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Encapsulation of anthocyanin-rich extract from blackberry residues by spray-drying, freeze-drying and supercritical antisolvent

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ABSTRACT

The extract obtained from blackberry (*Rubus fruticosus*) residues was encapsulated in polyvinylpyrrolidone (PVP) by three methods: the conventional spray-drying (SD) and freeze-drying (FD) techniques, and the new method of supercritical antisolvent (SAS), using CO_2 as antisolvent and ethanol as solvent of the organic solution (extract + PVP). The methods and their produced particles were evaluated in terms of precipitation yield, residual ethanol and moisture contents, anthocyanin concentration, antioxidant capacity, morphology, crystallinity and thermal stability. SD,FD and SAS achieved particles with good anthocyanin yields (above 76%), high antioxidant capacity (above 100 μmol TE/g particle) and were effective to concentrate anthocyanins in PVP without great degradation. Using SAS, particles with 1.42 mg ECy3Gl/g were achieved. Nevertheless, SAS particles presented high residual ethanol (8.17% w/w) and moisture (11.30% w/w), whereas in SD and FD particles these contents remained below 2 and 5%, respectively. Scanning electron microscopy revealed a spherical shape in the particles obtained by SD, while those produced by SAS and FD presented irregular agglomerates. The encapsulation processes were equivalent in terms of thermal protection of the extracts and they did not modify the crystallinity and thermal behavior of PVP. The SAS process achieved preferential precipitation of anthocyanins when compared to SD and FD, since supercritical CO_2 does not have any affinity to such compounds.

1. Introduction

Anthocyanins are phenolic compounds belonging to the flavonoid group, and represent the most important natural pigments in vascular plants. They are non-toxic, soluble in water and responsible for the blue, purple, violet and red colors of flowers, vegetables, fruits and their processed products [1]. Anthocyanins correspond to the glycoside or acyl-glycoside of anthocyanidins (also known as aglicones) and are located in the cell's vacuole, mainly in the fruits' peel [2]. These phytochemical compounds have attracted great interest due to their potential use as natural dye and biological properties, such as antioxidant, anti-inflammatory, anti-carcinogenic, anti-mutagenic and chemo-protective [3].

In the recent years, many researchers have shown that residues obtained from the processing of small fruits, such as blackberry, blueberry, grape, apple, pomegranate and strawberry are rich sources of anthocyanins [4]. Thus, the extraction of anthocyanins and other bioactive compounds from these residues is a promising strategy to recover and add value to food industry by-products, since most of them are currently discarded. Moreover, it is a mean to reduce the negative impact of their direct disposal in nature [5]. Therefore, the process is interesting in both economic and environmental points of view.

The low stability of anthocyanins and other bioactive compounds during their extraction, formulation, purification and storage has been increasingly a subject of interest to different researchers, who are in search of new forms of processing with minimal degradation [6].

The encapsulation of natural substances presents several advantages over the natural substance itself. First, they acquire controlled release behavior and are able to maintain their stability for longer periods, since they are less susceptible to degradation when in liquid extract [6]. Second, bioactive compounds marketed in the form of powder require less storage space than those marketed in liquid form [7].

Spray-drying (SD) is among the most applied techniques to encapsulate anthocyanins, besides being used to dry 80–90% of the encapsulated products [8]. Freeze-drying (FD) is also efficient to encapsulate anthocyanins, since it provides a porous non-shrunk structure that is particularly useful for thermally sensitive compounds. However, both

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techniques have drawbacks [9]. SD is not indicated for thermally sensitive compounds and is limited in terms of the choice of coating materials, which could have too high viscosity at high concentrations. FD produces non-uniform particles, requires large processing times, consumes too much energy and produces open porous structures that may not be effective barriers between the target compounds and the environment [8].

