EXTRACTION OF PHENOLIC COMPOUNDS AND ANTHOCYANINS FROM BLUEBERRY (*Vaccinium myrtillus* L.) RESIDUES USING SUPERCRITICAL CO_2

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ABSTRACT

Blueberries are considered the fruit with the highest antioxidant and polyphenol content, which is present in both peel and pulp. Supercritical fluid extraction (SFE) was applied to recover bioactive compounds from blueberry residues, which are usually discarded by industries after juice processing. SFE was performed with pure CO_2 and with ethanol and/or water as cosolvents. The extracts were evaluated in terms of yield, antioxidant activity, phenolics and anthocyanins. The SFE extractions with cosolvents showed good results in the condition of 90% CO_2, 5% water and 5% ethanol in terms of phenolic compounds, antioxidant capacity, and anthocyanins. The extract obtained from freeze-dried waste showed higher concentrations of phenolic compounds, anthocyanins and antioxidant activity, since the concentration of the mentioned compounds occurred in the freeze-drying process. Moreover, the target compounds are more concentrated in the peel than in the fruit pulp. The results of antioxidant activity and anthocyanins for the evaluated materials (fresh, oven-dried, and freeze-dried blueberry waste, and fresh blueberries) were similar or higher than those found in the literature, which can be explained by the difference of varieties. Sixteen anthocyanins were identified by Ultra Performance Liquid Cromathography (UPLC). The concentrations of anthocyanins by UPLC were lower than in the differential pH method.

INTRODUCTION

The blueberry fruit is rich in phenolic compounds, such as anthocyanins, which are antioxidant substances that help preventing degenerative diseases. Thus, blueberry is known as the "longevity fruit" and is used in food industries for the manufacture of juices and other products. In the group of small fruits that also covers strawberry, raspberry, and blackberry, blueberry is known as the most antioxidant-rich fresh fruit already studied, having a high content of polyphenols in both the peel and pulp. The method of supercritical fluid extraction (SFE) is gaining more space as alternative for the extraction from natural matter. In the food industry the advantages of the extracts obtained by this process are their natural origin, absence of residual organic solvent and possible use of mild temperatures.

This work aimed to recover phenolic compounds, antioxidants and anthocyanins from blueberry residues through SFE and added cosolvents, since the recovery of such allows the use of this waste, contributing to add value to this product and to minimize the negative impacts caused by its direct disposal in the environment.

MATERIALS AND METHODS

Raw material

A unique lot of 10 kg of blueberry (varieties Clímax, Bluegen, and Flórida) residues was purchased to prevent variations on lots during extractions. The material was composed of blueberry peel, seeds, and pulp. Part of the blueberry waste was submitted to freeze-drying in a bench top liophilizer at -42 ^{o}C (L101-LioTop/Liobrás) and another part was dried in an oven (Fanem, 320). A third part of the raw material was kept in its natural form, without drying. After drying, the products were packed in plastic containers and stored at -18 °C.

Characterization of blueberry waste and extracts: total phenolic content (TP), antioxidant activity (AA), anthocyanins by differential pH method (MA) and quantification of anthocyanins by Ultra Performance Liquid Cromathography (UPLC).

The blueberry residue was subjected to chemical characterization: soluble solids (Brix) by the titulometric method, acidity, pH, vitamin C, moisture [1], total polyphenols, antioxidant activity, and anthocyanins by differential pH method. The identification and quantification of the anthocyanins were performed by Ultra Performance Liquid Cromathography (UPLC) in Department of Analytical Chemistry of the University of Cádiz, Spain.

The TP content was determined spectrophotometrically using the Folin-Ciocalteu method, according to the methodology proposed by Singleton et al. [2]. AA was determined using two methods: 1) DPPH free radical sequestration (1,1-diphenyl-2-picrilhidrazil), following the methodology described by Brand-Williams et al. [3] and Mensor et al. [4], and 2) the free radical ABTS capture method, according to the procedure described by Rufino et al. [5]. MA was determined through the monomer pH differential methodology described by Giusti&Wrolstad [6]. Anthocyanins were identified by UPLC coupled to quadrupole-time-of-flight mass spectrometry (Q-ToF-MS) (Synapt G2, Waters Corp., Milford, MA, USA).

