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Extraction of antioxidant compounds from blackberry (*Rubus* sp.) bagasse using supercritical CO_2 assisted by ultrasound

José Luis Pasquel Reátegui[a], Ana Paula da Fonseca Machado[a], Gerardo F. Barbero[b], Camila A. Rezende[c], Julian Martínez[a,*]

[a] College of Food Engineering, Food Engineering Department, UNICAMP, 13083-862 Campinas, SP, Brazil
[b] Department of Analytical Chemistry, Faculty of Sciences, University of Cadiz, Agrifood Campus of International Excellence (CeiA3), P.O. Box 40, 11510 Puerto Real, Cádiz, Spain
[c] Institute of Chemistry, University of Campinas, UNICAMP, 13083-862 Campinas, SP, Brazil

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ABSTRACT

Supercritical carbon dioxide extraction (SFE) was performed to recover bioactive components from blackberry (*Rubus* sp.) industrial residues. Ultrasound was applied during the extractions in order to enhance rate and yield. Moreover, water and ethanol at different proportions were used as cosolvents to improve the extraction of polar compounds from the residues. The extraction global yields were measured at all performed conditions (pressure, temperature, ultrasound power and cosolvent). The extracts were evaluated in terms of their antioxidant activity, measured using two methods, phenolic content, monomeric anthocyanins, and anthocyanin profile. The application of ultrasound in SFE helped increasing the extraction rate at the beginning of the process, which could be observed on the extraction curves at 15 MPa, the lowest pressure applied. Scanning electron microscopy (FESEM) was used to analyze the blackberry bagasse undergoing SFE with and without ultrasounds and showed that ultrasound disturbs the cell walls, enhancing the release of the extractable compounds. The extracts have shown high antioxidant activity and phenolic contents when obtained at higher temperatures. Regarding anthocyanins, the use of water as cosolvent resulted in a significant increase. Four major anthocyanins were identified and quantified by ultra performance liquid chromatography (UPLC).

1. Introduction

Blackberry (*Rubus* sp.) is a fruit widely known for containing remarkable amounts of phenolic compounds, including anthocyanins, flavonols, chlorogenic acid, and procyanidins, which have high biologic activities and can provide benefits to human health, i.e., as dietary antioxidants [1]. Besides, blackberry has been used in food industries to produce juice, ice cream, yoghurt, and jellies. However, the processing of blackberries generate around 20% of residues, which are composed mainly of peel and seeds, and still contain high amounts of bioactive compounds [2]. Thus, the extraction of components from food wastes such as blackberries is of great interest to add value to materials that are typically discarded.

The quality of extracts obtained from some raw material is strongly related to the employed extraction technique, and can be evaluated through the chemical profile of the product. Supercritical technology is capable to extract specific compounds under specific combinations of temperature and pressure [3]. The extraction with supercritical fluids (SFE) aims to maximize the recovery and quality of the extracted material, and minimize the energy cost, since it is faster and more selective than conventional separation methods [4]. For low volatility compounds, the solubility in supercritical CO_2 decreases with increasing molecular weight and with polarity [5]. In these cases, solubility can be enhanced by adding liquid cosolvents at low concentrations. Carbon dioxide can be combined to ethanol and water to extract polar components, such as anthocyanins [6]. Moreover, the use of cosolvents can modify the properties of the solvent mixture, and help braking interactions between solute and solid matrix, improving the transport of solute from the solid pores to its surface [7,8].

SFE can also be enhanced by combining novel techniques to accelerate the process, such as microwaves and ultrasound. The application of ultrasound during SFE has been recently proposed as a mechanism to intensify the process and increase its yield [9]. Ultrasound of high intensity is based on the formation of high frequency ultrasonic waves that are able to promote cavitation in

* Corresponding author. Tel.: +55 19 35214046; fax: +55 19 35214027.
E-mail addresses: julian@fea.unicamp.br, jmartinez94@gmail.com (J. Martínez).

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the medium, due to cycles of expansion and compression of bubbles. Such cycles may lead to the rupture of cell walls of a vegetal substrate, favoring the penetration of the solvent and mass transfer. Nevertheless, when a liquid is under pressure, the acoustic intensity required to produce cavitation also increases, so a natural limitation is established on the application of ultrasound at high pressures [10,11]. Even so, cavitation has been observed in carbon dioxide at its subcritical state. Thus, ultrasound has been used to accelerate processes and reduce extraction times. Some published works show that the application of ultrasound during SFE affects both kinetics and yield. Balachandran et al. [12] studied the use of ultrasound in SFE of spicy compounds from ginger, and found a considerable increase in yield. Riera et al. [9] reported an increase of 20% in the production of almond oil by SFE by using ultrasound, against traditional methods. Santos et al. [13] observed an increase up to 77% in global yield when ultrasound was applied on SFE from red pepper (*Capsicum frutescens* L.).

Taking the mentioned information into account, this work explored the SFE from blackberry wastes, comparing the extracts obtained by conventional techniques to those extracted by SFE with and without ultrasound (SFE-US) using pure carbon dioxide (CO_2) and CO_2 with cosolvents.

2. Materials and methods

2.1. Reagents

The reference standard for anthocyanins was cyanidin chloride (assay ≥ 95% for HPLC, Sigma–Aldrich Co, Germany). The water was obtained from a Milli-Q water deionization system (Millipore, Bedford, MA, USA). Methanol and formic acid were HPLC grade and were obtained from Merck (Darmstadt, Germany).

2.2. Plant material

The raw material was the bagasse resulting from the processing of blackberry (*Rubus* sp.) pulp, donated by the company "Sítio do Bello", located in Paraibuna-SP, southeastern Brazil. The blackberry wastes were packed in dark plastic bags to protect samples from light, and stored in domestic freezer (Metalfrio, DA420, São Paulo-SP, Brazil) at −18 °C, until the experiments.

2.3. Sample preparation

The blackberry bagasse, with initial moisture of 69% determined by drying in stove (Model TE 395-1, Tecnal, São Paulo-SP, Brazil) at 105 °C for 4 h, until the sample mass was constant, according to the AOAC method (925.10) [14]. After drying, the sample was milled in a domestic blender for 30 s, in order to homogenize it and reduce particle size and increase the contact area between substrate and solvent to enhance mass transfer [5]. The solid particles of the sample were separated by size in a vertical vibratory sieve system (Series Tyler, Wheeling, WV) with sequential openings of 24, 32, 42, 60 and 250 Mesh. The material retained on each sieve was weighed in analytical balance (Bel engineering Piracicaba-SP, Brazil). The mean particle diameter (0.34 mm) was calculated from the sample masses retained in all sieves. The milled blackberry bagasse was packed in plastic bags and stored in domestic freezer at −18 °C.

2.4. Sample characterization

The characterization of the blackberry bagasse in terms of moisture, ashes, lipids, and proteins was performed according to the AOAC [14] official techniques.

2.4.1. Moisture

The moisture of fresh blackberry bagasse was determined according to the AOAC method 925.10 [14]. Approximately 5 g of sample were placed in aluminum recipients and dried in air circulation stove (TE 395-1, Tecnal, São Paulo-SP, Brazil) for 4 h. Next, the recipients were dried in desiccators at room temperature and weighed. The procedure was repeated until constant mass was achieved. All measurements were performed in triplicates.

The moisture content of the dried blackberry bagasse was determined through the Karl-Fisher method. 0.147 ± 0.02 mg of sample was pretreated at 170 °C for 20 min, and nitrogen flow rate of 50 mL min^{-1} in a Karl-Fisher titration system (Metrohm 701 KF Titrino, Riverview, FL) equipped with oven (832 KF Thermoprep, Riverview, FL).

2.4.2. Proximate composition

Residual minerals were determined by incineration at 550 °C, according to the AOAC method 972.15 [14]. Protein content (total nitrogen) was measured by the micro-Kjeldahl method using the factor 6.25 to convert nitrogen into protein (AOAC method 970.22 [14]). Total lipids were determined through extraction of the ethereal fraction with petroleum ether (Quimis, Brazil, Lot: 36128) as solvent by Soxhlet, according the AOAC method 963.15 [14].

2.5. Low pressure extractions

Extractions at low pressure were selected to be compared to SFE and SFE-US, with and without cosolvents, in terms of extract yield and composition (phenolics, antioxidant activity and anthocyanins).

2.5.1. Soxhlet

Soxhlet extraction was performed according to the AOAC method 963.15 [14], using ethanol (Êxodo Científica, Brazil, Lot: AE5761RA) as solvent. The extractions were performed in triplicates and the results were expressed in mean ± standard deviation.

2.5.2. Maceration

The maceration of blackberry bagasse was done in duplicates according to the method described by Rodriguez-Saona et al. [15]. The sample (5 g) was immersed in 160 mL of ethanol (P.A., Êxodo Científica, Brazil, Lot: AE5761RA). The mixture was left for 24 h with sporadic agitation. Next, the solution was filtered and evaporated, and the recovered extracts were stored in domestic freezer (−18 °C) until the analyses.

