EXTRACTION OF PHENOLIC COMPOUNDS FROM BLUEBERRY (*Vaccinium myrtillus L.*) RESIDUES USING SUPERCRITICAL CO_2 and PRESSURIZED WATER

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Abstract. This work explored the advantages of using subcritical water and supercritical carbon dioxide in the recovery of extracts containing phenolic compounds produced naturally in plants, from the residues of the blueberry (*Vaccinium myrtillus L.*). Supercritical CO_2 and pressurized water are alternatives to the use of toxic organic solvents or methods that apply high temperatures. Belonging to the group of small fruits, blueberries are considered the fruit with the highest antioxidant and polyphenol content, which is present in both peel and pulp. Resveratrol can be used in food and pharmaceutical industries due to its positive biological effects on the human body. Experiments were performed testing the lyophilized and fresh sample, since the compounds of interest present both nonpolar and polar characteristics. Therefore, the water content in the raw material was used to enhance the extraction, working as a co-solvent. The blueberry residues were donated by a juice industry from southern Brazil. Next, the influence of process parameters, such as temperature and pressure was evaluated in the supercritical extraction with CO_2 and the study of the behavior of various solvents under various conditions in the pressurized liquid extraction (PLE). The response variables were the global yield, composition and activity of the extracts. Moisture content was determined in the raw material. Antioxidant activity of the residues and extracts was measured using the methods: DPPH and ABTS. Total phenolic compounds were also determined. Anthocyanins were quantified by pH differential method.

Keywords: extraction, antioxidant, blueberry

1. Introduction

Blueberry is a tree species native from the northern hemisphere, belonging to the genus Vaccinium and family Ericaceae. Its fruit is rich in phenolic compounds, such as antocianic pigments, which are antioxidant substances that help prevention of degenerative diseases. Because of this, blueberry is known as "longevity fruit" and is used in food industries for the manufacture of juices and other products. In the group of small fruits that covers, among others, crops of strawberry, raspberry, blueberry and blackberry, the blueberry is classified as the most antioxidant-rich fresh fruit already studied, having a high content of polyphenols in both peel and pulp. Their availability, versatility, and variety of forms during almost all the year allow the blueberry to be embedded in a wide variety of formulations [1].

Flavonoids accumulate in the bark and leaves of the plants because its synthesis is stimulated by light. This may explain the possible difference in composition between fruits of the same plant, since the fruits that receive larger amounts of light tend to have a pronounced synthesis of these compounds [2]. First, it has been shown that resveratrol acts as fitoalexin, an antibiotic synthesized when the plant is subjected to stress, as the attack by pathogens, UV radiation or injury [3]. The second reason for the great interest of researchers in resveratrol is the possible benefits for human health, mainly due to its antioxidant properties and the decrease

in the incidence of cardiovascular disorders [3, 4, 5]. Phytochemical compounds recovery from solid wastes has been reported using conventional and alternative technologies. As an example of the first: Soxhlet extraction, extraction by maceration, extraction by infusion and vapor distillation; and second: supercritical fluid extraction (SFE), pressurized liquid extraction (PLE) and pressurized fluid extraction assisted with ultrasound. Extraction with supercritical fluids is gaining more space. In the food industry the great advantages of extracts obtained by this process are their natural origin, absence of residual organic solvent, and composition monitored by process selectivity. This trend of food industries is due to consumers that are increasingly health-conscious, driving the industry to look forward disease prevention [6]. The technique of pressurized liquid extraction (PLE), which is also known as accelerated solvent extraction (ASE), emerged as an alternative to the extraction and fractionation of natural products, through a clean technology and with the possibility of adjusting parameters to the selectivity of the process to a group of compounds to be extracted, which is a good option to add value to by-products of processing industry of blueberry. PLE using water as solvent is one of the most interesting methods, since water is non-toxic, non-flammable, environmentally safe and inexpensive [7, 8, 9]. PLE allows the rapid extraction of analytes in a closed and inert environment under high pressure and temperature. A great advantage of PLE over conventional extraction methods conducted at atmospheric pressure is that high pressure solvents remain in the liquid state, even when subjected to temperatures well above their boiling points, allowing, thus, working at high temperatures. These conditions enhance the solubility of analytes in the solvent and the desorption kinetics from solid matrices [10]. This work aimed to recover phenolic compounds from blueberry wastes through environmentally safe techniques, since their isolation 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.