Supercritical fluid extraction (SFE)

SFE tests were conducted in an extraction unit built at the Laboratory of High Pressure in Food Engineering (LAPEA/DEA-FEA/Unicamp, Brazil), detailed in Figure 1, according to procedures determined by Pascual-Martí et al. [7] and equipment limitations. The SFE unit is composed of an extraction cell of 300 mL, supporting pressures up to 45 MPa. The evaluated SFE pressures were 15, 20 and 25 MPa, at 40 ^{o}C. After selecting the best pressure conditions in terms of the extract's quality, SFE was performed using pure CO_2 as solvent, and also with water and ethanol as cosolvents. All the performed SFE conditions are reported in Table 2.

The analyses of TP, AA, MA, and UPLC described in the previous section were also performed on the SFE extracts obtained at various conditions.

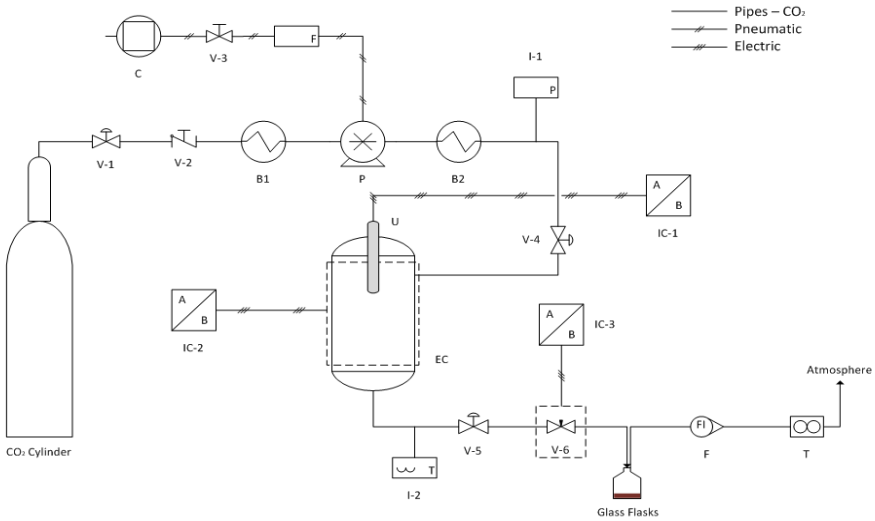

Figure 1. Diagram of the SFE unit with carbon dioxide; V-1, V-2, V-3, V-4, V-5 e V-6 – Control valves; V-6 – Micrometer valve; C- Compressor; F- Compressed air filter; B1 –Cooling bath; P- Pump; B2 – Heating bath; I-1 e I-2 – Pressure and temperature indicators, respectively; IC-1, IC-2 e IC-3 – Indicators and controllers of ultrasound power, temperature of extraction column and temperature of micrometer valve, respectively; EC – Extraction column ; U – Ultrasound probe; F – Rotameter; T –Flow meter.

RESULTS

Characterization of raw material

Physical and chemical characteristics of blueberry waste

Fruit soluble solids varied from 11.8° to 14°, acidity from 0.76%to 1.28%, and pH from 2.92 to 3.20. The moisture of the fresh residue (83.57%) is very close to that of fresh fruit, which is of 87.68%, reported by Silveira [8]. The drying of blueberry waste achieved a water reduction of around 70%, thus the freeze-dried and oven-dried sample's moistures were of 11.25% and 11.32%, respectively.

Total phenolic content, antioxidant activity, anthocyanins

Table 1. Phenolic content, antioxidant activity and anthocyanins in fresh, oven-dried and freeze-dried blueberry residues.