2.6. Supercritical CO_2 extraction assisted by ultrasound (SFE-US)

2.6.1. Preparation of the extraction bed

A standard procedure was adopted to prepare the extraction bed. A 300 mL extraction cell was used. One of the extremities of the cell was closed and a glass wool layer was put on its base to act as filter, to avoid the passage of fine particles that could obstruct the line. Then, 5 g of sample were added between two layers of glass beads to fill the entire cell volume.

2.6.2. SFE-US unit

The SFE-US unit is composed by a 300 mL stainless steel cell that supports pressures up to 45 MPa. The unit is equipped with a cooling bath (Marconi, model MA 184/E, Campinas, Brazil) to control the temperature of CO_2 at the pump (PP 111-VE MBR, Maximator, Nordhausen, Germany) suction, heating bath (Marconi, model MA 126/BD, Campinas, Brazil) and a heating electric jacket to control the temperature inside the cell, a cosolvent pump (Model series III, Laballiance, USA) with capacity up to 10 mL min^{-1}, a flow totalizer, thermocouples, and pressure gauges.

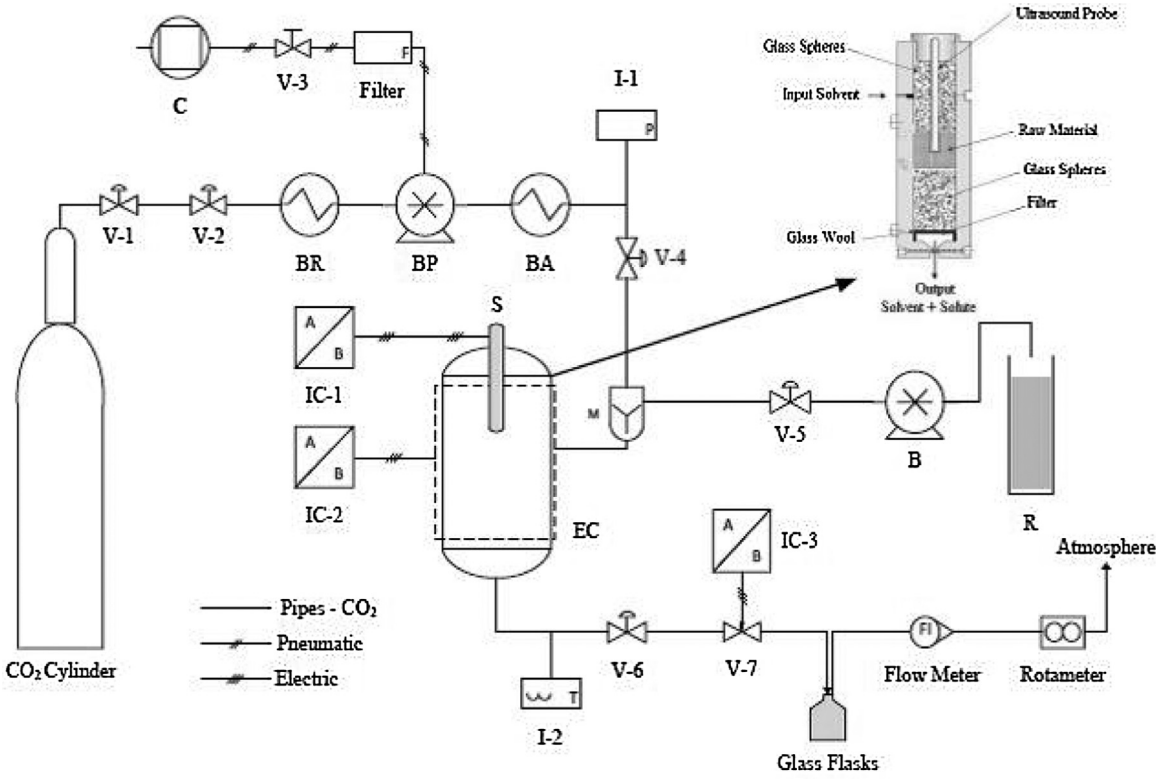

Fig. 1. Diagram of the SFE-US unit with cosolvents: V-1–V-6, control valves; V-7, micrometer valve; C, compressor; F, compressed air filter; BR, cooling bath; BP, pump (*Booster*); BA, heating bath; B, liquid pump (HPLC); R, fluid reservoir; M, mixer; I-1–I-2, pressure and temperature indicators, respectively; IC-1, indicators and controllers of ultrasound power; IC-2, temperature of extraction column and IC-3, temperature of micrometer valve; EC, extraction column; S, ultrasound probe.

An ultrasound probe with a 13 mm titanium end, coupled to a transducer (Unique Group, model DES500, Campinas, Brazil) is installed on the upper end of the extraction cell, and is operated from an ultrasound generator that can operate from 10 to 99% of its total power (800 W). Fig. 1 presents the operation diagram of the SFE-US unit, and the details of the SFE-US extraction cell, at the upper right.

2.7. Global yield (X_0)

The extraction global yield (X_0) was calculated by the ratio between total extracted mass (M_{ext}) and feed mass (F) in dry basis, according to Eq. (1).

$$X_0 = \frac{m_{ext}}{F} \times 100 \quad (1)$$

The SFE time was fixed in 120 min for the global yield experiments, so the solvent to feed ratio was of 400 kg solvent/kg feed. This time was defined after the observation the extraction curve obtained in a preliminary test performed with dry and milled sample at 15 MPa, 40 °C, and CO_2 flow rate of 2.77×10^{-4} kg/s. All the SFE experiments were performed in duplicates, and a Box–Behnken experimental design with three variables was adopted, with three levels defined according to the equipment limitations. The evaluated process variables were: temperature (40, 50 and 60 °C), pressure (15, 20 and 25 MPa), and ultrasound power (0, 200 and 400 W).

After each extraction, the vessel containing the extract was left for 10 min under ambient conditions to assure the complete removal of CO_2, then the extract was weighed in analytical balance (Bel Engineering, USA), and stored in domestic freezer (−18 °C) until the analyses.

Global SFE curves were built at some operational conditions, to verify the effect of ultrasound on the extraction kinetics. The extraction curves were fitted to a spline curve using three straight lines [16], and kinetic parameters were calculated.

2.8. SFE-US with cosolvents

The use of cosolvents in SFE-US was tested at the selected operation conditions, which were 15 MPa, 60 °C, and 200 W, defined based on the analyses described in Section 2.9.2. Five grams of fresh or dried and milled blackberry bagasse were submitted to SFE-US using water and ethanol as cosolvents, with a fixed CO_2 flow rate of 2.77×10^{-4} kg/s. The following ratios between cosolvent and CO_2 were used: 5% ethanol and 5% water for both fresh and dried-milled samples; 10% ethanol and 10% water only to dried and milled samples. Extraction times were about from 54 to 57 min, so the solvent to feed ratio was kept constant. All the experiments of SFE-US with cosolvents were performed in duplicates.

2.9. Chemical characterization of the extracts

For the analyses of total phenolics and antioxidant activity, 0.1 g of the extracts obtained by SFE with pure CO_2 was dissolved in 10 mL of methanol. Finally, the solution was stored in an amber flask under freezing (−18 °C) until the analyses.

2.9.1. Total phenolics

The total phenolic content of the extracts was determined using the spectrophotometric method of Folin Ciocalteu, described by Singleton and Rossi [17]. 2.5 mL of Folin–Ciocalteu reagent was added to a 0.5 mL aliquot of the methanolic solution extract. After 5 min, 2.0 mL of a 7.5% aqueous solution of sodium carbonate were added. The solutions remained 2 h at rest and protected from light, and then absorbance was measured at 760 nm in a spectrophotometer (Model U-310, Hitachi, Tokyo, Japan). The total phenolic

content was determined by interpolating the absorbance of the samples with the results obtained for a calibration curve built with gallic acid as standard (in concentrations from 0.01 to 0.08 mg/mL). Total phenolic content was expressed in mg gallic acid equivalent (GAE)/g extract.

2.9.2. Antioxidant activity

2.9.2.1. Method DPPH (1,1-diphenyl-2-picrylhydrazyl). The DPPH method is based on the capture of free radicals Brand-Williams et al. [18], with some modifications. A standard trolox curve was built in order to quantify the antioxidant activity in terms of inhibition percentage vs trolox concentration (μM). First, a 60 μM DPPH solution was prepared dissolving 4.8 mg of the radical DPPH in methanol. Next, the solution was homogenized and transferred to an amber flask to avoid degradation by light. A 0.1 mL aliquot of the extract solution was transferred to vials, then 3.9 mL of the DPPH solution were added, and the mixture was homogenized. A 0.1 mL of methanol in 3.9 mL DPPH was used as control solution. After remaining in dark for 4 h, the solutions had their absorbance measured at 515 nm in spectrophotometer (Model U-310, Hitachi, Tokyo, Japan). Therefore, the inhibition percentage of the radical DPPH was calculated according to Eq. (2) [19].

$$\%\text{Inhibition} = \frac{\text{Abs control} - \text{Abs control}}{\text{Abs control}} \times 100 \quad (2)$$

The antioxidant activity is expressed in μmol trolox equivalent (TE) per g extract. The use of the trolox equivalent envisaged the comparison between the antioxidant activities obtained by the methods DPPH and ABTS.

