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Pressurized liquid extraction of bioactive compounds from blackberry (*Rubus fruticosus* L.) residues: a comparison with conventional methods

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ABSTRACT

Extracts with antioxidant compounds were obtained from residues of blackberries (*Rubus fruticosus* L.) through pressurized liquid extraction (PLE). The influence of solvent type (water, acidified water pH = 2.5, ethanol and ethanol + water 50% v:v) and temperature (60, 80 and 100 °C) on global yield (X_0), total phenolics (TP), monomeric anthocyanins (MA) and antioxidant activity (AA) (by DPPH and ABTS) of the extracts was evaluated. Moreover, anthocyanins were identified and quantified by UHPLC-QToF-MS e UHPLC-UV-Vis, respectively. The best PLE condition was compared to conventional extractions (Soxhlet and maceration). Results showed positive influence of temperature on global yield, TP and AA. Ethanol + water as solvent at 100 °C was chosen as the best PLE condition, providing TP = 7.36 mgGAE/g fresh residue, MA = 1.02 mg C3GE/g fresh residue, AA = 76.03 μmol TE/g fresh residue and X_0 = 6.33%. Excepting MA, all other results were over those of conventional extractions. Four anthocyanins were identified by UHPLC in the extracts, and their higher yields were achieved with acidified water as solvent at 60 and 80 °C. PLE has proved to be a promising alternative to recover bioactive compounds from blackberry residues, as well as other food by-products.

1. Introduction

Blackberries (*Rubus spp.*, Rosaceae) have been appreciated by consumers, not only for their high nutritional value, but also for their benefits to physical and mental health (Ivanovic et al., 2014; Tavares et al., 2012). Besides high contents of fibers, vitamins, and essential minerals, blackberry is an important source of phenolic compounds, such as phenolic acids, tannins, elagitannins, flavonoids and anthocyanins (Elisia, Hu, Popovich, & Kitts, 2007; Hager, Howard, Liyanage, Lay, & Prior, 2008; Kaume, Howard, & Devareddy, 2011; Seeram et al., 2006). Polyphenols contribute to prevent degenerative diseases, and their effects on human health have been mainly attributed to their antioxidant properties (Sariburun, Şahin, Demir, Türkben, & Uylaşer, 2010; Serraino et al., 2003; Tate, Stanner, Shields, Smith, & Larcom, 2006). Anthocyanins are water-soluble natural dyes, responsible for the typical color of blackberries, and which have been considered as potential replacers of synthetic colorants, besides playing important role in human nutrition (Haminiuk, Maciel, Plata-Oviedo, & Peralta, 2012; Ivanovic et al., 2014; Li et al., 2012).

Blackberries are usually consumed as fresh, but may be processed and commercialized frozen or as concentrated juice. In the industry, blackberries are used to produce dietary supplements, juices, yoghurts, ice creams, jellies and other candies (Kafkas, Koşar, Türemiş, & Başer, 2006; Kaume et al., 2011). Researches have shown that agricultural and industrial residues from berries and other vegetable are good sources of bioactive compounds (Balasundram, Sundram, & Samman, 2006; Ignat, Volf, & Popa, 2011; Khanal, Howard, & Prior, 2010; Moure et al., 2001; Nile & Park, 2013). For example, in the peels of various fruits phenolics are found at concentrations higher than in the pulp (Balasundram et al., 2006; Cam & Hisil, 2010; Schieber, Stintzing, & Carle, 2001; Wijngaard, Hossain, Rai, & Brunton, 2012). From the economic point of view, industrial residues from vegetable materials are the best sourced of phenolic compounds (Vatai, Škerget, & Knez, 2009). The interest in the recovery of these by-products as raw material for processing new food, pharmaceutical and cosmetics is increasing, and represents an important activity, since it adds value to a food waste and reduces its disposal onto the environment.

The isolation of bioactive compounds, as polyphenols and anthocyanins, from vegetable matrices, is traditionally achieved through extraction with organic solvents with stirring and/or heating. However, traditional techniques generally consume long times, leading to degradation of target compounds during extraction, and require large amounts of organic solvents that, in some cases, are hazardous to human health and pollutant, needing to be separated from the extract and adequately discarded (Ivanovic et al., 2014; Roseiro et al., 2013).

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Therefore, pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE), appeared as alternative for the extraction and fractionation of natural products, since it allows faster extraction and reduces solvent consumption through a "green" technology. Moreover, in PLE one can adjust process parameters envisaging the process selectivity to a particular group of compounds (Carabias-Martínez, Rodríguez-Gonzalo, Revilla-Ruiz, & Hernández-Méndez, 2005). PLE using water and/or ethanol as solvents is even more promising, since these are GRAS (generally recognized as safe) solvents (Monrad, Howard, King, Srinivas, & Mauromoustakos, 2010; Wiboonsirikul & Adachi, 2008). It has been successfully used to extract thermally sensible phytochemicals from many vegetable sources (Luthria, 2008; Mustafa & Turner, 2011; Paes, Dotta, Barbero, & Martínez, 2014; Wijngaard, Ballay, & Brunton, 2012; Wijngaard, Hossain, et al., 2012). PLE allows fast extraction in closed and inert medium under high pressure and temperature. Once the extraction is performed at high pressures, the solvent can remain at liquid state (so-called subcritical), even when taken to temperatures much over their boiling points. Thus, PLE can operate at high temperatures, where solubility of the target compounds and desorption kinetics from the matrix are enhanced (Camel, 2001; Mustafa & Turner, 2011; Pronyk & Mazza, 2009).

The objective of this work was to obtain extracts containing bioactive compounds from residues discarded at the processing of blackberries (*Rubus fruticosus* L.), using PLE as extraction method. The influence of temperature and solvent type was evaluated, and chemical composition and antioxidant activity of the extracts were determined. The PLE results are compared to those obtained by traditional extraction techniques.

2. Materials and methods

2.1. Chemicals

The chemical compounds 1,1-diphenyl-2-picrylhydrazyl (DPPH); 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS); 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid (Trolox); potassium persulphate, monohydrated gallic acid and the anthocyanin standard (cyanidin chloride) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). Citric acid, hydrochloric acid, sodium hydroxide and sodium carbonate were purchased from Synth (São Paulo, Brazil), and ethanol and methanol were purchased from Êxodo Científica (São Paulo, Brazil). The Folin-Ciocalteu reagent was acquired from Dinâmica (São Paulo, Brazil), and petroleum ether was from Quemis (São Paulo, Brazil). Methanol (Merck, Darmstadt, Germany) and formic acid (Panreac, Barcelona, Spain) were HPLC grade. Ultrapure water was obtained with a purification system (Milli-Q, Millipore, Bedford, MA).