	TP (mg GAE*/g)	AA (DPPH) (µmol TE*/g)	AA (ABTS) (µmol TE*/g)	MAs (mg/100g)
Freeze-dried	57 ± 2	1284 ± 2	49.8 ± 0.7	301 ± 29
Oven-dried	11.7 ± 0.6	1082 ± 0.7	27 ± 1	134 ± 10
Fresh	66.5 ± 0.4	446.0 ± 0.7	17 ± 1	175 ± 17

*GAE- Gallic acid equivalent

It can be observed that the dry samples have higher TP than the fresh sample. This is due to an increase in the concentration of these compounds during the drying process. For the oven-dried sample, TP decreases when compared to the freeze-dried. This can be due to the loss of these compounds during heating [9]. The results for AA of fresh

blueberry residue measured by DPPH is half of that found by Reque [10], which reported 919.21 µmol TE/g in the residue, and 480.84 µmol TE/g in the blueberry juice. This difference can be explained by the variety used in each study, or even by the amount of juice in the residue. For the freeze-dried product, the AA increased 2.8 times. The result found in the fresh residue determined by ABTS is about three times larger than that found by Vendruscolo et al [11], which can be explained by differences in variety and crop. For the freeze-dried product, the AA increased 65%. In the oven-dried sample the value is lower than the freeze-dried sample, which can be explained by losses due to heating during the drying process. Regarding the anthocyanin content, rhe results for fresh blueberry residue were close to that found by White et al [12], who reported 121.4-362.5 mg anthocyanins/100g extract. These results were also reported by Reque [10], which found 375.48 mg/100 g, showing delfinidin (Df) as the major anthocyaninin in blueberry residues. For the freeze-dried samples the MA was about two times higher, and for the oven-dried sample the amount decreased to the amount of fresh sample.

Supercritical CO_2 extraction (SFE)

Preliminary extractions were performed from the fresh sample, fixing temperature at 40 °C and CO_2 flow rate in 1.4 x 10^{-4} kg/s. The extraction yields were 1.84%, 1.96% and 2.19% for pressures of 15, 20 and 25 MPa, respectively. The choice of the best condition of pressure and flow rate took into account the results of the chemical analyses performed on the extracts. The results justify the choice of pressure of 20 MPa, temperature of 40° C and the CO_2 flow rate of 1.4 x 10^{-4} kg/s for the SFEexperiments with cosolvents.

SFEwith cosolvents from fresh blueberryresidue

The results of the analyses of the extracts obtained by SFE with cosolvents are shown in Table 2. SFE with 50% acidified water as cosolvent achieved the highest yield. Seabra et al. [13] studied the SFE of elderberry pomace and found different yields using CO_2/ethanol/water, and took the same conclusions about the cosolvent proportions. They found lower yields (1.7%) in the proportions of cosolvents 90% CO_2/8% ethanol/2% water and higher yield (21.3%) in the proportion of 20% CO_2/40% etanol/40% water. SFE with 90% CO_2, 5% water and 5% ethanol gave satisfactory results in terms of the target components for all analyses. The combination of CO_2, water, and ethanol is more efficient in solubilization of phenolics, because ethanol is a bipolar molecule, thereby increasing the solubility of both nonpolar and polar compounds. Seabra et al. [13] also found the highest levels of phenolic compounds, extraction yields, and concentration of elderberry anthocyanins with combinations of CO_2, ethanol and water. The extract obtained with 50% acidified water showed lower antioxidant activity for both DPPH and ABTS analyses. This indicates that phenolic compounds contribute in the AA of the extracts analyzed, since they also presented the lowest value at such condition. Phenolic compounds are the main responsible for antioxidant activity in fruits. The presence of water as cosolvent was important to promote the extraction of antioxidants by increasing the solvent's polarity, and thus enhancing the recovery of aglycones, flavones, and polar flavonols. Regarding anthocyanins, the lowest concentrations were found in extracts obtained with ethanol as cosolvent, without water. The presence of water as cosolvent is important to increase the extraction of anthocyanins, which have high solubility in water, according to Metivier et al. [14].

Table 2. Global yield, total phenolics, antioxidant activity, and anthocyanins of SFE extracts from blueberry residues and fresh blueberries.

