential to be used with industrial purposes, since it can be extracted with pure water.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.05.031.

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