2.9.2.2. Method ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)). The ABTS method described by Re et al. [20], with some modifications, was used. Trolox was used as reference antioxidant, and prepared as standard solution in methanol. The ABTS reagent was dissolved in methanol to 7.0 mM, and reacted with a 140 nM solution of potassium persulphate to form the radical $ABTS^{+}$. The blue mixture was kept at dark at ambient temperature for 16 h. Then, the radical $ABTS^{+}$ was dissolved in methanol until the absorbance measured at 734 nm was 0.70 (±0.05). A 30 μL aliquot of the extract solution in methanol was transferred to vials, and next 3.0 mL of the radical $ABTS^{+}$ were added. Absorbance was measured at 734 nm after 6 min. To quantify the antioxidant activity, a standard trolox curve was built with concentration from 100 to 2500 μM. The antioxidant activities were expressed in μmol TE/g extract.

2.9.3. Monomeric anthocyanins

The anthocyanin content was determined through the differential pH method [21]. Two buffer solutions were prepared: 0.025 M potassium chloride pH 1.0 and 0.4 M sodium acetate pH 4.5. The adjustment of pH was made by adding concentrated HCl. The extracts of blackberry were diluted in these two buffer solutions. After 30 min of rest under dark and ambient temperature, absorbances were measured at 510 and 700 nm in spectrophotometer (Model U-310, Hitachi, Tokyo, Japan). The absorbance of anthocyanins was calculated with Eq. (3).

$$\text{Abs} = (\text{Abs}_{510} - \text{Abs}_{700})\text{pH}_1 - (\text{Abs}_{510} - \text{Abs}_{700})\text{pH}_{4,5} \quad (3)$$

The concentration of anthocyanins in the extracts was calculated with Eq. (4), considering the molar absorptivity (ε) of 26,900, and molecular weight of 449.2 g/mol for cyanidin-3-O-glucoside. The results are expressed as mg cyanidin-3-O-glucoside equivalent/g extract.

$$C_{\text{cyanidin 3-O-glucoside}}\ \text{mg/L} = \frac{\text{Abs.MW} \cdot \text{DF} \cdot 1000}{\varepsilon} \quad (4)$$

where Abs, absorbance; MW, molecular weight; DF, dilution factor, and ε, molar absorptivity.

2.9.4. Identification of anthocyanins by UPLC–QTOF-MS

Anthocyanins were identified by ultra-performance liquid chromatography (UPLC) coupled to quadruple-time-of-flight mass spectrometry (Q-ToF-MS) (Synapt G2, Waters Corp., Milford, MA, USA). 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} was 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^{-1}, desolvation temperature = 500 °C, cone gas flow = 10 L h^{-1}, 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).

2.9.5. Separation and quantification of anthocyanins by UPLC–UV–vis

The separation and quantification of anthocyanins were performed on an Elite UPLC 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. Anthocyanins were analyzed on a Halo™ C18 Hitachi LaChrom column (100 mm × 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 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.

2.10. Field emission scanning electron microscopy (FESEM)

The microstructure of the surface of blackberry bagasse was analyzed before and after the extraction procedures using a scanning electron microscope equipped with a field emission gun (FESEM – FEI Quanta 650). Samples were not dried after CO_2 extraction, just kept in a desiccator containing silica, under vacuum for 24 h prior to analysis. Then, samples were coated with gold (using a 40 mA current for 60 s) in a SCD 050 sputter coater (Oerlikon-Balzers, Balzers, Liechtenstein). These sputtering conditions resulted in a gold layer of 16 nm. Both equipments were available at the National Laboratory of Nanotechnology (LNNano) located in Campinas-SP/Brazil. Analyses of the sample surfaces were performed under vacuum, using a 5 kV acceleration voltage.

3. Results and discussion

3.1. Proximate composition of blackberry bagasse

Table 1 presents the proximate composition of blackberry bagasse. It can be noted that the moisture of the bagasse determined by the gravimetric method is quite lower than that of the fresh fruit, reported by Chim [22] for three varieties of blackberry (Guarani 87%, Tupy 88.3%, and Brazos 89.3%). The moisture that remains in the bagasse after drying (5.24%) may contribute to the further extraction of some polar components in SFE, since water in

Table 1
Composition of blackberry bagasse.

Analysis	Bagasse fresh	Bagasse dried and crushed
Moisture – gravimetric (%)	69.0 ± 0.06	–
Moisture – Karl Fisher (%)	–	5.24 ± 0.19
Ashes (%)	0.37 ± 0.01	1.62 ± 0.05
Lipids (%)	0.87 ± 0.00	11.35 ± 0.58
Proteins (%)	9.05 ± 0.01	14.31 ± 0.36

Results are mean ± standard deviation of experiments performed in triplicate.

small amounts can act as cosolvent. The contents of ash, lipids and proteins are higher for the dried material, as expected. Facco et al. [23] reported ash contents from 0.27 to 0.49% in fresh blackberries, which are near the values found in this work for fresh bagasse, but different from those found by Chim [22] for the mentioned varieties. Also classified as small fruit, blackberry has similar characteristics to blueberry. Besides the basic nutrients, blackberry has remarkable amounts of essential micronutrients as minerals, fibers, vitamins and various secondary metabolites of phenolic nature [23].

These differences may be related to the processes that modify the physical and chemical properties of the material, but also to environmental conditions of growth. The results show that the residue from blackberry pulp processing is still a good source of nutrients, which could be recovered and used on the manufacturing of novel food products.

3.2. Preliminary SFE tests with and without ultrasound

Preliminary SFE tests were performed on the dried and milled raw material. Next, the extraction bed was prepared as described in Section 2.6.1. CO_2 flow rate was 2.77×10^{-4} kg/s in all the experiments.

Table 2 shows the extract yields (X_0) obtained in the preliminary SFEs. A significant increase in X_0 is observed when ultrasound was applied, mainly at 15 MPa. The application of ultrasound can produce a cavitation effect near the cell walls [12], and also the release of soluble material onto the sample surface, leading to higher yields. Therefore, the extract yields achieved by SFE-US are quite higher than those of SFE without ultrasound at the same pressures and temperatures.

Fig. 2 shows SFE curves obtained at the conditions reported in Table 2, and the fittings obtained through the spline model. In SFE at 15 MPa, a clear increase in the extraction rate can be noted by the slopes of the curves (Fig. 2A and B) at the first 50 min, when SFE rate is constant and fluid phase convection is the main mass transfer mechanism. Such differences are not observed at 20 MPa (Fig. 2C), where the curves with and without ultrasound practically coincide. Possibly, the pressure of 20 MPa is high enough to provide the same effects of ultrasound, in terms of release of extractable material.

These observations are confirmed by the parameters calculated with the spline model, which are reported in Table 2. The extraction rate at the first extraction period (M_{cer}) increased when ultrasound was applied in SFE at 15 MPa, and decreased at 20 MPa. It can be noted, indeed, that the extraction yield (R_{cer}) and the extract concentration in CO_2 (Y_{cer}) also were increased with the application of ultrasound at 15 MPa, but not at 20 MPa.

On the other side, the slopes of the curves with and without ultrasound are close at the final part of extraction, which is controlled by diffusion. This indicates that ultrasound enhances convective mass transfer, and such enhancement may be achieved by releasing part of the extractable material to the solid surface, where it is directly accessible to CO_2.

3.3. Low pressure extractions

Table 3(A) shows the results obtained from the low pressure extractions with ethanol. It can be noted that Soxhlet provided higher yield than maceration, which can be due to solvent recycle and high temperature, which helps reducing the surface tension and viscosity, enhancing mass transfer of the solvent into the solid matrix [24–26]. Table 3(A) also shows that the use of ethanol as solvent increases the content of monomeric anthocyanins, phenolics and antioxidant activity, since most of these compounds are polar, and thus they are solubilized more easily in ethanol than in CO_2 [27].

3.4. SFE-US

Table 3(B) presents the results obtained for SFE-US at the conditions determined by the experimental design, for global yield, phenolic content, and antioxidant activity.