2. Materials and methods

2.1. Raw material

A unique lot of 10 kg of raw material was purchased to prevent variations on lots during all extractions. The material was acquired from "Orgânicos Pérolas da Terra", an industry located in southern Brazil. Part of the blueberry waste was submitted to freeze-drying in bench top freezer at - 42 ^{o}C (L101-LioTop/Liobrás) with time ranging from 48 to 72 hours. Another part was dried in an oven (Fanem, 320), ranging from 4 to 5 days at 40 °C. After drying, samples were ground in knife mill in order to blend them and reduce the resistance to mass transfer during the later extraction steps. After this step, the dried products were packed in plastic containers and stored at -18° C.

2.2. Characterization of the sample (residue)

The residue was subjected to chemical characterization by performing the following analyses: soluble solids by the titulometric method, acidity, pH, vitamin C, moisture [11], total polyphenols through the colorimetric method of Singleton & Rossi [12], antioxidant activity by DPPH and ABTS and anthocyanins by differential pH method. The same analyses were performed on the freeze-dried and oven-dried samples, excepting Brix, acidity, pH and vitamin C. The freeze-dried, oven-driedand fresh samples were always kept protected from light.

2.3. Total phenols content

The determination of total polyphenols was carried out through the Folin-Ciocalteu method, according to the procedure of Singleton et al. [13] and expressed in gallic acid equivalent (GAE)/g. The standard curve of gallic acid (99% >, Vetec, Brazil) was built according to the following procedure: a 1 mL aliquot of the extract was removed appropriately, diluted in ethanol or aqueous standard solutions of gallic acid (99% >, Vetec, Brazil, batch 0806387) (0-100 mg/L) and transferred to a 25 mL volumetric flask, containing 9 mL of water. The Folin-Ciocalteu reagent (1 mL) (Dynamic, Brazil) was added, and the mixture stirred. After 5 minutes, 10 mL of a 7% Na_2CO_3 (Reagents Carlo Erba, Italy) solution was added and the volume was completed with water. After 90 minutes at 23º C in the absence of light, the absorbance was measured at 750 nm in a spectrophotometer (UV-VIS 40 lambda, Perkin Elmer, USA). The reference solution used as blank in the spectrophotometer was prepared through the same way, with 1 mL of ultrapure water (Milli-Q). The

standard curve was obtained from tests in triplicate.

For the measurement of total phenolics in samples of dry extracts, these were initially diluted in ethanol (purity 99.5% v/v, Synth, Brazil) in proportion (20 mg/mL ethanol) from the diluted extract prepared aqueous dilution, taking proper amount and deposited in a 5 ml volumetric flask, made up to volume with ultrapure water (Milli-Q). The same procedure was followed as described above, if the absorbance value obtained remained within the range of the standard curve absorbance. All experiments were conducted in triplicate.

2.4 Antioxidant Activity (AA)

The antioxidant activity was assessed using the method of DPPH free radical sequestration (2.2 diphenyl-1-picrilhidrazil), following the methodology described by Brand-Williams *et al.* [14] and Mensor *et al.* [15] and by free radical ABTS capture according to the methodology described by Rufino *et al.* [16].

2.5. Anthocyanins by differential pH method

The determination of the total anthocyanin content was performed by monomer pH differential methodology described by Giusti & Wrolstad [17]. Filtration with filter paper was used to remove suspended solids and make possible to determine the abosrbance of the solution.

