EXTRACTION OF BIOACTIVE COMPOUNDS FROM BLACKBERRY RESIDUES USING SUPERCRITICAL CO_2 AND SUBCRITICAL LIQUIDS

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ABSTRACT

Blackberry (*Rubus spp*.) is a fruit rich in bioactive compounds, such as anthocyanins, which offer benefits to health. Residues from industrial processing of blackberries are good sources of anthocyanins, which are phenolic compounds known for their antioxidant activity. In order to recover this byproduct, extracts were obtained using supercritical carbon dioxide extraction assisted by ultrasound (SFE-US) and pressurized liquid extraction (PLE). In SFE-US a Box-Behnken design was proposed with three variables: temperature (40, 50 and 60 ^{o}C), pressure (15, 20 and 25 MPa) and ultrasound power (0, 200 and 400 W). CO_2 flow rate (2.77 x 10^{-4} kg/s) and extraction time (120 min) were kept constant. In PLE four different solvents (water, acidified water at pH 2.5, ethanol and ethanol+water 50 % v/v) and three temperatures (60, 80 and 100 ^{o}C) were used. Pressure (7.5 MPa), solvent to feed ratio (18.0 kg/kg residue) and extraction time (30 min) were kept constant. The extracts were evaluated in terms of global yield, total phenolics (TP), monomeric anthocyanins (MA) and antioxidant activity (AA – methods DPPH and ABTS). In both SFE-US and PLE, global yield, TP and AA increased with temperature. Anthocyanins were not identified in SFE-US extracts, and in PLE their concentrations decreased with temperature. Pressure and ultrasound had positive influence on the recovery of target compounds. The solvent type in PLE affected the extraction of bioactive compounds from blackberry waste. The optimized conditions for SFE were 60 ^{o}C, 15 MPa and 200 W, and for PLE were 100 ^{o}C, ethanol+water 50% v/v. The results for those conditions were, respectively: TP = 0.091 and 7.36 mg gallic acid equivalent/g residue; MA = 0 and 1.02 mg cianidin-3-glicoside equivalent/g residue; AA = 0.54 and 76.03 μmol trolox equivalent/g residue (DPPH) and 1.39 and 68.28 μmol trolox equivalent/g residue (ABTS), and global yield = 6.25 and 6.33%. From such results, it can be noted that both extraction techniques are good alternatives to extract bioactive compounds from blackberry residues.

INTRODUCTION

Blackberry (*Rubus spp.)*, a native fruit from the northern hemisphere, is one of the small berries most rich in antioxidants already studied, being thus a valuable source of

bioactive compounds. Besides its consumption as fresh fruit, it is industrially used in the fabrication of juices and other products [1]. However, the processing of blackberries generates around 10% of solid residue (bagasse) that is composed mainly by peel and seeds, and still contains a great percentage of phytochemicals of the fruit. The recovery of this residue as raw material for the processing of new food products is of great economic interest, and represents an important segment in industries, since it adds values to the byproduct and reduces the impact of its disposal on nature [2].

The recovery of phytochemicals from solid residues has been reported using conventional and alternative techniques. Conventional methods usually dispend much time, and may degrade target compounds during the extraction. Moreover, they require high amounts of organic solvents (such as ethanol, methanol, acetone, chloroform) that, in some cases, are hazardous to health and environment, needing to be separated from the extract and discarded properly. Therefore the techniques of supercritical CO_2 extraction (SFE) and pressurized liquid extraction (PLE) appear as alternatives to the extraction and purification of natural products, through clean methods and with the possibility of adjusting the selectivity to specific compounds by tuning process parameters. Both techniques allow fast extraction of the target compounds in closed systems under high pressure, and high temperatures in PLE. These conditions enhance the solubility of bioactive compounds in the chosen solvents, and their extraction kinetics from the solid substrates, improving the efficiency and yield of the extractions [3, 4, 5].

Based on such information, this work had the objective to obtain extracts rich in phenolic compounds from blackberry bagasse using SFE assisted by ultrasound and PLE as extraction techniques.

MATERIALS AND METHODS

SFE assisted by ultrasound

The SFE experiments were performed in a homemade unit composed by the following equipments: an extraction cell of 300 mL that supports pressures up to 45 MPa; an ultrasonic probe with 13 mm diameter titanium end, coupled to a transducer; compressor; pneumatic pump; cooling and heating baths to control temperature of the solvent throughout the process; a flow meter; thermocouples and pressure gauges; and block and micrometer valves.