2.2. Raw material

Blackberry residues were donated by the company Sítio do Bello, a juice processing industry located in Paraibuna, southeastern Brazil (23° 23' 9" S, 45° 39' 43" W). The residue came from the production of blackberry (variety Caingangue) pulp, and consisted of a bagasse (peel and seeds), which was collected, identified and stored in sealed plastic bags under freezing (−18 °C) until the extractions.

2.3. Pressurized liquid extraction (PLE)

2.3.1. Extraction unit

The schematic diagram of the PLE unit is presented in Fig. 1(a). The equipment is composed by a HPLC pump (LabAlliance, MY) that operates at flow rated from 0.01 to 10.0 mL.min^{-1}; two block valves to control the solvent flow; a 100 mL stainless steel extraction cell projected to operate at pressure and temperature up to 25 MPa and 250 °C, respectively; an electric heating jacket to keep the cell at the extraction temperature; a pressure gauge; a micrometer valve to establish the solvent flow rate; temperature indicator; and collection vessel. All pipes and connections were of stainless steel (1/16"and 1/8").

2.3.2. Extraction procedure

Approximately 5 g of fresh blackberry residue was placed between two glass sphere layers in the extraction cell, as shown in Fig. 1(b). In the cell outlet, besides a filter, a glass wool layer was added to avoid obstruction of the pipeline with particles in suspension. The PLE procedure was the following: (1) the extraction cell was heated for 25 min, until the process temperature was achieved; (2) the solvent was pumped into the extraction cell at the programmed flow rate, until pressure reached 7.5 MPa, which was the extraction pressure for all conditions; (3) the outlet block valve and the micrometer valve were opened, and extraction began; (4) extraction was performed for 30 min; (5) at the end of the extraction, the extract volume was measured, transferred to an amber vessel, and stored at −18 °C until the analyses.

To determine the effects of temperature and solvent type on the recovery of phenolics, anthocyanins and antioxidant activity, temperatures of 60, 80, and 100 °C were tested at the reported conditions, using pure water, pure ethanol, water + ethanol (50% v/v) and acidified water (pH = 2.5, adjusted by the direct addition of citric acid). Therefore, 12 different conditions were evaluated. The extraction pressure was of 7.5 MPa for all conditions. Besides pressure and extraction time, the solvent to feed mass ratio (S/F = 18 g solvent/g residue) was also kept constant. To achieve the constant S/F, different flow rates were used for each solvent (3.00 mL.min^{-1} for acidified water and pure water; 3.80 mL.min^{-1} for pure ethanol; and 3.35 mL.min^{-1} for water + ethanol), since their densities are not the same. All conditions were performed in duplicates, and their results are expressed in mean ± standard deviation.

2.4. Conventional extractions: Soxhlet and maceration

For the Soxhlet extraction, 5 g of fresh residue was placed inside a cellulose filter paper cartridge and extracted with 200 mL of solvent (ethanol and methanol) at 80 °C. Reflux was kept for 5 h. Maceration was performed according to the procedure described by Rodriguez-Saona and Wrolstad (2001). The residue was immersed in acidified methanol (0.01% v/v HCl) at room temperature during 24 h. Methanol and ethanol have been reported as efficient solvents to extract phenolics and antioxidants from vegetable substrates (Wijngaard, Ballay, et al., 2012; Wijngaard & Brunton, 2009; Wijngaard, Hossain, et al., 2012). After the extractions, the extract volumes were measured, transferred to an amber vessel, and stored at −18 °C until the analyses. The extractions were performed in triplicates, and their results are expressed in mean ± standard deviation.

2.5. Global yield (X_0)

The extraction global yields (X_0) obtained by PLE and the conventional methods were calculated by the ratio between the extract mass ($M_{extract}$) in dry basis and the mass of fresh residue fed into the extraction cell (F), as stated in Eq. (1). To determine $M_{extract}$ 8.0 mL of extract were dried at 105 °C in stove (FANEM, Mod. 320-SE, Brazil) until constant weight. Since the initial volume of the extract was known, the total extract mass could be calculated.

$$X_0 = \frac{M_{extract}}{F} x\ 100 \quad (1)$$

2.6. Chemical characterization of the extracts

2.6.1. Total phenolic content

The total phenolic content of the extracts obtained from blackberry bagasse by PLE, Soxhlet and maceration was determined through the

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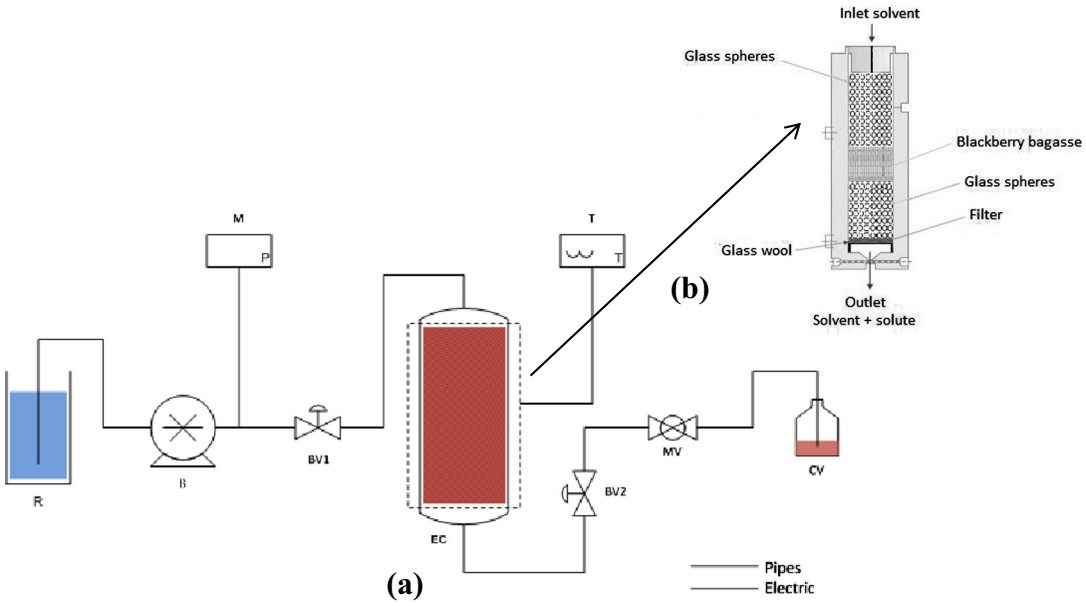

Fig. 1. Schematic diagram of the PLE unit. R: solvent reservoir; B: solvent pump; M: pressure gauge; BV1 and BV2: block valves; EC: extraction cell with heating jacket; MV: micrometer valve; T: temperature controller and indicator; CV: collection vessel.

spectrophotometric Folin-Ciocalteu method described by Singleton and Rossi (1965), with some modifications suggested by Singleton, Orthofer, and Lamuela-Raventós (1999) for vegetable extracts. Gallic acid was used as standard, and a methanolic solution 1.0 mg.mL^{-1} was diluted in methanol at various concentrations (0.01–0.08 mg.mL^{-1}) to build a standard curve. The reaction was performed by transferring 0.5 mL of extract sample or standard solutions into vials. Next, 2.5 mL of the Folin-Ciocalteu reagent (1:10) was added. After 5 min, 2.0 mL of aqueous sodium carbonate solution 7.5% was added. The vials were manually stirred for 30 s and rested protected from light at room temperature for 2 h. The absorbance of the solutions was measured at 760 nm in spectrophotometer (Hach, Mod.DR/4000, CO). All the experiments were performed in duplicates, and the results are expressed in mg gallic acid equivalent (mg GAE)/g of fresh residue (FR).