Sample	Solvents	P (MPa)	Yield (%)	TP (mg GAE/g)	AA (DPPH) (µmol TE/g)	AA (ABTS) (µmol TE/g)	MA (pH method) (mg/100 g)	Anthocyanins (UPLC) (mg/100 g)
Fresh residue	100% CO_2	15	-	36 ± 0.2^{b}	590 ± 0.4^{b}	13 ± 0.5^{b}	209 ± 4.7^{b}	-
	100% CO_2	20	-	41 ± 2^{a}	688 ± 0.8^{a}	14 ± 1.3^{a}	265 ± 8^{a}	-
	100% CO_2	25	-	28 ± 1.8^{c}	514 ± 0.2^{c}	10 ± 0.2^{c}	178 ± 9^{c}	-
	90% CO_2 + 10% H_2O	20	5.3 ± 1^{cd}	65 ± 4^{d}	1422 ± 11^{abc}	129 ± 1^{cd}	420 ± 31^{c}	343 ± 8.3
	50% CO_2 + 50% H_2O	20	8.4 ± 0.5^{b}	48 ± 4^{de}	1188 ± 116^{cd}	114 ± 11^{de}	291 ± 27^{cde}	228 ± 9.6
	90% CO_2 + 10% acidified H_2O (pH=2.0)	20	2.7 ± 0^{e}	46 ± 0.4^{de}	808 ± 77^{ef}	87 ± 7^{ef}	326 ± 12^{cd}	216 ± 0.3
	50% CO_2 + 50% acidified H_2O (pH=2.0)	20	16 ± 2^{a}	33 ± 3^{e}	639 ± 49^{f}	74 ± 7^{f}	291 ± 26^{cde}	164 ± 0.5
	90% CO_2 + 10% ethanol	20	3.3 ± 0.3^{de}	109 ± 9^{b}	1083 ± 103^{de}	89 ± 4^{ef}	134 ± 2^{e}	124 ± 88
	50% CO_2 + 50% ethanol	20	4.7 ± 0.5^{cd}	119 ± 2.4^{ab}	1141 ± 111^{cde}	97 ± 6^{def}	136 ± 1.4^{e}	123 ± 00
	50% CO_2 + 40% H_2O + 10% ethanol	20	6.9 ± 0.6^{bc}	62 ± 0.3^{d}	1292 ± 23^{bcd}	116 ± 11^{de}	214 ± 15^{de}	205 ± 0.1
	90% CO_2 + 5% H_2O + 5% ethanol	20	2.7 ± 0.3^{e}	134 ± 11^{a}	1658 ± 160^{a}	199 ± 20^{a}	1071 ± 64^{a}	808 ± 0.1
	90% CO_2 + 8% acidified H_2O (pH=2.0) + 2% ethanol	20	3.5 ± 0.1^{cde}	59 ± 5^{d}	1602 ± 42^{ab}	185 ± 9^{ab}	716 ± 65^{b}	674 ± 0.5
	95% CO_2 + 4% acidified H_2O (pH=2.0) + 1% ethanol	20	4.6 ± 0.4^{de}	88 ± 4^{c}	1604 ± 111^{ab}	159 ± 7^{bc}	709 ± 69^{b}	533 ± 0.3
Fresh residue (wet basis)	90% CO_2 + 5% H_2O + 5% ethanol	20	2.7 ± 0.3^{e}	3.6 ± 11^{a}	44.8 ± 160^{a}	5.4 ± 20^{a}	28.9 ± 64^{a}	21.8 ± 0.1
Freeze-dried residue (wet basis)		20	7.6 ± 0.8^{a}	3.5 ± 4^{a}	35 ± 19^{a}	1.3 ± 1^{b}	16.5 ± 111^{a}	14.2 ± 0.7
Fresh blueberry		20	3 ± 0.2^{b}	71 ± 6^{b}	949 ± 1^{b}	202 ± 1.8^{a}	305 ± 20^{b}	282 ± 3.3

Average values of duplicates. Different letters in the same column represent significant differences ($p < 0.05$). AA – Antioxidant activity; TP – Total phenolics; GAE - Gallic acid equivalent; TE – Trolox equivalent