3.4.1. Global yield (X_0)

The highest SFE global yield (9.87 ± 0.40%) was achieved at 25 MPa, 50 °C, and ultrasound power of 400 W. The increase of X_0 with pressure is explained by the increased density of CO_2, which enhances its solvation power [5]. Moreover, an increased X_0 is observed when ultrasound was applied during SFE. This behavior is consistent with those observed in former works [28]. According to Balachandran et al. [12], the increase of global yield can be attributed to the high extraction rates achieved in the process assisted by ultrasound. The application of ultrasound causes ruptures in the vegetal structure, promoting the release of compounds that were not formerly available, so the yield can be increased. Another possible effect of ultrasound in SFE is the release of extractable material onto the sample's surface [13]. In this case, the desorption of solute from the substrate to the solvent is enhanced, increasing mass transfer and yield. Also in Table 3, it can be observed that the lowest yield (6.25 ± 0.16%) was obtained at 15 MPa, 60 °C, and 200 W. Such results can be explained by the reduction of CO_2 density with decreased pressure, but also with the increase in temperature [29].

Comparing SFE with the low pressure extraction, it can be noted that Soxhlet with ethanol achieved higher yields, which can be

Table 2
Preliminary tests for supercritical CO_2 extraction with and without ultrasound: process conditions, model parameters and global yield.

Temperature (°C)	Pressure (MPa)	US Power (W)	t_{cer} (min)	$M_{cer} \times 10^8$ (kg/s)	$Y_{cer} \times 10^4$ (kg/kg CO_2)	X_{cer}	t_{fer} (min)	X_{fer}	X_0 (%)
40	15	0	42.48 ± 0.04	9.04	3.26	4.83 ± 0.35	126.55 ± 2.76	6.80 ± 0.27	7.13 ± 0.10
40	15	200	36.21 ± 5.50	13.0	4.69	5.99 ± 0.50	131.95 ± 9.12	8.58 ± 0.05	9.30 ± 0.35
50	15	0	44.40 ± 3.68	7.56	2.73	4.15 ± 0.01	130.05 ± 3.46	7.25 ± 0.11	8.45 ± 0.21
50	15	200	57.02 ± 2.52	9.76	3.52	6.57 ± 0.38	118.55 ± 11.95	8.75 ± 0.09	9.36 ± 0.14
50	20	0	26.69 ± 5.70	19.9	7.19	6.58 ± 0.64	94.93 ± 9.38	8.60 ± 0.08	9.48 ± 1.26
50	20	200	37.26 ± 5.80	16.4	5.92	7.03 ± 0.58	116.65 ± 10.08	9.42 ± 0.31	9.97 ± 0.30

t_{cer}, constant extraction rate period; M_{cer}, constant extraction rate; Y_{cer}, extract concentration in CO_2 at the constant extraction rate period X_{cer}, yield of the constant extraction rate period; t_{fer}, falling extraction rate period; X_{fer}, yield of the falling extraction rate period; X_0, global extraction yield.

Table 3
Extraction conditions for: (A) low pressure extractions, (B) SFE extraction with and without ultrasound, (C) SFE-US with cosolvents. For global yield, total phenolics, monomeric anthocyanins and antioxidant activity.

Low pressure extractions

Method	Sample	Solvent	Temperature (°C)	S/F (kg/kg)	Pressure (MPa)	US power (W)	X_0* (%)	MA*	TPC*	AA*	
										DPPH	ABTS
Soxhlet	Dried and crushed	Ethanol	50	31.6	–	–	14.58 ± 0.79[b]	4.84 ± 0.49[a]	4.25 ± 0.61[b]	68.40 ± 1.37[a]	95.70 ± 5.95[b]
Maceration	Dried and crushed	Ethanol	25	25.28	–	–	10.72 ± 0.25[a]	0.13 ± 0.01[b]	5.95 ± 0.08[c]	70.24 ± 4.09[a]	62.82 ± 2.93[a]

Supercritical CO_2 extraction assisted by ultrasound

Method	Sample	Temperature (°C)	Pressure (MPa)	US power (W)	Density of CO_2 (kg/m^3)	X_0 (%)	TPC	AA	
								DPPH	ABTS
SFE + US	Dried and crushed	40	15	200	780.23	8.00 ± 0.40	3.31 ± 0.14	16.36 ± 0.06	55.87 ± 1.47
SFE + US	Dried and crushed	60	15	200	604.09	6.25 ± 0.16	4.37 ± 0.17	25.94 ± 0.57	67.27 ± 0.03
SFE + US	Dried and crushed	40	25	200	879.49	8.37 ± 0.23	4.00 ± 0.12	17.10 ± 0.98	55.24 ± 0.09
SFE + US	Dried and crushed	60	25	200	786.55	8.51 ± 0.56	3.56 ± 0.21	17.56 ± 0.49	58.52 ± 4.35
SFE	Dried and crushed	40	20	0	839.81	7.86 ± 0.42	3.77 ± 0.06	21.57 ± 0.49	59.67 ± 1.30
SFE	Dried and crushed	60	20	0	723.68	8.31 ± 0.14	4.44 ± 0.30	21.24 ± 0.54	63.03 ± 1.99
SFE + US	Dried and crushed	40	20	400	839.81	8.99 ± 0.05	3.81 ± 0.12	19.63 ± 0.34	56.60 ± 0.20
SFE + US	Dried and crushed	60	20	400	723.68	8.58 ± 0.14	4.06 ± 0.01	22.52 ± 0.64	63.52 ± 0.07
SFE	Dried and crushed	50	15	0	699.75	6.84 ± 0.02	4.07 ± 0.24	24.85 ± 1.03	59.84 ± 0.28
SFE	Dried and crushed	50	25	0	834.19	8.65 ± 0.26	3.92 ± 0.16	23.90 ± 0.77	63.66 ± 3.61
SFE + US	Dried and crushed	50	15	400	699.75	7.94 ± 0.03	3.53 ± 0.19	23.00 ± 0.19	64.96 ± 1.70
SFE + US	Dried and crushed	50	25	400	834.19	9.87 ± 0.40	3.89 ± 0.11	19.76 ± 1.09	61.48 ± 2.52
SFE + US	Dried and crushed	50	20	200	784.29	8.88 ± 0.18	4.15 ± 0.17	18.91 ± 1.31	63.94 ± 1.40
SFE + US	Dried and crushed	50	20	200	784.29	8.92 ± 0.59	4.20 ± 0.25	18.95 ± 0.04	64.24 ± 0.73
SFE + US	Dried and crushed	50	20	200	784.29	8.95 ± 0.74	4.16 ± 0.02	18.68 ± 0.09	63.90 ± 0.23

SFE-US with cosolvents

Method	Sample	Cosolvent	Temperature (°C)	Pressure (MPa)	US power (W)	X_0* (%)	MA*	TPC*	AA*	
									DPPH	ABTS
SFE + US	Dried and crushed	CO_2:EtOH/90:10	60	15	200	18.25 ± 0.77[a]	2.20 ± 0.05[a]	12.73 ± 1.26[a]	53.43 ± 4.51[a]	53.05 ± 0.79[e]
SFE + US	Dried and crushed	CO_2:water/90:10	60	15	200	15.33 ± 0.05[b]	13.66 ± 0.07[b]	49.36 ± 0.27[b]	96.11 ± 4.49[b]	154.98 ± 1.83[a]
SFE + US	Dried and crushed	CO_2:EtOH/95:5	60	15	200	8.84 ± 0.10[c]	0.45 ± 0.03[c]	6.51 ± 0.27[c]	45.07 ± 2.20[c]	53.02 ± 0.07[e]
SFE + US	Dried and crushed	CO_2:water/95:5	60	15	200	7.58 ± 0.33[c]	5.13 ± 0.35[d]	33.05 ± 1.24[d]	74.36 ± 6.18[d]	112.59 ± 0.69[c]
SFE + US	Fresh	CO_2:EtOH/95:5	60	15	200	5.03 ± 0.16[d]	6.84 ± 0.31[d]	24.13 ± 1.62[e]	25.07 ± 1.44[e]	81.35 ± 0.48[d]
SFE + US	Fresh	CO_2:water/95:5	60	15	200	3.41 ± 0.18[e]	17.54 ± 0.07[e]	42.11 ± 3.96[f]	40.23 ± 1.07[f]	144.85 ± 3.05[b]

Results are expressed as mean ± standard deviation of the analysis. SFE = Supercritical fluid extraction; US, ultrasound; X_0, global yield (%); MA, monomeric Anthocyanins (mg cyanidin 3-O-glucoside/g of extract); TPC, phenolic content (mg EAG/g of extract); AA, antioxidant activity expressed as Trolox equivalent μmol TE/g of extract).

* Letters equal in the same column indicate that there is no significant difference at the level of 5% by the Tukey test.