2.6.2. Monomeric anthocyanins (MA)

The monomeric anthocyanin content (MA) of the PLE, Soxhlet and maceration extracts was determined by the differential pH method described by Giusti and Wrolstad (2001). The method consists in dissolving the samples in two buffer systems: potassium chloride pH 1.0 (0.025 M) and sodium acetate pH 4.5 (0.4 M). The extracts from blackberry bagasse were diluted into these solutions in a way that their absorbances measured in spectrophotometer (Hach, Mod.DR/4000, CO) from 510 to 700 nm were lower than 1000. The absorbance measures were performed 25 min after the dilutions. The anthocyanin concentration was calculated considering the molar absortivity (ε) of 26,900 L/cm.mol, molecular mass of 449.2 g/mol, which corresponds to cyanidin-3-O-glucoside, the major anthocyanin found in blackberries (Siriwoharn, Wrolstad, Finn, & Pereira, 2004), using Eq. (2). Results were expressed as mg cyanidin-3-O-glucoside equivalent (mg C3GE)/g of fresh residue (FR). All measurements were performed in duplicates.

$$AM\ \left({}^{mg}/_{L}\right) = \frac{A\ x\ MM\ x\ FD\ x\ 1000}{\varepsilon\ x\ 1} \qquad (2)$$

Where:

A $(Abs_{510\ nm} - Abs_{700\ nm})_{pH\ 1.0} - (A_{510\ nm} - A_{700\ nm})_{pH\ 4.5}$ = absorbance of the sample;
MM molecular mass of cyanidin-3-O-glucoside;
FD dilution factor;
l correction optic path factor (1 cm).

2.6.3. Identification of anthocyanins by UHPLC-QToF-MS

Anthocyanins in PLE extracts were identified by ultra-performance liquid chromatography (UHPLC) coupled to quadrupole-time-of-flight mass spectrometry (Q-ToF-MS) (Synapt G2, Waters Corp., Milford, MA, USA). The extracts were filtered through a 0.20 μm nylon syringe filter (Membrane Solutions, Dallas, USA) prior to chromatographic analysis. The injection volume was set to 3 μl. The chromatographic separation was performed on a reverse-phase C18 analytical column (Acquity UPLC BEH C18, Waters) of 2.1 mm × 100 mm and 1.7 μm particle size. For the identification of anthocyanins, water (2% formic acid) as solvent A and methanol as solvent B, as mobile phases at a flow rate of 0.4 mL.min^{-1} were used. The gradient employed was as follows: 0 min, 15% B; 3.30 min, 20% B; 3.86 min, 30% B; 5.05 min, 40% B; 5.35 min, 55% B; 5.64 min, 60% B; 5.94 min, 95% B; 7.50 min, 95% B. Total run time was 12 min, including 4 min for re-equilibration. The determination of the analytes was carried out using an electrospray source operating in positive ionization mode under the following conditions: desolvation gas flow = 700 L/h, desolvation temperature = 500 °C, cone gas flow = 10 L/h, source temperature = 150 °C, capillary voltage = 700 V, cone voltage = 30 V and collision energy = 20 eV. Full-scan mode was used (m/z = 100–800).

2.6.4. Separation and quantification of anthocyanins by UHPLC-UV-Vis

The separation and quantification of anthocyanins in PLE extracts were performed on an Elite UHPLC LaChrom (VWR Hitachi, Tokyo, Japan) consisting of an L-2200U Autosampler, an L2300 Column Oven, an L-2160U Pumps and an L-2420U UV-Vis Detector. The column oven was adjusted at 50 °C for the chromatographic. UV-Vis Detector was set at 520 nm for the analysis. The extracts were filtered through a 0.20 μm nylon syringe filter (Membrane Solutions, Dallas, USA) prior to chromatographic analysis. Anthocyanins were analyzed on a Halo™ C18 Hitachi LaChrom column (100 × 3 mm I.D., particle size 2.7 μm). A gradient method, using acidified water (5% formic acid, solvent A) and methanol (solvent B), working at a flow rate of 1.0 mL.min^{-1}, was employed for the chromatographic separation. The gradient

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employed was as follows: 0 min, 15% B; 1.50 min, 20% B; 3.30 min, 30% B; 4.80 min, 40% B; 5.40 min, 55% B; 5.90 min, 60% B; 6.60 min, 95% B; 9.30 min, 95% B; 10 min, 15% B. The UPLC method was used to obtain a calibration curve for cyanidin chloride ($y = 300568.88x - 28462.43$), which is the anthocyanidin standard commercially available for cyanidin anthocianins. Regression equation and correlation coefficient ($r^2 = 0.9999$) were calculated using Microsoft Office Excel 2010. The limit of detection (0.198 $mg.L^{-1}$) and quantification (0.662 $mg.L^{-1}$) were also calculated using Microsoft Office Excel 2010. The four anthocyanins present in blackberry residues were quantified using calibration curve for cyaniding chloride, taking into account the molecular weight of the anthocyanins analyzed. All analyses were carried out in duplicate. Results were expressed as milligrams of anthocyanins per g of fresh residue (FR).

2.7. Antioxidant activity (AA)

2.7.1. Method DPPH (1,1-diphenyl-2-picrylhydrazyl)

The antioxidant activity (AA) of the PLE, Soxhlet and maceration extracts was evaluated in terms of the ability of the antioxidants to sequestrate the stable radical DPPH•, with the method described by Brand-Williams, Cuvelier, and Berset (1995) with some adaptations. A Trolox standard curve was built (with concentrations from 50 to 1200 μM). In dark room, 0.1 mL of the extract diluted in methanol or the Trolox solutions were transferred to vials and mixed to 3.9 mL of a methanolic DPPH solution (60 μM). The vials were manually stirred for 30 s and rested in dark at room temperature for 30 min. The absorbance of the samples was measured in spectrophotometer (Hach, Mod.DR/4000, CO) at 515 nm. The sequestration percentage (%*Seq*) was calculated with Eq. (3).

$$(\%)Seq = 100\,x\left(\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right) \quad (3)$$

Where:

$Abs_{controle}$ is the initial absorbance of the methanolic DPPH solution;
$Abs_{amostra}$ is the absorbance of the reaction mixture (DPPH + sample).