Therefore, micronization techniques based on supercritical fluids, such as supercritical antisolvent (SAS) precipitation, have been proposed as alternatives to the mentioned conventional methods. The properties of supercritical carbon dioxide (SC-CO_2), which is versatile, inert, non-toxic, non-flammable, inexpensive, environmentally accepted and free from toxic residues in the final product, motivate its use as antisolvent. Moreover, the critical properties of CO_2(Tc = 31.1 °C and Pc = 7.38 MPa) are moderate when compared to other possible supercritical antisolvents, thus preventing thermal degradation and reducing operation costs [10]. Literature reports applications of SAS to precipitate and encapsulate several materials [11–13]. However, most of the works based on SAS employed toxic and environmentally unfriendly solvents, such as dichloromethane, dimethyl sulfoxide, ethyl acetate and acetone [14,15], because of their affinity with SC-CO_2. Nevertheless, these solvents are unsuitable for applications in food or pharmaceutical products.

The present work had the goal to encapsulate the extract obtained from blackberry (*Rubus fruticosus*) residues, a rich source of anthocyanins [4,16], in polyvinylpyrrolidone (PVP), using SAS with SC-CO_2 as antisolvent and ethanol as GRAS (Generally Recognized as Safe) solvent for the mixture of extract and PVP. Encapsulation should enhance the retention of anthocyanins in the particle, thus protecting these pigments from conditions and contributing to their application in food, pharmaceutics and cosmetics. To assess the effectivity of SAS, the conventional methods SD and FD were applied for comparison.

2. Materials and methods

This work was mostly performed in the Laboratory of High Pressure in Food Engineering (LAPEA/DEA/FEA/UNICAMP – Campinas/SP/Brazil) and in the Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA/UNICAMP – Paulínia/SP/Brazil). The chromatographic analyses were performed in the Department of Analytical Chemistry of the University of Cadiz, Spain.

2.1. Chemicals

Polyvinylpyrrolidone (PVP) (MW = 10,000 g/mol) (Sigma Aldrich Chemical Co., St. Louis, MO, USA) was chosen as a core material. Anthocyanin standard (cyanidin chloride), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) and potassium persulfate were also purchased fromSigma-Aldrich. Absolute ethanol, citric acid, glacial acetic acid, trihydrated sodium acetate, potassium chloride were purchased from Synth (São Paulo, SP, Brazil). Hexahydrated ferric chloride was purchased from Dinâmica (São Paulo, SP,Brazil) and carbon dioxide (99.9% pure) from White Martins Gases Industriais (Campinas, SP, Brazil). HPLC-grade methanol and formic acid, used in UHPLC, were purchased from Merck (Darmstadt, Germany) e Panreac (Barcelona, Spain), respectively. Ultrapure water was prepared in a purification system (Milli-Q, Millipore, Bedford, USA).

2.2. Preparation of blackberry residue

The residue obtained from industrial processing of blackberry was acquired from Sítio do Bello, a fruit processing company located in Paraibuna-SP, Brazil (23° 23′ 10″ S, 45° 39′ 44″ W). The residue was collected and stored in 5 kg plastic bags under freezing (−78 °C at Sítio do Bello and −18 °C at LAPEA) until lyophilization.

The fresh blackberry residue had moisture, Brix and pH of 30.65%, 8.60% and 3.38, respectively, and was composed of peel, seeds and edible pulp. It was lyophilized in a bench freeze dryer (Liotop, L101, São Paulo, SP,Brazil) and then ground in a domestic blender for 15 s, to homogenize and reduce particle size before extraction. Table 1 shows the proximate composition and the mean particle diameter (d_p) of the dried and ground residue prior to extraction.

It is worth mentioning that drying the residue was necessary, since SC-CO_2 used as antisolvent in SAS has very low affinity to water. Thus, the excess of water would reduce the drying efficiency and the particle formation.

2.3. Preparation of extract and polymeric solution

Anthocyanins were extracted by mechanical stirring for 90 min in a shaker (Marconi, MA140/CFT, Piracicaba-SP, Brazil), using 15 g of dried and ground blackberry residue and 350 mL of acidified ethanol (pH 3.0 adjusted by direct addition of citric acid). In acid media anthocyanins remain in their most stable form, cation flavilium, and have their extraction enhanced [17]. After extraction, samples were filtered (MF-Millipore™, pore size 0.45 μm) under vacuum and the liquid was recovered and stored at 4–6 °C until further use. The residue and solvent amounts were defined in order to achieve an extract with 1.50% total solids (*w*/*v*).