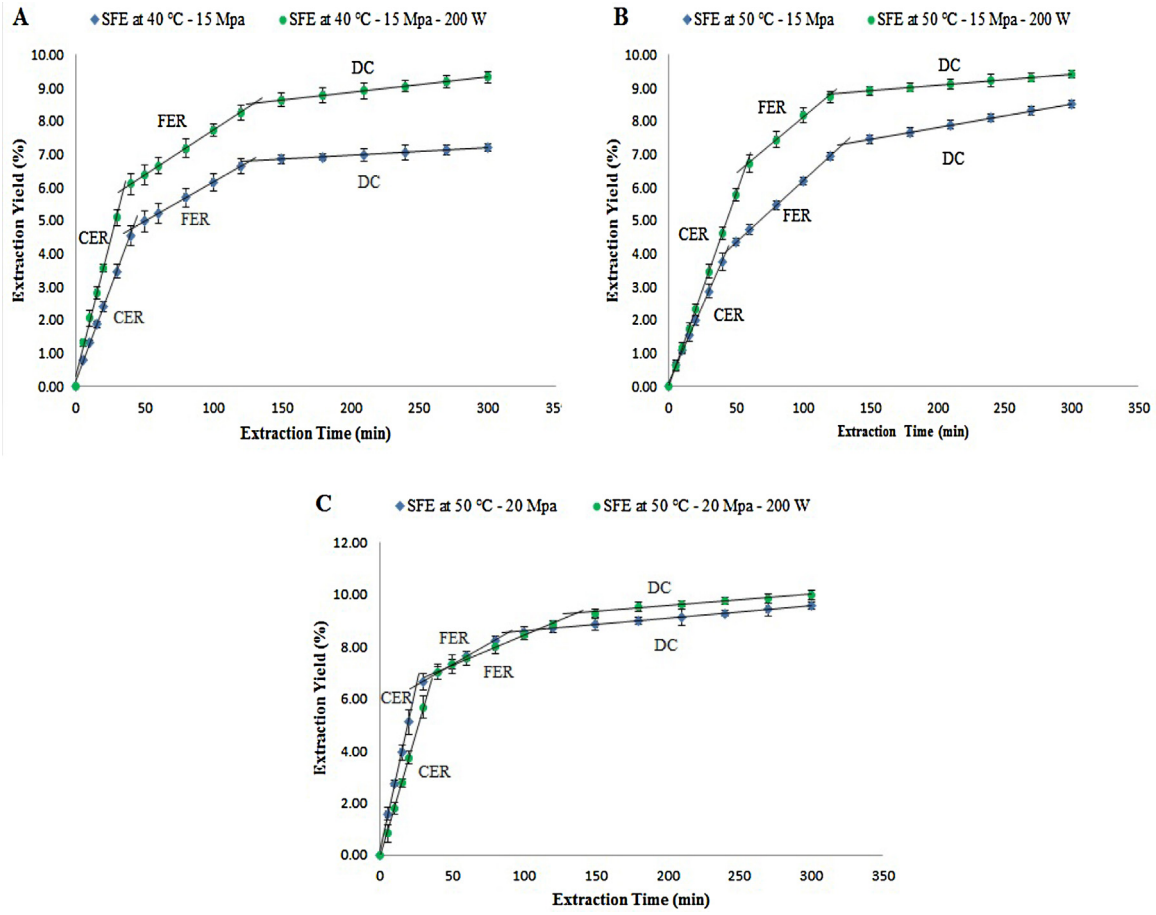

Fig. 2. Kinetics of SFE from blackberry bagasse at different pressures and temperatures – experimental data and model results.

attributed to the extraction of polar compounds that are not soluble in CO_2. Moreover, the longer extraction times and solvent to solid proportion also help increasing yield. The low pressure extraction methods are effective, but present disadvantages such as high required volume of solvent, long process times, and contamination of the extract with residual solvent [30].

3.5. Chemical composition of the extracts

3.5.1. Total phenolic content

Table 3(B) shows that the highest phenolic concentration (4.44 ± 0.30 mg GAE/g extract) was obtained with SFE at 20 MPa, 60 °C, without ultrasound. The energy released by ultrasonic waves may have degraded part of the phenolics. The phenolic content seems to increase with temperature in SFE, which means that such compounds are solubilized easier at high temperatures. According to Carrera et al. [31], high temperatures promote higher recovery of phenolic compounds. Moreover, the density of CO_2 increases with pressure and decreases with temperature, and the solvation power of CO_2 increases with density. Thus, at higher densities, other compounds could have been extracted, resulting in a slight reduction of the phenolics concentration in the extracts.

3.5.2. Antioxidant activity

The highest antioxidant activities obtained by the methods DPPH and ABTS were of 25.94 ± 0.57 and 67.27 ± 0.03 μmol TE/g extract, respectively, and were achieved at 15 MPa, 60 °C, and 200 W, as reported in Table 3(B). The increase of antioxidant activity was more pronounced at low pressures and high temperatures, indicating that the compounds responsible for the antioxidant activity are more soluble in high temperatures, and must not have suffered thermal degradation. Also, at lower pressures CO_2 may have been more selective as solvent, providing extracts with less additional compounds with no antioxidant potential.

3.5.3. Identification of anthocyanins by UPLC–QTOF-MS

The UPLC–QTOF-MS analysis identified four anthocyanins in the bagasse of blackberry. Table 4 presents the molecular ion and λ_{max} in the visible region of the identified compounds. From the UPLC–QTOF-MS analysis, it is possible to observe the chromatographic profile varying only the peak magnitudes, which is directly related to the concentrations of the anthocyanins. The major identified anthocyanins were cyanidin 3-O-glucoside (C3G) and cyanidin 3-O-rutinoside (C3R). Moreover, cyanidin 3-O-(6″-malonyl-glucoside) (C3MG) and cyanidin 3-O-(6″-dioxalyl-glucoside) (C3DG) were identified as derived from C3G with different acids. The UV–vis spectra of the identified compounds exhibit their maximum absorption wavelength, which is strictly related to the hydroxylation standard of the anthocyanins

Table 4
Characteristics of chromatographic, UV–visible spectrum and mass spectrometry obtained by UPLC–QTOF–MS.

Compound	Molecular ion – mass (m/z)	λ_{max} in the visible region (nm)
Cyanidin 3-O-glucoside	449	514
Cyanidin 3-O-rutinoside	595	514
Cyanidin 3-O-(6″-malonyl-glucoside)	535	517
Cyanidin 3-O-(6″-dioxalyl-glucoside)	593	518

[32]. According to Dao et al. [33], C3G and C3R are the main anthocyanins found in blackberries, as well as those found in this work for the bagasse. Another work performed in 18 blackberry cultivars also reported C3G as the major anthocyanin, besides C3R, cyanidin 3-O-xiloside, C3MG and C3DG as minor ones [34]. Thus, most of the anthocyanins reported in blackberries were also found in the bagasse from pulp processing, reinforcing the importance of recovering such waste. Moreover, C3G, the major anthocyanin in blackberry, is also mentioned as responsible for the antioxidant activity of this fruit [35], and for its effectiveness to prevent the oxidation of low density lipoproteins in human body [36].

3.5.4. Separation and quantification of anthocyanins by UPLC–UV–vis

Table 5 shows the concentrations of the anthocyanins C3G, C3R, C3DG and C3MG, found in the extracts obtained by SFE-US using ethanol and water as cosolvents, at 15 MPa, 60 °C and 200 W. The concentration of anthocyanins found by UPLC–UV–vis is lower than those measured by the pH differential method. Even so, the difference was quite small. According to Gouvêa et al. [37], even other substances could interfere in the pH differential method, explaining the observed difference.

3.6. SFE–US with cosolvents

Table 3(C) shows that the addition of ethanol and water in SFE-US from the dried and milled material leads to higher extraction yields. This behavior is related to the polar nature of the chosen cosolvents, which allows extracting components that would not be soluble in pure CO_2, which is non polar. The molecules of the cosolvent compete with the active sites of the substrate to interact with the extractable compounds. Therefore, the presence of cosolvents helps breaking the interactions between substrate and solute, which can be solubilized by the solvent mixture [7,38]. The increase of solubility caused by a cosolvent results from the formation of cosolvent–solute and solvent–cosolvent–solute groups [39]. A cosolvent with critical temperature lower than that of the supercritical fluid usually reduces the solubility of low volatility compounds, and the opposite occurs if the critical temperature of the cosolvent is higher [5]. Both cosolvents used in this work (ethanol and water) have critical temperatures higher than CO_2 (240.6 °C, 374.2 °C and 31.1 °C, respectively), so they enhance the solubility of many compounds present in the blackberry bagasse.

An increase in SFE-US yield is observed when ethanol was used as cosolvent, compared to water. Besides increasing yield, ethanol can be removed easier than water, so its application is widely reported [40]. This behavior can be explained by the enhanced solubility of polar compounds in the mixture CO_2 + ethanol. Moreover, the use of ethanol as cosolvent may have increased the number of extracted compounds, thus reducing the selectivity of the process. Although water is more polar than ethanol, the SFE yields with water as cosolvent were lower. Polarity is not the unique factor affecting extraction yield. The type of interactions between solvent and solute should also be comprehended.