The antioxidant activity of the extracts was expressed as μmol Trolox equivalent (mg TE)/g of fresh residue (FR). All measurements were performed in duplicates. The use of TE helps comparing the antioxidant activities obtained by DPPH and ABTS.

2.7.2. Method ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid))

The antioxidant capacity of the extracts from blackberry bagasse against the radical ABTS + • was evaluated according to Re et al. (1999) with some adaptations. The cation ABTS + • was produced by reacting 5 mL of ABTS aqueous solution (7 mM) with 88 μL of potassium persulphate solution (140 mM). The green-colored reaction mixture was stored in dark vessel at room temperature for 16 h, and then was diluted in ethanol to achieve an absorbance of 0.700 ± 0.050 at 734 nm. In dark room, 30 μL of the blackberry bagasse extract diluted in ethanol or the Trolox standard was transferred to vials and mixed with 3 mL of the resulting ABTS solution. After 6 min of reaction, the absorbance was measure in spectrophotometer (Hach, Mod.DR/4000, CO) at 734 nm. A standard Trolox curve was built with concentrations from 100 to 2000 μM. The antioxidant activity of the extracts was expressed as μmol Trolox equivalent (mg TE)/g of fresh residue (FR). All measurements were performed in duplicates.

2.8. Statistical analyses

All the results obtained for the extracts from blackberry bagasse were evaluated using analysis of variance (ANOVA) with significance level of 5% ($\alpha = 0.05$), followed by Tukey's test ($\alpha = 0.05$) to compare the means, using the software SAS version 9.2 for Windows (SAS Institute, Cary, NC). To determine the linear correlation between TP and AA the correlation coefficient of Pearson was calculated using Microsoft Office Excel 2010.

3. Results and discussion

Table 1 presents the global yields (X_0), total phenolic (TP) and monomeric anthocyanins (MA) contents and the antioxidant activities (AA–DPPH and ABTS) found for the Soxhlet, maceration and PLE extracts obtained from blackberry bagasse at the conditions described in Sections 2.3 and 2.4. The quantification of the anthocyanins identified by UPLC is reported on Table 2.

3.1. Global yield of PLE

The highest global yields of the PLE extracts were achieved with acidified water as solvent (12.10%, 14.27% and 14.99% at 60, 80 and 100 °C, respectively). With the other solvents, most of the global yields were statistically equal, near the mean value of 4.60%. Similar results were obtained by Ivanovic et al. (2014) for the blackberry variety "Cacanska Bestrna" with ultrasound assisted extraction with acidified ethanol as solvent, at moderate temperatures (25 and 40 °C). The great discrepancy found between X_0 with acidified water and other solvents occurs because acidifies media contribute to increase solubility of compounds present in the sample, such as carbohydrates (pectin, gums, hemicellulose) and proteins, and the used blackberry bagasse is rich in this groups of components, as can be verified in its proximate composition (Pasquel-Reátegui, Machado, Barbero, Rezende, and Martínez, 2014). Moreover, acid media help releasing substances from inside the vegetal tissues, and contribute with the hydrolysis and degradations of fibers and sugars into soluble compounds, which also lead to increased global yield (Ivanovic et al., 2014).

For all tested solvents X_0 increased with temperature, although differences were not significant in some cases. High temperatures favor the break of Van der Waals, hydrogen and dipole–dipole molecular bonds between extractable compounds and the vegetable substrate, thus reducing the required activation energy to their desorption. Indeed, viscosity and surface tension of the solvents decrease at higher temperatures, enhancing the penetration of the solvent into the matrix and a faster dissolution of the extract. Therefore, mass transfer rate is increased, resulting in higher global yield (Mustafa & Turner, 2011; Wiboonsirikul & Adachi, 2008; Wijngaard, Ballay, et al., 2012; Wijngaard, Hossain, et al., 2012).

Although acidified water as solvent has provided much higher yields, there was no gain in the quality (TP, MA and AA) of the extracts, if compared to the other solvents. Thus, these higher yields are due to the extraction of other non-phenolic compounds that do not have antioxidant activity, as will be detailed in Sections 3.2 and 3.3.

3.2. Chemical composition of the PLE extracts

3.2.1. Total phenolic content

As can be verified on Table 1(A), the lowest phenolic contents were found in the extracts obtained with acidified and pure water, both at 60 °C, and the highest TP were detected in the hydroethanolic extracts at 80 and 100 °C. Similar or higher contents are reported in extracts obtained from residues of jabuticaba and apple (Santos, Veggi, & Meireles, 2012; Wijngaard & Brunton, 2009). The effects of temperature and solvent were statistically significant at the level of 5%, as well as their interactions.

For all employed solvents, TP increased with temperature. As well as with global yield, this can happen because at high temperatures the solubility of phenolics increases, and the braking of chemical bonds, molecular diffusion and mass transfer rates is enhanced. Besides, the

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Table 1
Extraction conditions, global yield, total phenolic content, monomeric anthocyanins and antioxidant activity of the extracts obtained from blackberry bagasse through PLE, Soxhlet and maceration.