For the encapsulation, PVP was dissolved in the ethanolic extract to achieve a PVP:dry extract mass ratio of 5:2. The choice of PVP as core material was based on solubility tests performed with several polymers in ethanol and anthocyanin-rich extract, and also on some SD and SAS precipitation tests, besides the fact that PVO is a GRAS polymer with various medical, pharmaceutical and food applications [18].

2.4. Characterization of extract and PVP solution

2.4.1. Total solids and pH

The total solid content (TS) of the extract with and without PVP was determined gravimetrically, by drying *ca.*6.0 mL of extract in oven (FANEM, Mod. 315-SE, São Paulo, Brazil) at 70 °C until constant weight. TS was expressed in percentage (% - g total solids/100 mL extract).

The pH of the extracts with and without PVP was determined in a potentiometer (QUIMIS®, Mod.Q400AS, Brazil) that enables direct reading [19].

2.4.2. Monomeric anthocyanins

The monomeric anthocyanin content (MA) was determined using the differential pH method [20], with some modifications. First, the samples were diluted in two different buffer solutions: potassium chloride pH 1.0 (0.025 M) and sodium acetate pH 4.5 (0.40 M), both adjusted with concentrated HCl. The solutions' absorbances were measured at 510 and 700 nm in a spectrophotometer (Hach - Mod. DR/4000, Loveland, USA), which was calibrated using the buffer solutions. The samples were diluted to achieve absorbances from 0.100 to 0.900. MA was calculated with Eq.(1) and expressed as mg

Table1
Proximate composition and particle diameter of the lyophilized blackberry residue.

Analysis	Mean ± SD	Method
Moisture (%)	2.74 ± 0.93	AOAC (2002) [22]
Proteins (%)	9.83 ± 0.15	AOAC (2002) [22]
Lipids (%)	0.87 ± 0.01	Bligh and Dyer (1959) [23]
Ash (%)	0.51 ± 0.01	AOAC (2002) [22]
Fiber + Carbohydrates (%)	86.05 ± 0.24	By difference
d_p (μm)	638.95 ± 3.59	ASAE (1998) [22]

- d_p: particle mean diameter.
- Percentage (%) in dry basis
- Results expressed as mean ± standard deviation (SD).

cyanidin-3-*O*-glicoside equivalent (ECy3Gl)/g dry sample (ds).

$$MA = \frac{A\ x\ MM\ x\ FD\ x\ 1000}{\varepsilon\ x\ 1} \quad (1)$$

where:

MA = Monomeric anthocyanin content (mg ECy3Gl/g ds);
A = $(Abs_{510} - Abs_{700})_{pH1.0} - (Abs_{510} - Abs_{700})_{pH4.5}$;
MM = 449.2 g/mol – molecular mass of cyanidin-3-*O*-glucoside;
FD = dilution factor, (FD = 5);
1000 = conversion factor from g to mg;
ε = 26,900 L/cm.mol – molar absorptivity of cyanidin-3-*O*-glucoside;
l = optical path in cm.

2.4.3. Quantification of anthocyanins by UHPLC-UV–Vis

The anthocyanins identified in the extract were quantified in an ultra-performance liquid chromatography system (Elite UPLC LaChrom, VWR Hitachi, Tokio, Japan) consisting of an automatic sample injector (L-2200U), a column oven (L-2300), a pump (L-2160) and a UV–Vis detector (L-2420U). The chromatographic conditions were those described by Machado et al. [21]. Calibration curves were plotted within the 0 to 55 mg/L (ppm) range for each identified anthocyanin. Results were expressed as mg anthocyanin/g ds.

2.4.4. Antioxidant capacity

The antioxidant capacity (AC) of the extracts was determined through the DPPH and FRAP methods, described as follow, and expressed as μmol Trolox equivalent (TE)/g ds.