SFE with cosolvents of fresh and freeze-dried blueberry residue

The SFE extract obtained from fresh blueberry residue with 5% water and 5% ethanol as cosolvents showed the highest concentrations of TP, AA and MA among the tested conditions. Therefore, these cosolvent ratios were repeated in SFE from freeze-dried residue and fresh blueberries. Table 2 shows the yield, AA, MA and TP of these extractions. Considering all results in wet basis, the results for the freeze-dried residue were lower than for fresh residue. From this comparison, it can be stated that freeze-drying leads to the loss of target compounds. Morover, the target compounds are more concentrated in the peel than in the pulp, so the residues were expected to provide higher concentrations. Differences in the concentration of phenolic compounds are usual in cultivars of the same fruit, as reported by Malacrida and Motta [15], who noted that the variety of grape used in juice processing can be a cause of variation in the levels of phenolic compounds. This can be explained by the difference in varieties. It is also important to highlight thatthe recovery of anthocyanins in the extracts were higher than in the raw material, due to the concentration of these compounds in the extraction process.

Analysis of anthocyanins by UPLC

Sixteen anthocyanins were identified, evidencing the great complexity of the composition of extracts from bluberry wastes, and reinforcing the importance of using this byproduct in novel formulations. The sixteen anthocyanins identified in the blueberry residue are delphinidin 3-O-galactoside, delphinidin 3-O-glucoside, cyanidin 3-O-galactoside, delphinidin 3-O-arabinoside, cyanidin 3-O-glucoside, petunidin 3-O-galactoside, cyanidin 3-O-arabinoside, petunidin 3-O-glucoside, peonidin 3-O-galactoside, petunidin 3-O-arabinoside, peonidin 3-O-glucoside, malvidin 3-O-galactoside, peonidin 3-O-arabinoside, malvidin 3-O-glucoside, malvidin 3-O-arabinoside and malvidin 3-O-xyloside. Among these, the major anthocyanin was cyanidin 3-O-glucoside. All of these anthocyanins were simultaneously identified in the raw materials and blueberry extracts obtained by SFE.

Table 2 presents the quantification of total anthocyanins by UPLC-UV-Vis in blueberry residues and their extracts obtained by SFE. It can be noted that the anthocyanin contents obtained by the differential pH method are higher than those of UPLC-UV-Vis. This happens because the diferential pH method overestimates the amount of anthocyanins, due to the possible presence of other substances that interfere in the absorbance of the extracts, as stated by Gouvea [16] in the quantification of anthocyanins in açai. Cho et al. [17] found concentrationsof anthocyanins between 140 and 823 mg/100g in analyses performed by HPLC. This variation is due to different varieties of fruit, but the values were mostly near 140mg/100g, confirming the results found in the raw material. Gao and Mazza [18] found lower contents of anthocyanins (from 109.0 to 112.0 mg/100g), which can be attributed to differences in environmental, harvest, and fruit ripening conditions. Bunea et al [19] found values of anthocyanins ranging from 101 to 195 mg/100g in different blueberry varieties. Prior et al. [20] reported a minor amount of anthocyanins in wild blueberry varieties compared to cultivated blueberries. In the workof Bunea et al. [19] profiles of all varieties showed that the major anthocyanin is delphinidin, followed by malvidin and petunidin. Gouvea [16] also determined the anthocyanin content of cranberry extract, which has the cyanidin 3-O-glucoside anthocyanin in higher concentration, the majority as recommended by the differential pH method. Comparing the values, the author noted that for this extract, the results of the analysis by pH differential and HPLC have the same order of magnitude and similar values, since the quantification performed by the

first methodology provides values of total monomeric anthocyanin, that is both majoritarian as well as other lower concentration are quantified, while the second method gives the sum of the values of concentration of major anthocyanins, noting that both techniques are efficient for the quantification of anthocyanins in extracts of blueberry, confirming the results obtained in this study. The concentration of anthocyanins in the extract from the freeze-dried residue is lower than those of fresh blueberry in wet basis, confirming that some anthocyanins could have been degraded in the freeze-drying process.