(A) PLE

T (°C)	Solvent	X_0 (%)	TP (mg GAE/g FR)	MA (mg C3GE/g FR)	AA	
					DPPH (μmol TE/g FR)	ABTS (μmol TE/g FR)
60	Water	3.56 ± 0.71 ^{Aa}	2.39 ± 0.21 ^{Aa}	0.88 ± 0.01 ^{Aa}	14.25 ± 0.74 ^{ABa}	21.26 ± 0.74 ^{Aa}
60	Water pH 2.5	12.10 ± 0.49 ^{Ba}	1.93 ± 0.21 ^{Aa}	0.95 ± 0.08 ^{Aa}	12.25 ± 0.21 ^{Aa}	31.14 ± 6.35 ^{ABa}
60	Ethanol	3.23 ± 0.08 ^{Aa}	3.18 ± 0.62 ^{ABa}	1.25 ± 0.03 ^{Ba}	24.33 ± 4.40 ^{Ba}	32.04 ± 6.75 ^{ABa}
60	Ethanol (50%)	3.85 ± 0.37 ^{Aa}	5.23 ± 0.83 ^{Ba}	1.40 ± 0.02 ^{Ba}	37.04 ± 2.80 ^{Ca}	49.24 ± 3.32 ^{Ba}
80	Water	4.16 ± 1.47 ^{Aa}	3.78 ± 0.03 ^{Ab}	0.79 ± 0.08 ^{Aa}	33.22 ± 0.08 ^{Aa}	36.46 ± 0.72 ^{ABab}
80	Water pH 2.5	14.27 ± 0.37 ^{Bb}	4.46 ± 0.25 ^{Ab}	0.99 ± 0.05 ^{Aba}	36.30 ± 0.78 ^{Ab}	43.97 ± 0.79 ^{BCa}
80	Ethanol	4.23 ± 0.28 ^{Ab}	3.72 ± 0.60 ^{Aa}	1.39 ± 0.02 ^{Bb}	31.40 ± 4.38 ^{Aa}	31.48 ± 5.18 ^{Aa}
80	Ethanol (50%)	5.19 ± 0.08 ^{Ab}	5.51 ± 0.80 ^{Aa}	1.08 ± 0.21 ^{Aba}	46.38 ± 1.28 ^{Ab}	52.10 ± 3.32 ^{Cb}
100	Water	6.39 ± 0.36 ^{Aa}	4.97 ± 0.51 ^{Ab}	0.65 ± 0.10 ^{Aba}	42.85 ± 5.45 ^{Ab}	42.79 ± 6.70 ^{ABb}
100	Water pH 2.5	14.99 ± 0.05 ^{Cb}	5.34 ± 0.46 ^{Ab}	0.38 ± 0.04 ^{Ab}	40.40 ± 3.34 ^{Ab}	51.29 ± 2.94 ^{ABa}
100	Ethanol	4.46 ± 0.24 ^{Bb}	4.12 ± 0.23 ^{Aa}	0.93 ± 0.03 ^{BCc}	36.61 ± 0.24 ^{Aa}	31.07 ± 4.60 ^{Aa}
100	Ethanol (50%)	6.33 ± 0.04 ^{Ac}	7.36 ± 0.18 ^{Ba}	1.02 ± 0.11 ^{Ca}	76.03 ± 1.05 ^{Bc}	68.28 ± 2.68 ^{Bb}

(B) Comparison of extraction methods

Method	X_0 (%)	TP (mg GAE/g FR)	MA (mg C3GE/g FR)	AA	
				DPPH (μmol TE/g FR)	ABTS (μmol TE/g FR)
Maceration	5.02 ± 0.07 $^{\underline{A}}$	3.66 ± 0.05 $^{\underline{A}}$	1.21 ± 0.02 $^{\underline{AB}}$	29.04 ± 2.18 $^{\underline{A}}$	46.09 ± 1.15 $^{\underline{A}}$
Soxhlet Methanol	5.25 ± 0.17 $^{\underline{A}}$	4.64 ± 0.01 $^{\underline{A}}$	1.33 ± 0.01 $^{\underline{AB}}$	39.55 ± 2.87 $^{\underline{B}}$	60.19 ± 3.30 $^{\underline{B}}$
Soxhlet Ethanol	5.51 ± 0.27 $^{\underline{A}}$	4.10 ± 0.71 $^{\underline{A}}$	1.68 ± 0.23 $^{\underline{B}}$	42.79 ± 1.19 $^{\underline{B}}$	63.10 ± 4.58 $^{\underline{B}}$
PLE (100 °C, Ethanol (50%))	6.33 ± 0.04 $^{\underline{B}}$	7.36 ± 0.18 $^{\underline{B}}$	1.02 ± 0.11 $^{\underline{A}}$	76.03 ± 1.05 $^{\underline{C}}$	68.28 ± 2.68 $^{\underline{B}}$

Results are expressed as their mean ± standard deviation.
Equal capital letters in the same column indicate that there is no difference between solvents at the same temperature, at the level of 5% of significance, according to Tukey's test.
Equal lowercase letters in the same column indicate that there is no difference between temperatures for the same solvent, at the level of 5% of significance, according to Tukey's test.
Equal underlined capital letters in the same column indicate that there is no difference between extraction methods, at the level of 5% of significance, according to Tukey's test.

reductions of viscosity and surface tension of the solvent contribute to promote the contact between solvent and solutes (Mustafa & Turner, 2011; Wiboonsirikul & Adachi, 2008). Similar behaviors are related in various works, showing that from 40 to 120 °C there is a growth on the extraction of phenolics with subcritical solvents (Cam & Hisil, 2010; Cha et al., 2010; Hassas-Roudsari, Chang, Pegg, & Tyler, 2009; Howard & Pandjaitan, 2008; Ju & Howard, 2003; Ju & Howard, 2005; Karacabey, Mazza, Bayindirli, & Artik, 2012; Monrad et al., 2010; Srinivas, King, Monrad, Howard, & Zhang, 2011; Truong, Hu, Thompson, Yencho, & Pecota, 2012; Wijngaard, Ballay, et al., 2012; Wijngaard & Brunton, 2009). According to Wijngaard, Ballay, et al. (2012), Wijngaard, Hossain, et al. (2012), temperature is one of the most important parameters for the extraction of polyphenols by PLE.

Summarizing, the solvents that extracted more phenolics are, in decreasing order: ethanol + water (50% v/v) > acidified water (pH = 2.5) > pure ethanol > pure water. Only at 60 °C this order is not observed. Therefore, a positive synergism between temperature and solvent is noted, and contributes to the enhancement of the extraction of phenolics.

Many works have shown that PLE is more efficient in the extraction of phenolics from bagasse and other vegetable sources when mixtures of solvents are used, such as methanol or ethanol in water, instead of pure solvents (Kim, Park, & Lim, 2009). This observations agrees with those who suggest that either highly polar or non polar liquids are not good extraction solvents (Vizzotto & Pereira, 2011). Phenolic compounds of blackberry, as well as of other berries, have moderately polar nature. Pure water combined to other organic solvents (mainly methanol and ethanol) may create a moderately polar medium, favoring the extraction of polyphenols. Moreover, while one of the solvents (ethanol) can enhance the solute's solubility, the other (water) helps in the desorption of the solute from the substrate (Mustafa & Turner, 2011). The use of ethanol also reduces the boiling point of the mixture and affects its polarity, so the concentration of ethanol has strong effect on the polyphenol yield (Wijngaard & Brunton, 2009).

Finally, the acidification of the solvent also increases the efficiency of the extraction of bioactive compounds (Ivanovic et al., 2014). It can be verified in this work when phenolic contents of the extracts obtained with pure water are compared to those extracted with acidified water

Table 2
Concentration of the anthocyanins identified in the PLE extracts obtained with different solvents and temperatures.