As observed for SFE from blackberry bagasse, other works report remarkable enhancements in SFE yield by using ethanol as cosolvent at low concentrations. Luengthanaphol [41] compared yield and antioxidant activity of extracts (*Tamarindus indica* L.) from tamarind seeds obtained by SFE with pure CO_2 and with 10% ethanol as cosolvent, and verified that SFE of antioxidants is significantly improved with ethanol. Kitzberger et al. [42] observed an increase in the SFE yield from shiitake from 1.01% to 3.81% by using 15% ethanol as cosolvent.

The contribution of ethanol and water as cosolvents in the recovery of phenolics, anthocyanins, and the antioxidant activity of the extracts can be observed in Fig. 3. In general, both cosolvents had positive influence on the extraction of the mentioned compounds, and the effect of water as cosolvent was clearly higher than ethanol.

In SFE-US with cosolvents the highest anthocyanin content (17.54 ± 0.07 mg cyanidin 3-O-glucoside/g extract) was obtained using 5% water for the fresh sample. Tena et al. [43] and Murga et al. [44] reported that adding cosolvent to CO_2 helps improving the extraction yield of some compounds, such as anthocyanins, phenolics, and antioxidant activity. It can be noted in Table 3(C) that the cosolvent ratio affects the anthocyanin concentration in the extracts, indicating that interactions between solute and substrate must have been broken and replaced by cosolvent molecules [7]. Thus, the anthocyanin yield increased with the cosolvent concentration [45]. This is clear in the extractions from dried and crushed samples, where raising the water ratio from 5 to 10% strongly increased the anthocyanin concentration, due to the enhancement of the solute/cosolvent interactions that raise solubility [7]. The same effect is also noted in the extractions with ethanol, although the anthocyanin recovery was lower than with water. Thus, water as cosolvent not only increases SFE yield, but is also the most adequate solvent since is ecologically safe and cheap.

Regarding phenolic compounds, the best results of SFE-US were found using 10% water in the dried and crushed sample and 5% water in the fresh sample. As expected, water extracts phenolics efficiently, and the extracts with most phenolics also presented the highest antioxidant activities measured by DPPH and ABTS, evidencing some correlation between phenolics and antiradical capacity [46,47]. However, the relation shows that phenolics are not the unique responsible for the antioxidant activity of the extracts. Vegetable substrates contain several phenolic components with different antioxidant activities, and the synergism between antioxidants in a mixture makes their activity dependent of concentrations, structure and their chemical interactions [48].

It can also be observed in Table 3(C) that the method ABTS is more effective in the detection of antioxidant compounds than DPPH. The method DPPH is widely applied to determine the antioxidant activity in extracts and isolated compounds, such as phenolics, anthocyanins, flavonols and cumarins [49,50]. Every method provides precise and reproducible results, but the antioxidant activities may differ significantly from one method to other. Thus, most methods provide partial results regarding antioxidant activity of complex extracts [51,52].

The addition of water as cosolvent at 10% provides the highest antioxidant activities by both methods. For the extracts obtained by SFE-US with pure CO_2 the antioxidant activities were quite lower, indicating again that this property is intimately related to phenolics, which are polar compounds that can hardly be extracted with a nonpolar solvent like CO_2.

Finally, evaluating the extracts obtained by SFE-US with cosolvent, one can conclude that to achieve the best yields it is preferable to use 10% ethanol, which provided yields eight times higher than with pure CO_2. Since ethanol is a slightly polar solvent, its addition as cosolvent allowed the dissolution of polar substances that were not extracted with pure CO_2. In terms of anthocyanins, phenolics, and antioxidant activity, it is better to use 10% water as cosolvent. The increase of such compounds may be due to the low solubility of water in CO_2, which may lead to the coexistence of two phases. In this case, a liquid phase containing water as major component would help extracting preferentially phenolics and anthocyanins. Nevertheless, if SFE is performed on the fresh sample, 5% water is more recommendable, since the water content of the sample seems to work as cosolvent and enhance the extraction of anthocyanins. Moreover, the recovery of anthocyanins could have been enhanced due to pH reduction in the presence of CO_2 and water, since anthocyanins are usually more stable in acidified media [53–55]. Summarizing, the differences between SFE-US with and without cosolvents can be attributed to the changes in

Table 5
Concentration of anthocyanins of the extracts of blackberry obtained by UPLC–UV–vis.

Sample	Cosolvent	C3G mg/g of extract	C3R mg/g of extract	C3MG mg/g of extract	C3DG mg/g of extract	Total
Dried and crushed	CO_2:EtOH/90:10	0.523 ± 0.05	0.044 ± 0.00	0.034 ± 0.00	0.041 ± 0.01	0.641 ± 0.06
Dried and crushed	CO_2:Water/90:10	5.697 ± 0.38	0.422 ± 0.00	0.218 ± 0.01	0.256 ± 0.01	6.594 ± 0.40
Dried and crushed	CO_2:EtOH/95:5	0.194 ± 0.01	0.031 ± 0.00	0.021 ± 0.00	0.027 ± 0.00	0.273 ± 0.01
Dried and crushed	CO_2: Water/95:5	3.909 ± 0.49	0.289 ± 0.03	0.166 ± 0.02	0.188 ± 0.02	4.551 ± 0.56
Fresh	CO_2:EtOH/95:5	4.443 ± 0.02	0.286 ± 0.00	0.149 ± 0.00	0.196 ± 0.01	5.075 ± 0.03
Fresh	CO_2: Water/95:5	8.067 ± 0.19	0.649 ± 0.03	0.256 ± 0.01	0.338 ± 0.02	9.309 ± 0.25

Total anthocyanins level expressed in terms of the sum of the anthocyanins C3G, C3R, C3MG and C3DG per gram of extract.
Results expressed by its mean (±) standard deviation.

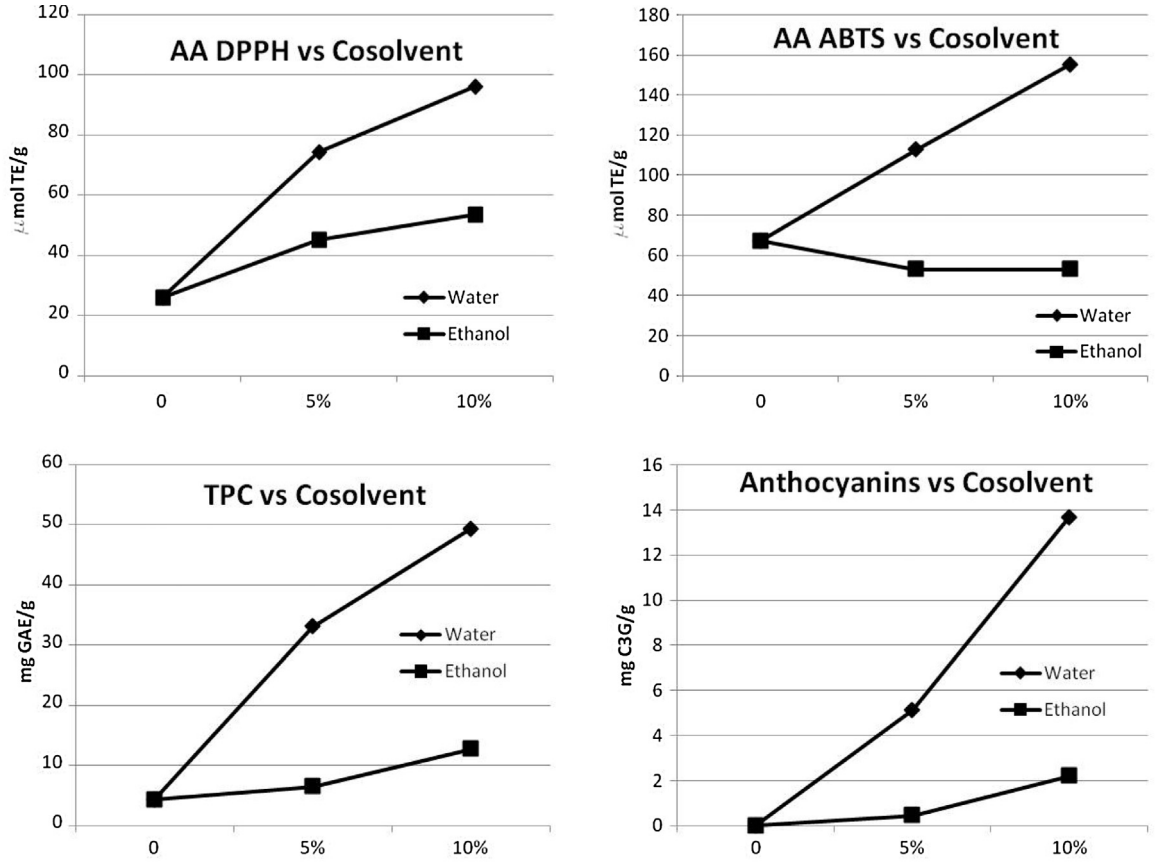

Fig. 3. Effect of the cosolvent concentrations in the antioxidant activity (AA DPPH and AA ABTS), phenolic (TPC) and anthocyanin contents of SFE extracts from dried blackberry bagasse obtained at 15 MPa, 60 °C and ultrasound power of 200 W.