2.4.4.1. DPPH. The capture of the free radical DPPH (1,1-diphenyl-2-picrylhydrazine) was evaluated according to Brand-Williams et al. (1995) [22]. In the dark, 0.1 mL of samples diluted in ethanol or Trolox standard solutions were mixed with 3.9 mL of ethanolic DPPH solution (60 μM). The mixtures were stirred in Vortex (Phoenix Luferco, AP56, Araraquara –SP, Brazil) and incubated in the dark for 30 min at room temperature. Sample absorbances were measured at 515 nm in a spectrophotometer (Hach, Mod.DR/4000, Loveland, USA), which was calibrated with ethanol. A calibration curve was plotted with Trolox concentrations from 50 to 1000 μM.

2.4.4.2. FRAP. The FRAP antioxidant capacity of the extracts was evaluated as described by Silva et al. (2013) [23], with some adaptations. The FRAP reagent was obtained from the mixture of sodium acetate buffer (0.3 M, pH 3.6), TPTZ(2,4,6-tris(2-pyridyl)-s-triazine) solution (10 mM) and ferric chloride (20 mM) in the proportion 10:1:1 (v:v:v). In the dark, 90 μL of the diluted samples or Trolox standard solutions were mixed with 270 μL of ultrapure water and 2.7 mL of FRAP reagent in tubes. Samples were homogenized in tube stirrer and heated in a bath (Novatecnica®, NT281, Brazil) at 37 °C. After 30 min their absorbance was read at 595 nm in spectrophotometer (Hach, Mod.DR/4000, Loveland, USA) calibrated with FRAP reagent. A calibration curve was plotted with Trolox concentrations from 50 to 100 μM.

2.4.5. Total reducing sugars

The total reducing sugar content (TRS) of the extracts was determined through the colorimetric methods described by Miller [24] and Nelson [25]. Two reagents were prepared: Somogyi-NelsonI (SN-I) and Somogyi-NelsonII (SN-II). 1.0 mL of the sample or standard was pipetted into an assay tube, where 2.0 mL of the SN-I reagent was added. The tubes were stirred and heated in boiling water for 6 min, and then 2.0 mL of SN-II was added, the mixture was stirred and let to rest for 5 min at room temperature. Finally, 25 mL of distilled water was added. The samples' absorbances were read at 540 nm in spectrophotometer (Hach, Mod. DR/4000, Loveland, USA). TRS was expressed as g glucose equivalent (GE)/g dry extract (de).

2.5. Encapsulation processes

2.5.1. Spray-drying (SD)

To produce particles through SD, the PVP solution (3.75% in ethanol) and the extract from blackberry residue containing PVP (5:2 – PVP:extract) were dried in a bench scale spray-dryer (Mini Spray-Dryer, BÜCHI B-290, Flawil, Switzerland). Compressed nitrogen was used as drying fluid at 0.5–0.8 MPa. The solutions were fed into the spray-dryer by a peristaltic pump and atomized through a 0.7 mm diameter injection nozzle. The solution and nitrogen flow rates were 3.0 mL/min and 536 L/h, respectively. The total volume of the injected solution was 200 mL and the inlet and outlet temperatures were 105 and 80 °C, respectively, controlled by PT-100 sensors. The resulting dry material was collected in a high performance cyclone separator and nitrogen was removed through an aspiration system with 90% vacuum. Absolute ethanol was aspersed to clean the spray-drier between the experiments, which were carried out in triplicate. The dry particles were collected, weighed and stored in the dark at −18 °C to prevent degradation before analyses, since particles were highly hygroscopic and could be degraded in desiccator. The operating conditions of the process were defined based on data published in the literature [26,27] and, mainly, on preliminary tests carried out in the equipment.

2.5.2. Freeze-drying (FD)

To produce dry particles through FD, the solutions were first evaporated (Marconi, MA-120, São Paulo, SP,Brazil) to completely remove ethanol. The samples were then suspended in water and frozen in glass recipients at −18 °C. Next, they were subjected to freeze-drying in a bench scale freeze-dryer (Liotop L101 (São Carlos, SP,Brazil). The process was performed under strong vacuum (≈20 μHg) and controlled room temperature (24 °C). The freeze-dried material was converted into a thin powder through maceration. The dry particles were then stored in the dark at −18 °C until the analyses. The experiments were performed in triplicate.