T (°C)	Solvent	C3G (mg/g FR)	C3R (mg/g FR)	C3MG(mg/g FR)	C3DG (mg/g FR)	Total (mg/g FR)
60	Water	0.4239 ± 0.1586^{AB}	0.0524 ± 0.0126^{AB}	0.0095 ± 0.0079^{A}	0.0162 ± 0.0116^{A}	0.5019 ± 0.1906^{AB}
60	Water pH 2.5	0.7649 ± 0.2502^{AB}	0.0702 ± 0.0301^{AB}	0.0214 ± 0.0001^{A}	0.0299 ± 0.0050^{A}	0.8864 ± 0.2853^{AB}
60	Ethanol	0.3160 ± 0.0864^{A}	0.0221 ± 0.0040^{A}	0.0118 ± 0.0019^{A}	0.0150 ± 0.0032^{A}	0.3649 ± 0.0954^{A}
60	Ethanol (50%)	0.4141 ± 0.0284^{AB}	0.0272 ± 0.0007^{A}	0.0152 ± 0.0007^{A}	0.0191 ± 0.0010^{A}	0.4755 ± 0.0293^{AB}
80	Water	0.4949 ± 0.2507^{AB}	0.0522 ± 0.0151^{AB}	0.0125 ± 0.0091^{A}	0.0190 ± 0.0126^{A}	0.5786 ± 0.2874^{AB}
80	Water pH 2.5	1.0565 ± 0.3886^{B}	0.0954 ± 0.0312^{B}	0.0219 ± 0.0129^{A}	0.0371 ± 0.0180^{A}	1.2108 ± 0.4507^{B}
80	Ethanol	0.2473 ± 0.0152^{A}	0.0182 ± 0.0001^{A}	0.0103 ± 0.0003^{A}	0.0132 ± 0.0008^{A}	0.2891 ± 0.0165^{A}
80	Ethanol (50%)	0.3764 ± 0.0377^{AB}	0.0266 ± 0.0018^{A}	0.0138 ± 0.0011^{A}	0.0174 ± 0.0011^{A}	0.4341 ± 0.0418^{AB}
100	Water	0.3458 ± 0.2239^{A}	0.0420 ± 0.0176^{AB}	0.0101 ± 0.0058^{A}	0.0162 ± 0.0092^{A}	0.4141 ± 0.2566^{AB}
100	Water pH 2.5	0.2701 ± 0.0170^{A}	0.0338 ± 0.0015^{A}	0.0091 ± 0.0019^{A}	0.0159 ± 0.0010^{A}	0.3289 ± 0.0155^{A}
100	Ethanol	0.1786 ± 0.0097^{A}	0.0155 ± 0.0004^{A}	0.0081 ± 0.0004^{A}	0.0113 ± 0.0005^{A}	0.2135 ± 0.0110^{A}
100	Ethanol (50%)	0.3239 ± 0.0420^{A}	0.0255 ± 0.0005^{A}	0.0117 ± 0.0013^{A}	0.0164 ± 0.0011^{A}	0.3775 ± 0.0449^{A}

Results are expressed as their mean ± standard deviation.
Equal capital letters in the same column indicate that there is no difference between the conditions PLE extraction, at the level of 5% of significance, according to Tukey's test.
FR = fresh residue.

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at 80 and 100 °C. Acids in the solvents help braking cell membranes, thus enhancing the release and solubilization of some compounds (Ju & Howard, 2003).

3.2.2. Monomeric anthocyanins (MA)

Table 1(A) shows that the higher monomeric anthocyanin yields were achieved at the conditions in which ethanol + water or pure ethanol were used as solvents, at 60 and 80 °C. Similar results were found for PLE from jabuticaba bagasse (Santos et al., 2012) and in other extraction methods from fresh blackberries (Fan-Chiang & Wrolstad, 2005; Ferreira, Rosso, & Mercadante, 2010; Siriwoharn et al., 2004; Wu et al., 2011).

The increase of temperature generally decreased the anthocyanin content of the extracts, although the decrease was significant only for the extracts obtained with acidified water (Galanakis, Goulas, Tsakona, Manganaris, & Gekas, 2013). Therefore, a negative synergism between high temperatures and acid solvent must have contributed on the degradation of anthocyanins. This is confirmed by the ANOVA analysis, which showed a significant effect of the interaction temperature*solvent on the anthocyanin concentration. Acids at high temperatures possibly resulted in the hydrolysis of acylated components and sugar residues, inactivating the chemical structure of anthocyanins (Ju & Howard, 2003; Ju & Howard, 2005; Monrad et al., 2010). Temperature itself is well known as a factor affecting the stability of anthocyanins (Patras, Brunton, O'Donnell, & Tiwari, 2010), whose degradation can happen by two mechanisms (Simpson, 1985): (1) hydrolysis of the 3-glucoside to form the unstable aglicone, and (2) hydrolytic opening of the heterocyclic ring, forming chalcone, which is a colorless anthocyanin structure that, at high temperatures, is degraded into an insoluble brown-colored phenolic compound. In fact, the extracts obtained in this work at 80 and 100 °C had color varying from red to brown, indicating some anthocyanin degradation.

The mean MA content found in this work corresponds to 27% of the TP of the extracts, agreeing with levels found in other blackberry cultivars (Siriwoharn et al. (2004), where cyanidin-3-O-glucoside was the major anthocyanin (as also verified in this work, see Section 3.2.3). Other works report higher MA/TP ratios, reaching up to 90% (Howard & Pandjaitan, 2008; Ju & Howard, 2003, 2005; Monrad et al., 2010; Wijngaard & Brunton, 2009). Nevertheless, these works used residues from other fruits, different solvents, and PLE conditions. Indeed, the discrepancy may be consequence of higher extraction of thermally stable compounds from blackberry bagasse, such as procyanidins and phenolic acids. The solubility of phenolic acids (gallic, cafeic, pherulic and coumaric) increase with temperature (García-Marino, Rivas-Gonzalo, Ibáñez, and García-Moreno, 2006).

3.2.3. Identification and quantification of anthocyanins by UHPLC

Four anthocyanins were simultaneously and tentatively identified in the extracts obtained from blackberry residues by PLE: two major (cyanidin-3-O-glucoside (C3G) and cyanidin-3-O-rutinoside (C3R)) and two minor (cyanidin-3-O-(6"-malonyl-glucoside) (C3MG) and cyanidin-3-O-(6"-dioxalyl-glucoside) (C3DG)). These results are similar to those found in fresh blackberries and their extracts obtained by conventional and alternative techniques (Fan-Chiang & Wrolstad, 2005; Ferreira et al., 2010; Siriwoharn et al., 2004; Stintzing, Stintzing, Carle, & Wrolstad, 2002; Wu et al., 2011; Zou, Wang, Gan, & Ling, 2011; Zou et al., 2012).