2.6. Supercritical Extraction (SFE)

SFE experiments were carried out in the unit described in Figure 1, which operates up to a maximum pressure of 35 MPa and maximum solvent flow rate of 1.0×10^{-4} kg/s. The extraction bed has 3.41 cm of internal diameter and 46 cm long. This unit consists of a high-pressure pump for solvent (Thermo Separation Products, model 2000, Florida, USA); two programmable thermostatic baths (PolyScience, Niles, 9510 model USA and Marconi, model MA-184, Piracicaba, SP, Brazil), responsible for the maintenance of stainless steel extractor temperature and CO_2 pump heads, respectively; a flow totalizer (LAO, model G 0.6 ± 0.001 m3, São Paulo, SP); thermocouples; and three pressure gauges (Record, (50.0 ± 0.5) MPa, São Paulo, SP). A column of perforated teflon equivalent to about 70% of the volume of the extractor may be inserted at the base of the same volume, decreasing the pressurizing. This unit can operate with the capacity of 415 cm^3 [18].

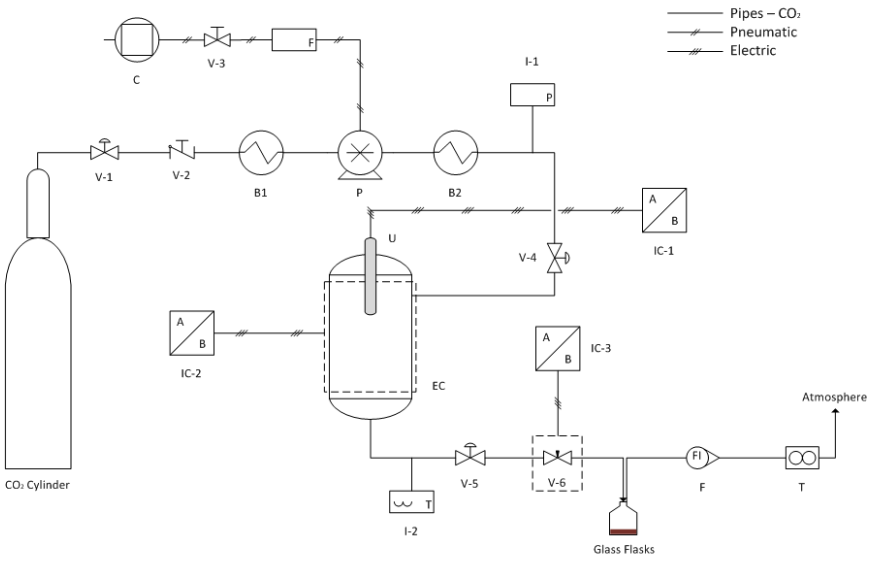

Figure. 1. Diagram of the supercritical extraction unit with carbon dioxide; V-1, V-2, V-3, V-4, V-5 e V-6 – Control valves; V-6 – Micrometer valve; C- Compressor; F- Compressed air filter; B1 –Cooling bath; P- Pump; B2 – Heating bath; I-1 e I-2 – Pressure and temperature indicators, respectively; IC-1, 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.

2.7. Pressurized liquid extraction (PLE)

The extraction unit used in PLE experiments is composed of three different extraction cell volumes (5, 50 or 100 mL) coated by electric heating; a reservoir to supply the solvent; a HPLC pump, which operates with flow rates in the range of 0.001-10.0 mL/min; a pressure gauge; a shut-off valve that controls the flow of solvent; a temperature indicator; a back-pressure valve to control the pressure and a collection flask. This unit works in temperature and pressure ranges of 25 – 180° C and 0.5 - 40 MPa, respectively. Figure 2 shows the schematic diagram of this home-made PLE unit. Figure 3 shows the unit, which was assembled in the Laboratory of Supercritical Technology, Extraction, Fractionation and Identification of Plant Extracts (LASEFI-DEA/FEA-Unicamp).