2.5.3. Supercritical antisolvent (SAS)

2.5.3.1. SAS equipment. The SAS encapsulation of anthocyanins was performed in the unit schematically presented in Fig. 1, which was designed and assembled in LAPEA. It is composed by a 723 mL stainless steel (AISI 316) jacketed precipitation cell (7.0 cm internal diameter x 18.8 cm height); two piping lines entering on the cell's top to supply CO_2 and organic solution at the work pressure and temperature; a pipeline leaving the cell's bottom to remove the CO_2 + solvent mixture; a pneumatic pump (Maximator®, MO37-S, Nordhausen, Germany) and a HPLC pump (Jasco, PU2080, Tokyo, Japan) to feed SC-CO_2 and the organic solution, respectively; a compressor to operate the pneumatic pump; an injection system coupled in the top of the cell, to spray the liquid solution into it; a stainless steel filter (1 μm pore size) on the cell's bottom, to collect the precipitated material; a vessel to collect the solvent and compounds removed by SC-CO_2; a gas totalizer to measure the total CO_2 volume used in each experiments; block valves; an electric-heated micrometer valve to control the antisolvent flow rate; safety valves; heating and cooling baths; and instruments to measure pressure and temperature. The pipes and connections were made of stainless steel with outer diameters of 1/4″, 1/8″ and 1/16″. Teflon gaskets were used to seal the precipitation cell.

2.5.3.2. Conditions and SAS operation. The SAS experiments were performed with pure PVP solution and with a solution containing extract from blackberry residue and PVP (1:2.5, w:w), using SC-CO_2 as antisolvent at the following conditions: temperature (T) = 40 °C; pressure (P) = 10 MPa; solution flow rate (Q_{sol}) = 0.5 mL/min; CO_2 flow

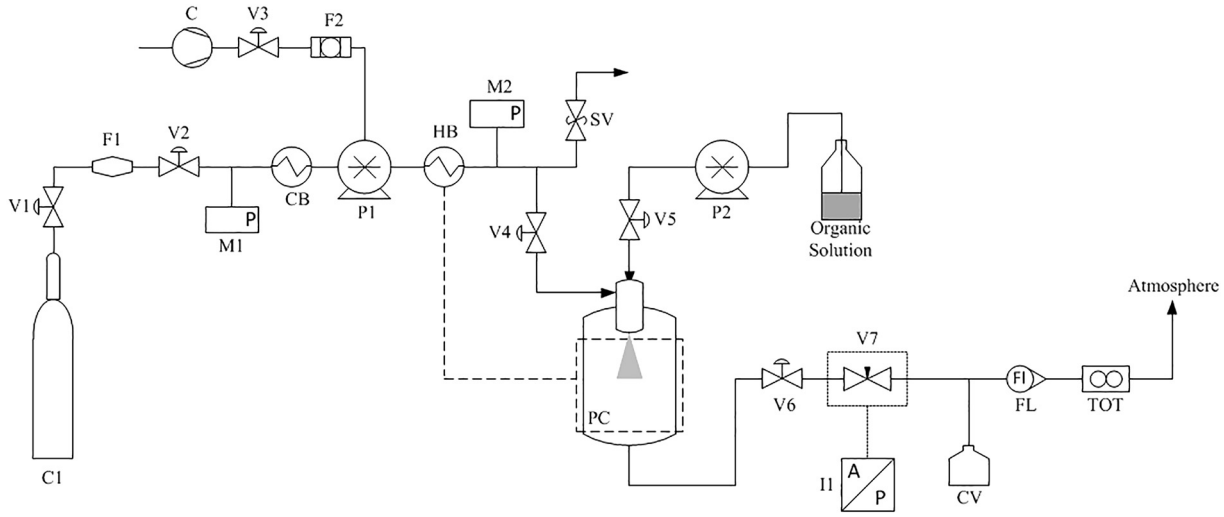

Fig. 1. Schematic diagram of the SAS unit. C1– CO_2 reservoir; F1– CO_2 filter; M1 and M2– pressure gauges; CB– cooling bath; C– compressor; V1, V2, V3, V4, V5 e V6– block valves; F2– compressed air filter; P1– pneumatic pump; HB– heating bath; P2– HPLC pump; SV– safety valve; PC– precipitation cell; V7– micrometer valve; I1– temperature indicator; CV– collect vessel; FL– flow meter; TOT– gas totalizer.