The UHPLC-UV-Vis (520 nm) chromatogram representing the four detected anthocyanins is illustrated in Fig. 2, as well as the mass spectrum data obtained by UHPLC-QToF-MS. Peak 1 (retention time (t_R) = 1.70 min) revealed a molecular ion ($[M]^+$) at 449 m/z and a fragmented ion at 287 m/z, indicating that it is a derived from cyanidin. The neutral loss of 162 mass units (U) corresponds to a molecule of hexose. Therefore, peak 1 was tentatively identified as cyanidin-3-O-glucoside, by comparison with literature data (Fan-Chiang & Wrolstad, 2005; Stintzing et al., 2002). Peak 2 (t_R = 2.15 min) produced a molecular ion at 595 m/z and a fragmented ion at 287 m/z, which again coincides with cyanidin, resulting in the loss of rutinose (308 U). This description corresponds to cyanidin-3-O-rutinoside. Peak 3 (t_R = 3.37 min) to the molecular ion at 535 m/z and fragmented ion at 287 m/z, identical to that of cyanidin, and the difference between both molecules is 248 U, which corresponds to malonylglucoside. Thus, peak 3 was identified as cyanidin-3-O-(6"-malonyl-glucoside). Finally, peak 4 (t_R = 3.80 min) showed a molecular ion at 593 m/z and again a fragmented ion of 287 m/z, resulting in the loss of 292 U, being identified as cyanidin-3-O-(6"-dioxalyl-glucoside) (Ferreira et al., 2010; Rosso et al., 2008; Stintzing et al., 2002; Wu & Prior, 2005; Wu et al., 2011; Zou et al., 2012).

The concentrations of the anthocyanins C3G, C3R, C3MG and C3DG present in the extracts at all performed conditions are on Table 2. It can be noted that the anthocyanin content obtained by UHPLC-UV-Vis is quite lower than those obtained by the spectrophotometric method. Spectrophotometric analyses may overestimate the anthocyanin content, since other compounds can absorb at the wavelength (λ) established for the differential pH method.

The higher anthocyanin yields were achieved with acidified water at 60 and 80 °C. Moderately acidified solvents enhance both release and solubilization of anthocyanins from fruit tissues, and also their stabilization, since these pigments are mainly found in the flavillic cation form, which is the most stable anthocyanin structure in aqueous medium (Dai, Gupte, Gates, & Mumper, 2009; Jackman, Yada, Tung, & Speers, 1987). At 100 °C the anthocyanin content is the acidified aqueous extract decreased, as also observed with the differential pH method. This reduction confirms the negative synergism between high temperatures and acid solvents.

Comparing to other data (Fan-Chiang & Wrolstad, 2005; Siriwoharn et al., 2004; Torre & Barritt, 1977; Zou et al., 2011), the concentration of the four identified anthocyanins in the PLE extracts from the bagasse was slightly lower. However, the mentioned works were performed with different blackberry cultivars, and the fresh fruit instead of its bagasse.

3.3. Antioxidant activity (AA) of the PLE extracts

There is a great variability among the antioxidant activities reported in the literature for PLE extracts, which is due not only to the method used to determine AA, but also to the form that AA is expressed. Indeed, the variations are consequence of the operational extraction conditions, solvent, type and variety of material, crop season, and pretreatments applied to the samples.

The results on Table 1(A) indicate that both methods (DPPH and ABTS) employed to determine AA of the PLE extracts provided the same profiles, being the values of ABTS slightly higher than those of DPPH. Similar trends were observed in other works with blackberry and its residues (Ozgen, Reese, Tulio, Scheerens & Miller, 2006; Pasquel-Reátegui et al., 2014; Pérez-Jiménez et al., 2008).

The highest AA were found in the blackberry bagasse extracts obtained with ethanol + water (50% v/v) at 80 and 100 °C, the same conditions that achieved the highest TP. High AA values were also found with acidified water at the same temperatures, with no significant difference from the former.

AA increases with temperature for all the applied solvents, as also reported for other fruit by-products (Howard & Pandjaitan, 2008; Ju & Howard, 2003, 2005; Monrad et al., 2010; Santos et al., 2012; Wijngaard & Brunton, 2009). As mentioned in previous sections, the increase of temperature enhanced TP yield, but decreased the anthocyanin content. Thus, it can be stated that other phenolic compounds than anthocyanins, such as procyanidins, phenolic acids and others, are possibly the main responsible for the increase of AA with temperature. Fig. 3 shows the correlation coefficients of Pearson (r) between AA measured by DPPH and ABTS and TP were r = 0.9650 and r = 0.9360, respectively, confirming that AA is strongly related to TP. However,

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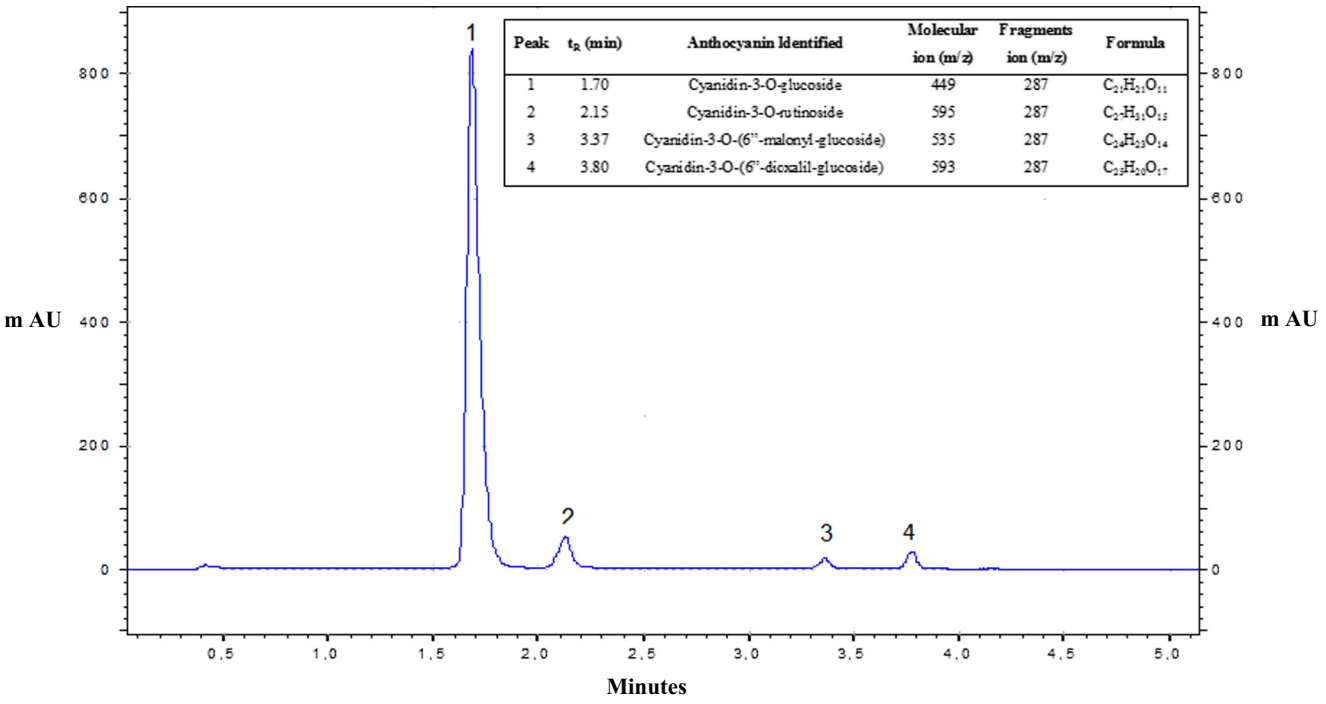

Fig. 2. Representative UPLC-UV-Vis (520 nm) chromatogram and mass spectra data of the four anthocyanins identified in the PLE extracts from blackberry bagasse.

this is not verified for anthocyanins, whose content is weakly related to AA ($r < 0.1$ for both methods).