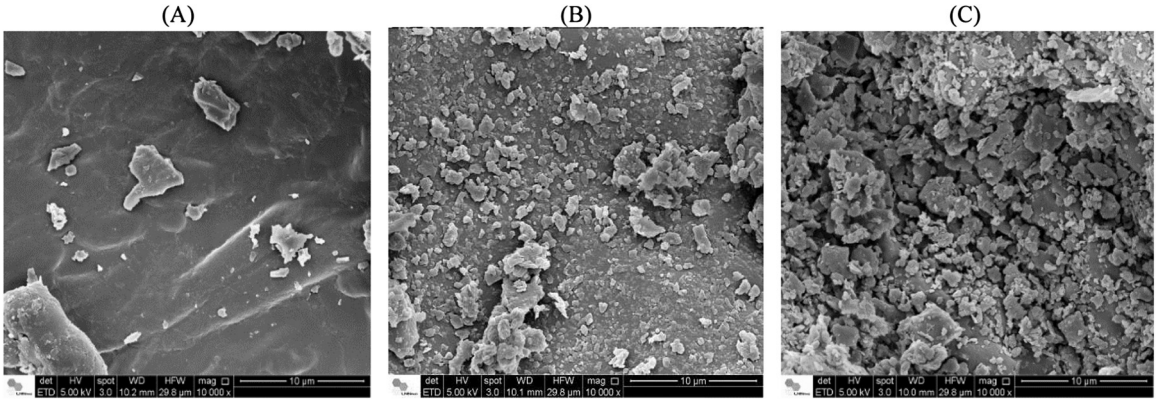

Fig. 4. Images obtained by scanning electron microscopy on the surface of blackberry bagasse particles under different conditions of extraction: (A) SFE without ultrasound at 50° C and 25 MPa; (B) SFE + US at 40° C, 15 MPa and a 200 W ultrasound power; (C) SFE + US at 50° C, 25 MPa and a 400 W ultrasonic power.

cosolvent concentration, type of pretreatment, and extraction method [56].

3.7. Morphological analysis

The effect of ultrasound on the physical structure of the blackberry bagasse was evaluated through FESEM. As it can be observed in Fig. 4, ultrasonic vibration effectively changes the surface of the blackberry bagasse particles. Samples undergoing only SFE extraction present a much more homogeneous and clean surface (Fig. 4A), indicating that supercritical CO_2 alone did not cause important morphological changes. In contrast, samples exposed to ultrasound exhibit an irregular surface, covered by particle fragments. Moreover, an effect of the ultrasound power can also be noticed by comparing Fig. 4B and C, with a greater amount of particles deposited under 400 W than at a 200 W power. The deposition of fragments on the surface is probably related to the removal of material from the internal parts of the cell wall, followed by its deposition on the surface during the extraction process. It should contribute to increase the accessibility of these particles to the solvent, which is in agreement with the relatively higher extraction yields in these samples. It is important to notice that the extraction process does not seem to cause fissures or rupture on the sample surface, since it keeps its integrity under the particulate deposits.

4. Conclusions

The recovery of blackberry bagasse to be used as raw material for extraction is a promising activity, due to the high amount of valuable compounds that remain in this industrial residue. The influence of ultrasound on SFE was significant at the pressure of 15 MPa, contributing to enhance the extraction kinetics and increase its global yield, without compromising the quality of the extracts. The enhancement of mass transfer with ultrasound is caused by changes on the structure of the substrate, which were observed by the FESEM image analyses. Specifically, the increase of particles stuck onto the surface of blackberry bagasse must have contributed to reduce the barriers to both solvent and extractable compounds.

The addition of water and ethanol as cosolvents in SFE-US increased the global yield, as well as the contents of phenolics, anthocyanins, and the antioxidant activity of the extracts. Two major (cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside) and two minor (cyanidin 3-O-(6″-dioxalyl-glucoside) and cyanidin 3-O-(6″-malonyl-glucoside)) anthocyanins were identified and quantified. The polar nature of water and ethanol and the acidification of the solvent mixture helped the solubilization of the anthocyanins.

The results presented in this work indicate that coupling ultrasound with SFE processes can be a feasible mean to improve extractions, reducing the required time and thus operational cost, to achieve yields and quality that make the process viable. When using liquids as cosolvents, an additional operation step is needed to separate them from the extracts, and other coupled techniques could be integrated to SFE, such as adsorption or membrane separation, in order to purify the final product. Indeed, as well as blackberries, other residues of fruit processing are certainly rich in bioactive compounds, so SFE-US is a promising method to add value to these byproducts.

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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.supflu.2014.07.019.

References

[1] A. Moure, J.M. Cruz, D. Franco, J.M. Domıńguez, J. Sineiro, H. Domıńguez, M.A. José Núñez, J.C. Parajó, Natural antioxidants from residual sources, Food Chemistry 72 (2001) 145–171.
[2] I. Ignat, I. Volf, V.I. Popa, A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables, Food Chemistry 126 (2011) 1821–1835.
[3] G. Brunner, Supercritical fluids: technology and application to food processing, J. Food Engineering 67 (2005) 21–33.
[4] J.M. Del Valle, J.M. Aguilera, Review: high pressure CO_2 extraction, Fundamentals and Applications in the Food Industry 5 (1999) 1–24.
[5] G. Brunner, Gas Extraction: An Introduction to Fundamentals of Supercritical Fluids and the Application to Separation Processes, vol. 1, Steinkopff, Darmstadl, Alemnha, 1994, pp. 387.
[6] T. Vatai, M. Škerget, Ž. Knez, Extraction of phenolic compounds from elder berry and different grape marc varieties using organic solvents and/or supercritical carbon dioxide, J. Food Engineering 90 (2009) 246–254.
[7] J. Hollender, J. Shneine, W. Dott, M. Heinzel, H.W. Hagemann, G.K.E. Gotz, Extraction of polycyclic aromatic hydrocarbons from polluted soils with binary and ternary supercritical phases, J. Chromatography A 776 (1997) 233–243.
[8] I. Dalmolin, M.A. Mazutti, E.A.C. Batista, M.A.A. Meireles, J.V. Oliveira, Chemical characterization and phase behaviour of grape seed oil in compressed carbon dioxide and ethanol as co-solvent, J. Chemical Thermodynamics 42 (2010) 797–801.
[9] E. Riera, Y. Golás, A. Blanco, J.A. Gallego, M. Blasco, A. Mulet, Mass transfer enhancement in supercritical fluids extraction by means of power ultrasound, Ultrasonics Sonochemistry 11 (2004) 241–244.
[10] J. Berlan, F. Trabelsi, H. Delmas, A.M. Wilhelm, J.F. Petrignani, Oxidative degradation of phenol in aqueous media using ultrasound, Ultrasonics Sonochemistry 1 (1994) S97–S102.
[11] D.L. Goldfarb, H.R. Corti, F. Marken, R.G. Compton, High-pressure sonoelectrochemistry in aqueous solution: soft cavitation under CO_2, J. Physical Chemistry A 102 (1998) 8888–8893.
[12] S. Balachandran, S.E. Kentish, R. Mawson, M. Ashokkumar, Ultrasonic enhancement of the supercritical extraction from ginger, Ultrasonics Sonochemistry 13 (2006) 471–479.
[13] P. Santos, A.C. Aguiar, G.F. Barbero, C.A. Rezende, J. Martínez, Supercritical carbon dioxide extraction of capsaicinoids from malagueta pepper (*Capsicum frutescens* L.) assisted by ultrasound, Ultrasonics Sonochemistry (2014), http://dx.doi.org/10.1016/j.ultsonch.2014.05.001 (in press).
[14] AOAC. Official methods of analysis of AOAC iternational. Association of Official Analytical chemists, 1999.
[15] L.E. Rodriguez-Saona, R.E. Wrolstad, Extraction, isolation and purification of anthocyanins, in: Current Protocols in Food Analytical Chemistry, John Wiley & Sons, Inc., New York, NY, 2001.
[16] Determination of the solubility of extracts from vegetable raw material in pressurized CO_2: a pseudo-ternary mixture formed by cellulosic structure + solute + solvent, J. Supercritical Fluids 22 (2002) 21–36.
[17] V.L. Singleton, J.A. Rossi, Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents, American J. Enology and Viticulture 16 (1965) 144–158.
[18] W. Brand-Williams, M.E. Cuvelier, C. Berset, Use of a free radical method to evaluate antioxidant activity, Food Science and Technology 28 (1995) 25–30.
[19] T. Kulisic, A. Radonic, V. Katalinic, M. Milos, Use of different methods for testing antioxidative activity of oregano essential oil, Food Chemistry 85 (2004) 633–640.
[20] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radical Biology and Medicine 26 (1999) 1231–1237.
[21] M.M. Giusti, R.E. Wrolstad, Characterization, and measurement of anthocyanins by UV–visible spectroscopy, in: R.E. Wrolstad, S.J. Schwartz (Eds.), Current Protocols in Food Analytical Chemistry, John Wiley & Sons, Inc., New York, NY, 2001.
[22] J.F. Chim, Caracterização de Compostos Bioativos em Amora-preta (*Rubus* sp.) e Sua Estabilidade no Processo e Armazenamento de Geléias Convencional e Light, Universidade Federal de Pelotas, UFPEL, Brazil, 2008.
[23] E.M.P. Facco, G.E. Hirsch, M. Bagetti, T. Emanuelli, Estudo da compoisição centezimal e valor nutricional fde diferentes variedades de amora preta (*Rubus* sp.), in: XV. Seminário Latino americano e do Caribe de Ciência e Tecnologia de Alimentos, Belo Horizonte, 2008.