rate (Q_{CO2}) = 11.5 g/min; injected solution volume (V_{sol}) = 45 mL; removal time of ethanol from the particles (t_{rem}) = 75 min. These conditions were based on preliminary tests, limitations of the equipment and on the works of Reverchon et al. [28], Rossmann et al. [29] and Visentin et al. [11]. The experiments were performed in triplicate.

The following operation procedure was adopted: CO_2 was fed into the precipitation cell until work pressure and temperature were achieved. Once P,T and Q_{CO2} were stabilized, the organic solution was pumped into the cell at the defined flow rate through a coaxial nozzle with inner diameter of 0.007″ (0.178 mm) until V_{sol} was completed. Then, the system was maintained at the operation conditions for 75 min, to remove the residual solvent from the precipitated particles. Finally, the chamber was slowly decompressed for 75 min and opened. The particles were collected, weighed and stored in glass recipients in the dark at −18 °C until the analyses, to prevent degradation and water sorption.

2.6. Characterization of processes and particles

2.6.1. Global particle yield

The global particle yield (GPY) was defined as the mass ratio between the collected particles at the end of each process and the total solid amount in the organic solution.

2.6.2. Residual ethanol and moisture

The residual ethanol content (RE) of the particles was determined by the mass decrease of 0.5 g samples kept at 40 °C for 24 h. The particles' moisture was gravimetrically determined in a vacuum oven (Marconi, MA030/12, SP,Brazil) at 70 °C until constant weight was achieved [19].

2.6.3. Anthocyanin content and antioxidant capacity

The monomeric (MA) and individual anthocyanin contents and the antioxidant capacity (AC –DPPH and FRAP) of the particles were determined as described in Sections 4.2.4.2, 4.2.4.3 and 4.2.4.4, respectively. The quantifications were performed from the samples (around 50 mg) diluted in 10 mL of absolute ethanol.

2.6.4. Anthocyanin precipitation yield

The anthocyanin precipitation yield (APY) was defined as the mass ratio between encapsulated anthocyanins and the total monomeric anthocyanins contained in the feed solution.

2.6.5. Crystallographic structure

The crystallographic structure of the dry materials was analyzed by X-ray diffraction (XRD) in a diffractometer (Shimadzu, XRD-7000, Tokyo, Japan). Around 20 mg particles were placed on glass plates and inserted in the diffractometer. Analyses were carried out with X-ray radiation CuKα (λ = 1.54056 Å) with a 40 kV tension generator at angle variation (2θ) from 5° to 60° at a scan rate of 2°/min. Data were analyzed with the software OriginPro version 8.5 (OriginLab, Massachusetts, USA).

2.6.6. Thermal stability

The thermal stability of the particles was investigated in a thermogravimetric analyzer (TGA) (TA Instruments SDTQ600). Around 2–5 mg of dried particles were analyzed in a temperature range from 25 to 550 °C with a 10 °C/min heating rate under a synthetic air flow. Data were analyzed with the software OriginPro version 8.5 (OriginLab, Massachusetts, USA).

2.6.7. Glass transition temperature

The glass transition temperature (T_g) of the particles (2–5 mg) was determined using differential scanning calorimetry (DSC) in a calorimeter (TA Instruments, DSC-Q100, New Castle, USA) under argon flow. Thermal curves were obtained as follow: heating from 25 to 200 °C at 10 °C/min; isothermal for 2 min; cooling to 0 °C at 10 °C/min; isothermal for 2 min; heating to 200 °C at 10 °C/min.