Another possible reason for the increase of AA with temperature is the formation of products from Maillard's reaction, which have antioxidant potential, such as carboxymethyl-lysine, hydroxymethylfurfural, pentosidine, carboxyethyl-lysine, pirraline, glyoxal-lysine dimer, methylglyoxal-lysine dimer, glucosepane, so AA could increase at temperatures from 80 to 140 °C due to these components. Heated amino acids and sugars mixtures can lead to formation of melanoidins with high antioxidant capacity (Yilmaz & Toledo, 2005) and brown color, like observed in the PLE extracts obtained at 100 °C from blackberry bagasse. As observed in this work, AA also increases with temperature in hydroethanolic extracts from other sources, as spinach (Howard & Pandjaitan, 2008), where again AA correlates with the darkling of the samples.

3.4. Comparison of the extraction methods

Among the tested solvents and temperatures of PLE, the best extraction condition chosen was ethanol + water (50% v/v) as solvent at 100 °C. As already reported, this condition provided the highest phenolic content and antioxidant activity, and although its anthocyanin recovery was not the highest, MA was close to the highest achieved, with statistically equal value according to Tukey's test. Moreover, the global yield achieved was higher than in other conditions, excepting those with acidified water. Therefore, the selected PLE condition was compared to conventional extraction techniques (Soxhlet and maceration) from the same material and using methanol and ethanol as solvents.

The results presented in Table 1(B) show that PLE (in the best extraction condition) is quite more effective than the conventional methods on the extraction global yield and phenolic content. The PLE extract has also presented higher antioxidant activity. The combined application of high pressure and temperature in PLE has proven to be effective in the recovery of anthocyanins and phenolics from various sources when compared to traditional methods (Howard & Pandjaitan, 2008; Ju & Howard, 2003, 2005; Monrad et al., 2010; Santos et al., 2012; Wijngaard & Brunton, 2009). However, PLE achieved the lowest anthocyanin content, which can be attributed to the negative effect of temperature, as discussed in Sections 3.2.2 and 3.2.3.

The data on Table 1(B) show an additional advantage of PLE over traditional methods. The extraction time required by PLE to achieve

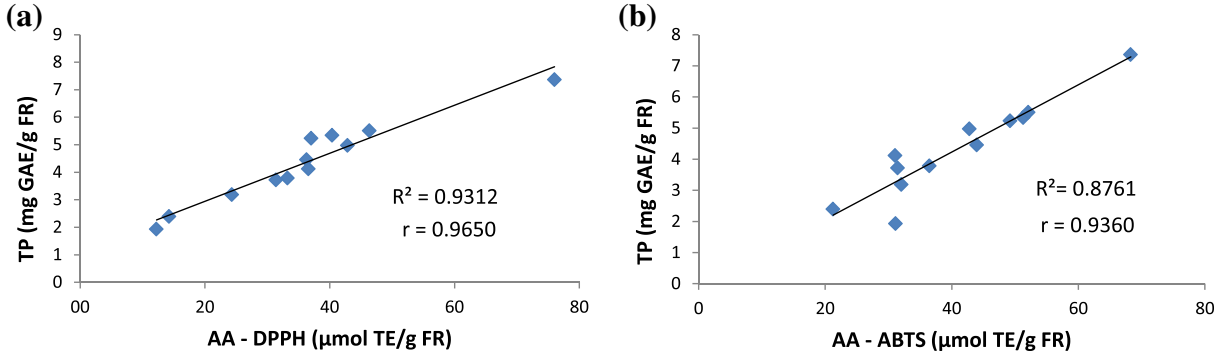

Fig. 3. Correlations between antioxidant activity (AA) and total phenolic content (TP) of the extracts obtained from blackberry bagasse by PLE at different conditions. (a) AA measured through DPPH and (b) AA measured through ABTS. (*) GAE = gallic acid equivalent, TE = trolox equivalent e FR = fresh residue.

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similar or even higher yields than traditional methods is much lower, which would reduce the operational cost in industrial scale. Although the initial investment to build a high pressure extraction plant may be high, it is generally compensated by a significant reduction of operational cost, mainly because the solvent consumption and energy expense of the involved unit operations are much lower (Farías-Campomanes, Rostagno, & Meireles, 2013; Rosa & Meireles, 2005; Santos, Veggi, & Meireles, 2012; Veggi, Cavalcanti, & Meireles, 2014). PLE (water + ethanol, 100 °C) achieved, in 30 min, higher global yield than Soxhlet and maceration in 5 and 24 h, respectively, with increased TP and AA. As a consequence, the solvent consumption is also reduced, from 40 g solvent/g fresh residue (Soxhlet) and 20 g solvent/g fresh residue (maceration) to 18 g solvent/g fresh residue in PLE.

4. Conclusions

Bioactive compounds, such as polyphenols and anthocyanins, can be recovered from residues of the industrial processing of blackberry pulp through pressurized liquid extraction (PLE), a rapid and environmentally clean technique. Results have indicated that PLE is a feasible alternative to conventional methods for the extraction of valuable components from blackberry bagasse and other food by-products. The solvent type and temperature have strong influence on the recovery of phenolics and anthocyanins. Solvent mixtures with moderate polarity at high temperatures (80 to 100 °C) are more efficient to extract phenolics. Acidified media at moderate temperatures are also efficient. Considering the phenolic and anthocyanin content and the antioxidant activity, the best PLE condition was ethanol + water (50% v/v) as solvent at 100 °C. The PLE extracts have high antioxidant activities, which make them potentially applicable as food ingredients, pharmaceuticals, and in cosmetic formulations. The high antioxidant activity is strongly related to the phenolic content of the extracts.

Two major (cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside) and two minor (cyanidin-3-O-(6"-malonyl-glucoside) and cyanidin-3-O-(6"-dioxalyl-glucoside) were identified and quantified in the PLE extracts. However, the anthocyanin content is weakly related to the antioxidant capacity.

PLE stands as a promising technique for the recovery of bioactive compounds from blackberry bagasse, as well as other residues from fruit and vegetable processing. Further steps are needed in order to purify the extracts and make them commercially viable. In this context, clean separation techniques should be evaluated and coupled to PLE to enhance the product's quality and applicability.

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