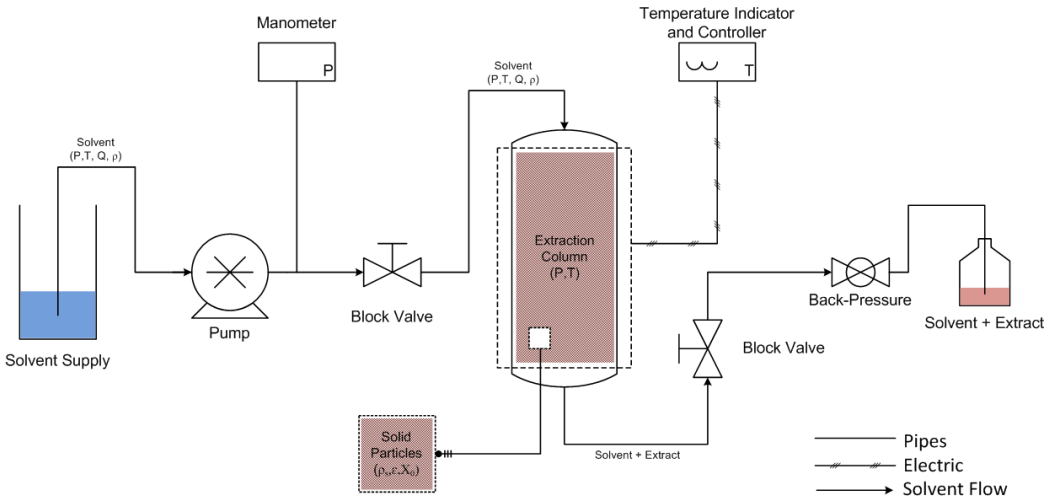

Figure 2 – Schematic diagram of the home-made pressurized liquid extraction unit. P – Pressure (MPa); T – Temperature (°C); Q - Flow rate solvent (mL/min); ρ – solvent density (kg/m³); ρ_s – solid particles density (kg/m³); ε - particles solids porosity (ad.); X_0 – Solid particles global yield.

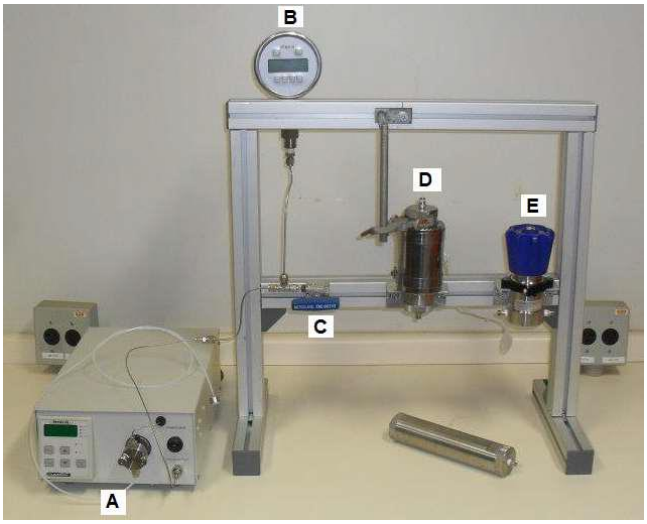

Figure 3 - PLE unit: A - HPLC pump; B - Gauge; C - shut-off valve; D - extraction cell with heating; E - back pressure valve.

2.8. Extraction conditions of SFE and PLE

SFE was performed with CO_2 at 40° C, solvent flow rate of 3 L/minute and pressures of 15, 20, 25 and 30 MPa. The extractions were performed in a cell of 50 ml, using 50 g of fresh sample. The PLE extractions were performed by fixing the temperature, pressure and flow rate at 40° C, 20 MPa and 10 mL/min, respectively, and varying the solvent composition. The extractions were performed in a 5 ml cell and in duplicate. The feed mass was of 20 g for fresh, and 4g for freeze-dried sample. The conditions of temperature and pressure have been set considering data from Pascual-Martí *et al.* [19] and limitations of the equipment. Table 1 shows the performed extraction conditions.