2.6.8. Surface morphology

The particle surface morphology was analyzed in a scanning electron microscope equipped with a field emission gun (FESEM – FEI®Quanta 650, Hillsboro, USA). Samples were analyzed under vacuum at an acceleration voltage of 4 kV. Prior to imaging, samples were fixed on stubs using double-sided carbon adhesive tape and then coated with gold in a sputter coater (BAL-TEC, SCD 050, Balzers, Liechtenstein). A great number of images were obtained with different magnification at different areas of the samples, to ensure the reproducibility of the results.

2.7. Statistical analysis

Most analyses were performed in triplicate and their results were expressed as mean ± standard deviation. The results of the quantitative analyses (GPY, RE, MA, AC, APY, individual anthocyanins and moisture) were evaluated by Tukey's test, using the software Minitab version 16

Table 2
Characterization of the ethanolic extract of blackberry residue with and without PVP in terms of total solids, pH, total reducing sugars, monomeric and individual anthocyanins and antioxidant capacity, as determined by DPPH and FRAP.

Analysis	Extract	
	Without PVP	With PVP
TS (%)	1.50 ± 0.01	5.25 ± 0.02
pH	3.09 ± 0.06	4.70 ± 0.15
Spectrophotometric analyses		
TRS (g GE/g de)	0.69 ± 0.01	0.21 ± 0.01
MA (mg Cy3GlE/g de)	4.31 ± 0.06	1.05 ± 0.01
DPPH (μmol TE/g de)	331.64 ± 1.45	98.07 ± 0.83
FRAP (μmol TE/g de)	496.47 ± 1.85	120.68 ± 1.67
UHPLC analyses		
Cy3Gl (mg/g de)	3.62 ± 0.02	0.90 ± 0.02
Cy3Ru (mg/g de)	0.32 ± 0.01	0.04 ± 0.01
Cy3MG (mg/g de)	0.10 ± 0.01	0.03 ± 0.01
Cy3DG (mg/g de)	0.17 ± 0.01	0.04 ± 0.01
Total (mg/g de)	4.21 ± 0.03	1.01 ± 0.02

- TS: total solid content TRS: total reducing sugars; MA: monomeric anthocyanins; GE: glucose equivalent; de: dry extract; Cy3GlE: cyanidin-3-*O*-glucoside equivalent; TE: Trolox equivalent; Cy3Gl: cyanidin-3-*O*-glucoside; Cy3Ru: cyanidin-3-*O*-rutinoside; Cy3MG: cyanidin-3-*O*-malonyl-glucoside; Cy3DG: cyanidin-3-*O*-dioxalyl-glucoside.
- Results expressed as mean ± standard deviation.

(Minitab 16.1.0, Minitab Inc., State College, PA, USA). The adopted significance level was 5% (p-value < 0.05) and the Pearson correlation coefficient (r)was calculated using Microsoft Office Excel 2010 to verify the linear correlation between AC and MA, and among other different parameters analyzed to characterize the techniques SD,FD and SAS and the particles produced by them.

3. Results and discussion

3.1. Characterization of the extracts

As can be noted in Table 2, the mechanical agitation used to produce an ethanolic extract with 1.5% global yield from blackberry residue was efficient, achieving high anthocyanin content and antioxidant capacity. High MA and AC were also found in the extract of jussara by Carvalho et al. [30] using the same extraction method. Four anthocyanins were identified in the extracts: cyanidin-3-*O*-glucoside (Cy3Gl) and cyanidin-3-*O*-rutinoside (Cy3Ru) in higher amounts, and cyanidin-3-*O*-malonyl-glucoside (Cy3MG) and cyanidin-3-*O*-dioxalyl-glucoside (Cy3DG) in minor contents. The extract presented high TRS, which represented 69% of the total solids of the extract without PVP. This characterizes the extract as a material rich in simple carbohydrates [31,32], thus indicating its great complexity. It is also observed that the values of TRS,MA and AC were lower for the solution containing PVP than for the solution without PVP. This is the result of the addition of the PVP polymer, which leads to the dilution of the soluble solids present in the ethanolic extract.