CONCLUSIONS

SFE extractions with cosolvents showed good results in the condition of 90% CO_2, 5% water and 5% ethanol in terms of phenolic compounds, antioxidant acrtivity, and anthocyanins. The extracts obtained with acidified water showed lower amount of antioxidants and phenolics. The extract from the residues showed higher concentrations of phenolic compounds, anthocyanins and antioxidant activity than the fresh fruit, indicating that the compounds of interest are more concentrated in the peel than in the fruit pulp. The results of antioxidant activity and anthocyanins for evaluated materials were considered similar or higher than those found in the literature, which can be explained by the difference of varieties. The results of quantifications of anthocyanins by UPLC were lower when comparing with differential pH method. The recovery of bioactive compounds from blueberry residues shows that valuable products can be developed from this and other fruit processing byproducts, giving background to more investigation on supercritical fluid technologies applied to food residues.

REFERENCES

[1] A.O.A.C., Association of Official Analytical Chemists., Arlington, **1998.**
[2] SINGLETON, V. L., ORTHOFER, R., LAMUELA-RAVENTOS, R. M., Methods in Enzymology, Vol. 299, **1999**, p. 152.
[3] BRAND-WILLIAMS, W., CUVELIER, M. E., BERSET, C., Food Science and Technology, Vol. 28, **1995**, p. 25.
[4] MENSOR, L. L., MENEZES, F. S., LEITÃO, G. G., REIS, A. S., SANTOS, T. C. D., COUBE, C. S., LEITÃO, S. G., Phytotherapy Research, Vol. 15, **2001**, p. 127.
[5] RUFINO, M. S. M., ALVES, R. E., DE BRITO, E. S., PEREZ JIMENEZ, J., SAURA-CALIXTO, F., MANCINI-FILHO, J., Food Chemistry, Vol. 121, **2010**, p. 996
[6] GIUSTI, M.M., WROLSTAD, R. E., Biochemical Engeneering Journal, Vol. 14, **2003**, p. 227.
[7] PASCUAL-MARTI´, M. C., SALVADOR, A., CHAFER, A., BERNA, A.,Valencia, Spain, **2001**.
[8] SILVEIRA, N. G. A. da, VARGAS, P. N., ROSA, C. S. da, Alim. Nutr., Vol. 18, **2007**, p. 365.
[9] SEVERO, J., GALARÇA, S. P., AIRES, .R. F., CANTILLANO, R. F. F., ROMBALDI, C. V., SILVA, J. A., Braz. J. Food Technol., II SSA, **2009**.
[10] REQUE, P. M., Master thesis, Federal University of Rio Grande do Sul, Brazil, **2012.**
[11] VENDRUSCOLO, J. L. M., SILVA, R., TORALLES, R., Braz. Journal of Food Tech., **2011**.
[12] WHITE, B. L., HOWARD, L. R., PRIOR, R. L., Food Chemistry, Vol. 58, **2010**, p.4030.
[13] SEABRA, I. J., ELGA, M. A., BRAGA, M., BATISTA, M. T., SOUSA, H. C. de, The Journal of Supercritical Fluids, **2010**, p. 145.
[14] METIVIER, R. P.; FRANCIS, F. J., CLYDESDALE, F. M., Journal of Food Science, Vol. 45, **1980**, p. 1099.
[15] MALACRIDA, C., R.; MOTTA, S., Sci Food Technology, Vol. 25, **2005**, p. 659.
[16] GOUVÊA, A.C.M.S., Masther Thesis, Federal University of Rio de Janeiro, Brazil, **2010**.
[17] CHO, M. J., HOWARD, L. R., PRIOR, R. L., CLARK, J. R., J Sci Food Agric., Vol. 84, **2004**, p. 1771.
[18] GAO, L., MAZZA, G., Journal of Food Science, Vol. 59, **1994**, p. 1057.
[19] BUNEA, A., RUGINA, D., SCONT, Z., POP, R. M., PINTEA, A., SOCACIU, C., TABARAN, F., GROOTAERT, C., STRUIJS, K., VANCAMP, J., Phytochemistry, Vol. 95, **2013**, p. 436.

[20] PRIOR, R. L., CAO, G., MARTIN, A., SOFIC, E., MCEWEN, J., O'BRIEN, C., LISCHNER, N., EHLENFELDT, M., KALT, W., KREWER, G., MAINLAND, C. M., J. Agric. Food Chem., Vol. 46, **1998**, p. 2686.