Table 1: PLE conditions

Extraction	Fresh sample	Extraction	Freeze dried sample
1	100% ethanol	6	100% ethanol
2	50% ethanol and 50% water	7	50% ethanol and 50% water
3	100% acid water (pH: 2.0)	8	100% acid water (pH: 2.0)
4	50% ethanol and 50% acidified water (pH: 2.0)	9	50% ethanol and 50% acidified water (pH: 2.0)
5	100% acetone		

2.9 Soxhlet Extraction

Blueberry residue samples were submitted to Soxhlet extraction in accordance with the methodology described by Institute Adolfo Lutz [20]. About 5 g of raw material and 150 mL of solvent were introduced in a Soxhlet extractor. Hexane (96%, Merck, Sao Paulo, Brazil) and methanol (96%, Merck, Sao Paulo, Brazil) were used as solvents at their boiling points during 6 hours. Soxhlet was performed as conventional extraction technique in order to compare the yield and the quality of extracts with those found in extractions by PLE and SFE.

3. Results and discussion

3.1. Physico-chemical characteristics of raw material

The physico-chemical characteristics of the blueberry waste are presented in Table 2.

Table 2. Physico-chemical characteristics of blueberry waste

Quality attributes	Results
ºBrix (soluble solids)	10.33
Acidity (% citric acid)	0.48
Ratio (brix/acidity ratio)	21.2
pH	3.33
Vitamin C (mg/100g)	74.75

It is observed that in the blueberry residue is the physico-chemical characteristics are similar to those of fresh fruit, described by Redies [21]. Fruit soluble solids vary from 11.8 to 14, acidity from 0.76 to 1.28, and pH from 2.92 to 3.20.

3.2. Moisture

The fresh residue's moisture is very close (83.57%) to that of fresh fruit, which is of 87.68%, reported by Silveira [22]. Dry waste achieved a reduction of around 70% water, thus the freeze-dried and oven-dried sample's moistures were of11.25% and 11.32%, respectively.

3.3. Total phenolic content

Table 3 presents the results of phenolic compounds obtained for the blueberry wastes.

Table 3 Phenolic compounds in fresh, oven-dried and freeze-dried raw material

	Phenolic compounds results (mg GAE*/g)
Freeze-dried	65.52
Oven-dried	56.59
Fresh	10.36

*GAE- Gallic acid equivalent

One can observe that the dried samples have higher amounts of phenolic compounds than the fresh sample. This is due to an increase in the concentration of these compounds during the freeze-drying process. For the oven-dried sample, the amount of phenolic compounds decreases when compared to freeze-dried. This is due to the loss of these compounds during heating [23]. Heat loss can occur because flavonoids accumulate in the bark and leaves of the plants, sincetheir synthesis is stimulated by light. This may explain the possible difference in composition between fruits of a same plant, that is, the fruits that receive a larger amount of light tend to have a pronounced synthesis of these compounds [2].

3.4. Antioxidant Activity

DPPH (Determination of Total antioxidant activity by capturing the free Radical DPPH) The result of the antioxidant activity of fresh blueberry residue showed 446 µmol TE/g. This result is half of that found by Reque [24], 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 amount increased 2.8 times, resulting in 1284.41 µmol TE/g. The same occurred with the oven-dried sample, which was of 1082.15 µmol TE/g.

ABTS (Determination of total antioxidant activity by radical ABTS capture The antioxidant activity of fresh blueberry residue determined by ABTS was of 17.7 µmol TE/g. This result is about 3 times larger than that found by Vendruscolo [25], and can be explained by differences in variety and crop. For the freeze-dried product, the quantity increased 65 %, resulting in 49.84 µmol TE/g. In the oven-dried sample, the antioxidant activity was 26.7 µmol TE/g. This value is lower than the freeze-dried sample, which can be explained by heat losses during the drying process.

3.5. Anthocyanins

The concentration of anthocyanins in the extracts was determined by the differential pH method, which is based on structural transformations of anthocyanins as a function of pH generating colored solutions. The flavilic cation, of red colour, is the predominant form in pH 1.0 while the carbinol, colorless, predominates at pH 4.5. So, following this method are made spectrophotometric measurements of anthocyanins in solutions of pH 1.0 and 4.5, at a wavelength around 500-550 nm (maximum absorption of anthocyanins) and 700 nm, to correct any errors related to the scattering of light, since the colloidal suspensions may be extracts. The fresh sample presented anthocyanin content of 338.99 mg/100 g, which was close to that found by White et al [26], who reported 121, 4 - 362.5 mg/100 g of anthocyanins.