[24] M. Markom, M. Hasan, W.R.W. Daud, H. Singh, J.M. Jahim, Extraction of hydrolysable tannins from *Phyllanthus niruri* Linn: effects of solvents and extraction methods, Separation and Purification Technology 52 (2007) 487–496.
[25] S. Mazzutti, S.R.S. Ferreira, C.A.S. Riehl, A. Smania Jr., F.A. Smania, J. Martínez, Supercritical fluid extraction of *Agaricus brasiliensis*: antioxidant and antimicrobial activities, J. Supercritical Fluids 70 (2012) 48–56.
[26] Z. Xu, J.S. Godber, Comparison of supercritical fluid and solvent extraction methods in extracting γ-oryzanol from rice bran, J. American Oil Chemists' Society 77 (2000) 547–551.
[27] L. Casas, C. Mantell, M. Rodríguez, A. Torres, F.A. Macías, E. Martínez de la Ossa, Extraction of natural compounds with biological activity from sunflower leaves using supercritical carbon dioxide, Chemical Engineering J. 152 (2009) 301–306.
[28] Y. Gao, B. Nagy, X. Liu, B. Simándi, Q. Wang, Supercritical CO_2 extraction of lutein esters from marigold (*Tagetes erecta* L.) enhanced by ultrasound, J. Supercritical Fluids 49 (2009) 345–350.
[29] L.M.A.S. Campos, E.M.Z. Michielin, L. Danielski, S.R.S. Ferreira, Experimental data and modeling the supercritical fluid extraction of marigold (*Calendula officinalis*) oleoresin, J. Supercritical Fluids 34 (2005) 163–170.
[30] A.A. Maul, Fluidos supercríticos: situação atual e futuro da extração supercrítica, Biotecnologia Ciência e Desenvolvimento 2 (1999) 42–46.
[31] C. Carrera, A. Ruiz-Rodríguez, M. Palma, C.G. Barroso, Ultrasound assisted extraction of phenolic compounds from grapes, Analytica Chimica Acta 732 (2012) 100–104.
[32] Q. Tian, M.M. Giusti, G.D. Stoner, S.J. Schwartz, Characterization of a new anthocyanin in black raspberries (*Rubus occidentalis*) by liquid chromatography electrospray ionization tandem mass spectrometry, Food Chemistry 94 (2006) 465–468.
[33] L.T. Dao, G.R. Takeoka, R.H. Edwards, Improved method for the stabilization of anthocyanidins, J. Agriculture and Food Chemistry 46 (1998) 3564–3569.
[34] H.J. Fan-Chiang, R.E. Wrolstad, Anthocyanin pigment composition of blackberries, J. Food Science 70 (2005) C198–C202.
[35] S.Y. Wang, L. Bowman, M. Ding, Methyl jasmonateb enhances antioxidant activity and flavonoid content in blackberries (*Rubus* sp.) and promotes antiproliferation of human cancer cells, Food Chemistry 107 (2008) 1261–1269.
[36] M.T. Satué-Gracia, M. Heinonen, E.N. Frankel, Anthocyanins as antioxidants on human low-density lipoprotein and lecithin–liposome systems, J. Agricultural and Food Chemistry 45 (1997) 3362–3367.
[37] A.C.M.S. Gouvêa, M.C.P. Araujo, S. Pacheco, J.O. Neto, R.L. Godoy, J.S. Rosa, Cyanidin-3-glucoside and cyanidin-3-rutinoside anthocyanins standards isolation by HPLC from freeze-dried açaí (*Euterpe oleraceae* Mart.), in: Congreso Internacional de Ciencia y Tecnología de los Alimentos, Córdoba, Argentina, 2009, p. 184.
[38] C. Lutermann, E. Willems, W. Dott, J. Hollender, Effects of various binary and ternary supercritical phases on the extraction of polycyclic aromatic hydrocarbons from contaminated soils, J. Chromatography A 816 (1998) 201–211.
[39] J. Ke, B. Han, H. yan, Hydrogen bonding of some organic acid in supercritical CO_2 with polar cosolvents, J. Supercritical Fluids 11 (1997) 53–60.
[40] L.M.A.S. de Campos, F.V. Leimann, R.C. Pedrosa, S.R.S. Ferreira, Free radical scavenging of grape pomace extracts from Cabernet sauvingnon (*Vitis vinifera*), Bioresource Technology 99 (2008) 8413–8420.
[41] S. Luengthanaphol, Extraction of antioxidants from sweet Thai tamarind seed coat – preliminary experiments, J. Food Engineering 63 (2004) 247–253.
[42] C.S.G. Kitzberger, R.H. Lomonaco, E.M.Z. Michielin, L. Danielski, J. Correia, S.R.S. Ferreira, Supercritical fluid extraction of shiitake oil: curve modeling and extract composition, J. Food Engineering 90 (2009) 35–43.
[43] M.T. Tena, A. Ríos, M. Valcárcel, Supercritical fluid extraction of t-resveratrol and other phenolics from a spiked solid, J. Analytical Chemistry 361 (1998) 143–148.
[44] R. Murga, R. Ruiz, S. Beltran, J.L. Cabezas, Extraction of natural complex phenols and tannins from grape seeds by using supercritical mixtures of carbon dioxide and alcohol, J. Agricultural and Food Chemistry 48 (2000) 3408–3412.
[45] P. Tonthubthimthong, P.L. Douglas, S. Douglas, W. Luewisutthichat, W. Teppaitoon, L. Pengsopa, Extraction of nimbin from neen seeds using supercritical CO_2 and a supercritical CO_2–methanol mixture, J. Supercritical Fluids 30 (2004) 287–301.
[46] D.O. Kim, S.W. Jeong, C.Y. Lee, Antioxidant capacity of phenolic phytochemicals from various cultivars of plums, Food Chemistry 81 (2003) 321–326.
[47] S. Sellappan, C.C. Akoh, G. Krewer, Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries, J. Agricultural and Food Chemistry 50 (2002) 2432–2438.
[48] A. Djeridane, M. Yousfi, B. Nadjemi, D. Boutassouna, P. Stocker, N. Vidal, Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds, Food Chemistry 97 (2006) 654–660.
[49] M. Leja, A. Mareczek, G. Wyzgolink, J. Klepacz-Baniak, K. Czekonska, Antioxidative properties of bee pollen in selected plant species, Food Chemistry 100 (2007) 237–240.
[50] H. Vogel, M. Gonzales, F. Faini, I. Razmilic, J. Rodriguez, J. San Martin, F. Urbina, Antioxidant properties and TLC characterization of four Chilean haplopappus-species known as bailahuén, J. Ethnopharmacology 97 (2005) 97–100.
[51] S. Arabshahi-Delouee, A. Urooj, Antioxidant properties of various solvent extracts of mulberry (*Morus indica* L.) leaves, Food Chemistry 102 (2007) 1233–1240.
[52] C. Delgado-Andrade, J.A. Rufián-Henares, F.J. Morales, Assessing the antioxidant activity of melanoidins from coffee brews by different antioxidant methods, J. Agricultural and Food Chemistry 53 (2005) 7832–7836.
[53] J.S. Barnes, H.P. Nguyen, S. Shen, K.A. Schug, General method for extraction of blueberry anthocyanins and identification using high performance liquid chromatography–electrospray ionization-ion trap-time of flight-mass spectrometry, J. Chromatography A 1216 (2009) 4728–4735.
[54] A. Castañeda-Ovando, M.d.L. Pacheco-Hernández, M.E. Páez-Hernández, J.A. Rodríguez, C.A. Galán-Vidal, Chemical studies of anthocyanins: a review, Food Chemistry 113 (2009) 859–871.
[55] R.L. Jackman, R.Y. Yada, M.A. Tung, R.A. Speers, Anthocyanins as food colorants: a review, J. Food Biochemistry 11 (1987) 201–247.
[56] M.E. Cuvelier, H. Richard, C. Berset, Antioxidative activity and phenolic composition of pilot-plant and commercial extracts of sage and rosemary, J. American Oil Chemists' Society 73 (1996) 645–652.