The SFE experiments were performed in duplicates and evaluated based on a Box-Behnken design with three variables (temperature, pressure, and ultrasound power) and three levels: temperature (40, 50 and 60 °C), pressure (15, 20 and 25 MPa), and power (0, 200 and 400 W). In all conditions the SFE time (120 min) and CO_2 flow rate (2.77 x 10^{-4} kg/s) were kept constant.

PLE

The PLE extractions were performed in a homemade unit composed by the following parts: a stainless stell extraction cell of 100 mL with metal filter in the outlet; an electric heat jacket used to keep extraction temperature at the set value; a HPLC pump; block and micrometer valves; thermocouples and pressure gauges.

The PLE experiments were done at three temperatures (60, 80 and 100 °C) and four different solvents (water, ethanol, water+ethanol 50% v/v and acidified water pH = 2.5). All other process conditions were kept constant (pressure = 7.5 MPa, extraction time of 30 min, solvent to substrate mass ratio = 18), totalizing 12 different conditions that were performed in duplicates.

Analyses of the extracts
Both SFE and PLE extracts were evaluated in terms of the global extraction yield (X_o) and their chemical composition (total phenolic content (TP), monomeric anthocyanins (MA), and antioxidant activity (AA) measured by the methods DPPH and ABTS)

Global extraction yield. X_o was determined as the ratio between total extracted mass and blackberry bagasse mass fed, according to Equation (1):

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

Total phenolic content. TP was determined thorough the spectrophotometric method of Folin-Ciocalteau, described by Singleton and Rossi [6], with some adaptations to vegetal extracts, suggested by Singleton et al. [7]. All the assays were performed in duplicates and the results were expressed in mg gallic acid equivalent (GAE)/g residue.

Monomeric anthocyanins. MA were quantified by the differential pH method described by Giusti and Wrolstad [8]. The results are expressed in mg cyanidin 3-O-glucoside/g residue. All the experiments were performed in duplicates.

Antioxidant activity. AA was determined by the methods DPPH and ABTS. All the assays were performed in duplicates, and the results are expressed in μmol Trolox equivalent (TE)/g residue. In the DPPH (1,1-diphenyl-2-picrylhydrazyl) method the capacity of the antioxidant compounds to sequestrate the stable radical DPPH• was determined according to the method described by Brand-Williams et al.[9]. For the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) method, the antioxidant capacity of the extracts was analyzed against the radical ABTS+•, as reported by Re et al. [10].

RESULTS

Tables 1 and 2 show the results obtained at the different conditions using PLE and SFE with and without ultrasound, respectively. As can be observed, the subcritical extracts are richer than the supercritical ones in antioxidant components. This happens because these compounds usually have more affinity with polar solvents, such as water and ethanol, than CO_2, which is nonpolar. One can also note that anthocyanins were not recovered in the SFE extracts, and their concentration was reduced in PLE when temperature increased, indicating that high temperatures must have degraded part of the anthocyanins. Regarding the other results (X_0, TP and AA), the increase of temperature lead to better yields. Moreover, the solvent type in PLE also affected the extraction of phenolics and anthocyanins. In Table 1, a clear correlation between TP and AA can be noted. Since blackberries are reported as having high contents of phenolics compounds with antioxidant activity, one can presume that AA of the extracts is due to those phenolics, but not to anthocyanins, which appear in lower concentrations even in extracts with high AA.
In SFE, ultrasound and pressure had positive influence on the recovery of the target compounds, increasing the extraction rate and yield. The best condition for SFE was 60 ºC, 15 MPa and 200 W, and for PLE it was 100 ºC, with water+ethanol 50% v/v. At these conditions, the response variables were, respectively: TP = 0.09 and 7.36 GAE/g residue; MA = 0 and 1.02 cyanidin 3-O-glucoside/g residue; DPPH = 0.54 and 76.03

μmol TE/g residue; ABTS = 1.39 and 68.28 μmol TE/g residue; and X_0 = 6.25% and 6.33%.

Excepting the absence of anthocyanins in the SFE extracts, all the other conditions provided good recoveries in antioxidants, phenolics and anthocyanins, indicating that SFE and PLE may be feasible techniques to obtain bioactive compounds from fruit residues. Indeed, the solvents used in PLE can be used in SFE, as modifiers to enhance the extraction of polar components. Water and ethanol mixed to supercritical CO_2 at ratios below 10% usually change the solvation power significantly.

Table 1. Extraction conditions for PLE. Results for global yield, total phenolics, monomeric anthocyanins and antioxidant activity.