These results were also reported by Reque [24], which found 375.48 mg/100 g, showing the majority the anthocyanin Df (Delfinidin) in residues of blueberry. For the freeze-dried samples the amount of anthocyanins was about 4 times larger, 1348 mg/100 g, and for the oven-dried sample the amount decreased 3 times compared to the amount of fresh sample, achieving 1104 mg/100 g. It is important to note that, even with heating, the oven drying did not degradate anthocyanins present in the extract, because the temperature used in this procedure was 40° C, which is not aggressive to those compounds.

3.6. Soxhlet Extraction

For comparison, conventional extractions were performed with the Soxhlet method. Mixtures of freeze-dried blueberry+hexane, fresh residue +hexane, freeze-dried+methanol, oven-dried+methanol, and fresh+methanol were used.

The extraction with hexane was not efficient, with no extract recovery for any sample. With methanol, extraction occurred with both samples, resulting in extracts with properties exposed in Table 4. Methanol was removed using a rotatory evaporator (Heidolph, model Laborota 4001, Viertrieb, Germany) under vacuum (Heidolph, model Rotavac/Rotavac control, Viertrieb, Germany). The overall yields of the extractions with methanol were 57% in the oven-dried sample, 67.8% in the freeze-dried sample, and 33.66% in the fresh sample.

Analyzing the results in Table 4, it can be affirmed that the extraction was efficient, and in all the analyses, the results were higher than those found in their raw materials. With the extraction, the recovery of antioxidants was higher.

Table 4. Results of the analyses of total phenolics, DPPH, ABTS and antioxidant activity of anthocyanins in the Soxhlet extracted samples.

	Total Phenolics (mg GAE*/g)	DPPH (µmol TE*/g)	ABTS (µmol TE*/g)	Anthocyanins (mg/100g)
Freeze-dried	95.25	1898.20	80.5	1724.16
Oven-dried	90.07	1701.93	79.7	868.34
Fresh	29.36	309.41	69.0	642.91

*GAE- gallic acid equivalent *TE –Trolox equivalent

3.7. Pressurized Liquid extraction (PLE)

PLE usually requires less time (extraction time varies from 5 to 30 minutes) and less consumption of solvents that standard techniques [27]. Therefore, the PLE extractions were held in 15 minutes. Freeze-dried and fresh samples were used.The amount of fresh sample used in the extractions was 20 g and of freeze-dried sample was 4 g. The dried/fresh sample ratio was chosen considering dry basis. The use of solvents and their combinations were chosen based in previous works [28,29]. After extraction the extracts were evaporated at 30°C under vacuum to obtain pure extract.

Table 5. Results of the analyses of yield, total phenolics and yield of freeze-dried and fresh samples extracted by PLE

	Phenolics Total (mg GAE/g) and yield (%)					
Solvents	Experiment	Yield	Fresh	Experiment	Yield	Freeze-dried
100% ethanol	1	3.91	87.36	6	4.23	101.71
50% ethanol e 50% water	2	4.34	89.82	7	5.43	99.33
100% acidified water (pH 2,0)	3	6.77	80.58	8	7.98	64.37
50% acidified water (pH 2,0) 50% ethanol	4	5.79	73.23	9	5.23	83.86
100% acetone	5	4.01	59.65			

Note that the phenolic contents for both freeze-dried and fresh samples increased when compared to the raw material. Also, there was no difference in the amount of phenolic compounds between experiments of each sample, excepting the experiment with acetone, where the result was lower.