					AA[d]	
T (ºC)	**Solvent**	**X_0[a]**	**TP[b]**	**MA[c]**	**DPPH**	**ABTS**
60	Water	3.56 ± 0.71	2.39 ± 0.21	0.88 ± 0.01	14.25 ± 0.74	21.26 ± 0.74
60	Water pH 2,5	12.10 ± 0.49	1.93 ± 0.21	0.95 ± 0.08	12.25 ± 0.21	31.14 ± 6.35
60	Ethanol	3.23 ± 0.08	3.18 ± 0.62	1.25 ± 0.03	24.33 ± 4.40	32.04 ± 6.75
60	Ethanol (50%)	3.85 ± 0.37	5.23 ± 0.83	1.40 ± 0.02	37.04 ± 2.80	49.24 ± 3.32
80	Water	4.16 ± 1.47	3.78 ± 0.03	0.79 ± 0.08	33.22 ± 0.08	36.46 ± 0.72
80	Water pH 2,5	14.27 ± 0.37	4.46 ± 0.25	0.99 ± 0.05	36.30 ± 0.78	43.97 ± 0.79
80	Ethanol	4.23 ± 0.28	3.72 ± 0.60	1.39 ± 0.02	31.40 ± 4.38	31.48 ± 5.18
80	Ethanol (50%)	5.19 ± 0.08	5.51 ± 0.80	1.08 ± 0.21	46.38 ± 1.28	52.10 ± 3.32
100	Water	6.39 ± 0.36	4.97 ± 0.51	0.65 ± 0.10	42.85 ± 5.45	42.79 ± 6.70
100	Water pH 2,5	14.99 ± 0.05	5.34 ± 0.46	0.38 ± 0.04	40.40 ± 3.34	51.29 ± 1.94
100	Ethanol	4.46 ± 0.24	4.12 ± 0.23	0.93 ± 0.03	36.61 ± 0.24	31.07 ± 4.60
100	Ethanol (50%)	6.33 ± 0.04	7.36 ± 0.18	1.02 ± 0.11	76.03 ± 1.05	68.28 ± 2.68

* Results expressed by mean ± standard deviation.

[a] Global extraction yield expressed in percentage (100*g/g residue).

[b] Total phenolic content expressed in gallic acid equivalents (GAE) per gram of residue.

[c] Content of monomeric anthocyanins expressed in cyanidin- 3 O-glucoside equivalent (C3GE) per gram of residue.

[d] Antioxidant activity expressed in μmoL Trolox equivalent (TE) per gram of residue.

Table 2. Extraction conditions for: (A) low pressure extractions, (B) SFE extraction with and without ultrasound, (C) SFE-US with cosolvents. Results for global yield, total phenolics, monomeric anthocyanins and antioxidant activity.

(A) Low pressure extractions

Method	Sample	Solvent	Temperature (°C)	Pressure (MPa)	US Power (W)	X_0 (*) (%)	MA (*)	TP (*)	AA (*) DPPH	AA (*) ABTS
Soxhlet	Dried and crushed	Petroleum ether	50	-	-	11.35±0.58[a]	-	5.60±0.02[a]	52.60±3.94[b]	62.13±0.29[a]
Soxhlet	Dried and crushed	Ethanol	50	-	-	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	-	-	-	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]

(B) SFE assisted by ultrasound

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

(C) SFE-US with cosolvents

Method	Sample	Cosolvent	Temperature (°C)	Pressure (MPa)	US Power (W)	X_0 (*) (%)	MA (*)	TPC (*)	AA (*) DPPH	AA (*) 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:água / 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 extract); TPC = Phenolic content (mg EAG/g extract); AA = Antioxidant activity expressed as Trolox equivalent µmol TE/ g extract). (*)Letters equal in the same column indicate that there is no significant difference at the level of 5% by the Tukey test.

CONCLUSION

This work has shown that blackberry bagasse is a good source of bioactive compounds, as phenolics and anthocyanins, which can generate products with high antioxidant activity to be applied as food ingredients and cosmetics. Environmentally clean techniques, such as SFE and PLE, are feasible alternatives to the extraction of valuable compounds from blackberry bagasse, as well as from wastes from many other food processes. Taking into account the differences between the SFE and PLE extracts, both techniques can be explored as sequential procedures in the same production unit, in order to obtain two or more types of extracts, with different properties. Moreover, ultrasound has proved to enhance SFE, and could be also tested in PLE.

The advantages of SFE and PLE, with ultrasound, can be explored together, by means of SFE with CO_2 and water and/or ethanol as modifiers, to improve the recovery of bioactive compounds. Moreover, future investigations should be addressed in order to optimize the purification of the extracts, using supercritical fluid processing or other clean technologies.

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