Table 6. Results of the analyses of antioxidant activity of extracts obtained by PLE from freeze-dried and fresh samples

DPPH and ABTS (µmol TE/g)

Solvents	Experiment	Fresh DPPH	Fresh ABTS	Experiment	Freeze fried DPPH	Freeze dried ABTS
100% ethanol	1	1622.85	73.17	6	1862.72	102.5
50% ethanol e 50% water	2	1632.99	65.34	7	1764.74	42.84
100% acidified water (pH 2,0)	3	643.12	30.83	8	1369.47	23.84
50% acidified water (pH 2,0) 50% ethanol	4	1531.64	72.50	9	1413.39	49.83
100% acetone	5	1315.42	62.50			

The antioxidant activities of the PLE extracts were higher that those of raw material for both fresh and freeze-dried sample. Comparing experiments, for the fresh sample only Experiment 3 performed below expectations, which can be explained by the low ability of water to recover nonpolar components. For both fresh and freeze-dried samples experiments with 100% ethanol, antioxidant activity measured by DPPH and ABTS were the highest. In these extracts, both polar and non-polar fractions are present, due to the chemical nature of the solvent. Therefore the extraction with ethanol promoted the extraction of more antioxidant compounds.

Table 7. Results of the analysis of anthocyanins of extracts obtained by PLE from freeze-dried and fresh samples

Anthocyanins (mg/100g)

Solvents	Experiment	Fresh	Experiment	Freeze dried
100% ethanol	1	1001.93	6	1870.28
50% ethanol e 50% water	2	1127.17	7	1895.32
100% acidified water (pH 2,0)	3	2220.95	8	2379.56
50% acidified water (pH 2,0) 50% ethanol	4	2621.72	9	2797.06
100% acetone	5	634.56		

Analyzing Table 7, one can observe that extraction by PLE is efficient in recovering anthocyanic components, since the anthocyanin contents of Experiments 3 and 4 were higher that that of the raw material. Where the conditions were favorable for extraction (acid water), the amount of extracted anthocyanins was 6.5 times larger. The best result was the combination of solvents in Experiment 4 (acidified water and ethanol), where the anthocyanin content increased 7.5 times compared to the raw material. As for the freeze-dried sample, the same happened for Experiments 8 and 9, but the increase was 1.7 and 2 times greater, respectively. The experiment with acetone was not efficient for the extraction of components anthocianins.

3.8. Supercritical CO_2 extraction (SFE)

Preliminary tests were conducted using extraction from the fresh sample, fixing the temperature at 40° C and the flow of CO_2 in 3 L/minute. First, SFE was tested at a pressure of 15 MPa. Under these conditions, after 20 minutes elapsed, no material was extracted. The same was repeated with the 30 MPa. At 25 MPa, the extraction was minimal and should be repeated in future studies. Subsequently, the extraction was performed under the same conditions, but at the pressure 20 MPa . In the first 5 minutes of extraction, the extract began to leave, along with water. This water comes from the fresh product, which presents more than 80% moisture. It is important to mention that, in SFE with CO_2, the water present in the sample behaves as a co-solvent, helping to extract nonpolar compounds that cannot be recovered with pure CO_2. The extract was then rotaevaporated under vaccum to remove water. The results of the SFE analyses are presented in Table 8.

Table 8. Results of the analyses of total phenolics, DPPH, ABTS, antioxidant activity and yield of anthocyanins in SFE samples

	Yield (%)	Phenolics Total (mg GAE/g)	DPPH (µmol TE/g)	ABTS (µmol TE/g)	Anthocyanins (mg/100g)
Fresh sample	3.25	56.15	991.10	26.50	1085.83

Observing the results in Table 8, one can say that SFE obtained good extracts comparing to the conventional Soxhlet extraction. On the other side, compared with the PLE extracts, one can affirm that PLE with solvent combinations was more advantageous in terms of amount of phenolic compounds, antioxidant activity and anthocyans components. Further works in this sense shall be done, in order to evaluate the feasibility of SFE with water and/or ethanol as modifiers, as well as the use of ultrasound to enhance extraction rate and yield.

4. Conclusions

Pressurized liquid extraction is an efficient technique on the extraction of phenolics, antioxidants, and anthocyanins from blueberry wastes. The results of the analyses of experiments with Soxhlet extractions were important to compare the extracts and their results are worse than those of PLE and SFE. The performed SFE experiments are at a preliminary stage, but they already indicate that the water content of the sample works as a co-solvent, being useful to enhance the extraction of the target compounds.. Future SFE extractions with association of CO_2 and other solvents are needed to achieve more comprehensive results.

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