An increase in the pH of the extract (initially pH = 3.09) is observed with the addition of PVP(pH = 7.0), resulting in a moderately acid solution with brown to red color. Color change is a consequence of the modifications in the chemical conformation of anthocyanin molecules. The pH exerts strong influence on anthocyanins' color and stability: at pH from 1.0 to 3.0, anthocyanins have red color with their most stable conformation, the flavilium cation, whereas at pH around 4.5 carbinol structure prevails and anthocyanins turn colorless. [17,33]. This explains the loss of red intensity in the extracts containing PVP.

3.2. Evaluation of SD, FD, SAS and particle composition

Table 3 presents the global particle yield, residual ethanol and moisture contents, monomeric anthocyanins, their precipitation yield and the antioxidant capacity of the particles obtained by SD,FD and SAS.

3.2.1. Global particle yield

As can be observed in Table 3, FD achieved the lowest loss of solids, followed by SD and SAS. In the latter processes, GPY is usually reduced due to particle adherence to the chamber wall and other surfaces of the equipment, dragging of particles by the drying fluid and, specifically in the SAS process, chemical affinity between the drying fluid and the target compounds. For instance, Villanueva-Bermejo et al. [13], in the precipitation of ethanolic corn extract by SAS, observed higher yields in the product recovered after the expansion of CO_2 than in the one collected in the precipitation cell. Visentin et al. [11] showed that low SAS yields can be obtained when small particles (<1 μm) are not retained in the filter at the cell's bottom. GPYs close to those obtained here were found by Devakate et al. [34], Maury et al. [35], Patel et al. [26], Haj-Ahmad et al. [27] and Osorio-Tobón et al. [36]. GPY near 100% is expected in FD, since this process and the equipment design make the loss of any compound, other than water and volatile compounds, almost impossible.

3.2.2. Residual ethanol and moisture

The residual ethanol and moisture contents of the particles produced by SAS were above twice those achieved in SD and FD, contradicting the results reported in other SAS processes. For instance, the particles obtained by SAS (integrated in the same line with pressurized liquid extraction) by Zabot and Meireles [37] contained RE and moisture of 2.0% and 3.6%, respectively. The high viscosity of the PVP solution may explain the results found in this work, since it affects negatively the process fluid dynamics [38], and therefore the mass transfer between ethanol and SC-CO_2 [39,40]. The ineffective drying of the solid precipitates explains the coalescence of SAS particles, observed in the SEM images (Fig. 2C). It is believed that a solution with lower polymer content can produce particles, *by* SAS, with more favorable physical characteristics. However, a lower ratio between the biopolymer and the active compound to be encapsulated may not ensure that the latter is well protected [41]. The temperature and flow rate of the organic solution have been shown to be a major influence on the residual solvent content and, consequently, on the degree of agglomeration of the

Table 3
Global particle yield (GPY), residual ethanol (RE) and moisture contents, monomeric anthocyanins (MA), their precipitation yield (APY) and the antioxidant capacity (DPPH or FRAP) of the particles obtained by SD, FD and SAS.

Samples	GPY (%)	RE (%)	Moisture (%)	MA (mg Cy3GlE/g dp)	APY (%)	Antioxidant capacity	
						DPPH (μmol TE/g dp)	FRAP (μmol TE/g dp)
SD	50.56 ± 0.79^{A}	2.10 ± 0.06^{A}	5.27 ± 0.01^{A}	1.36 ± 0.08^{A,B}	76.26 ± 2.82^{A}	109.52 ± 3.82^{A}	183.12 ± 1.28^{A}
FD	98.86 ± 0.13^{B}	0.95 ± 0.52^{A}	3.80 ± 0.83^{A}	1.05 ± 0.04^{A}	92.76 ± 1.20^{B}	113.60 ± 2.88^{A}	188.48 ± 2.75^{A}
SAS	63.23 ± 1.43^{C}	8.17 ± 0.02^{B}	11.03 ± 0.36^{B}	1.42 ± 0.07^{B}	77.53 ± 2.57^{A}	98.05 ± 3.69^{A}	154.93 ± 1.22^{B}

- dp: dry particle.
- Results expressed as mean ± standard deviation.
- Uppercase letters in the same column indicate no significant difference at 5% level according to Tukey's test.

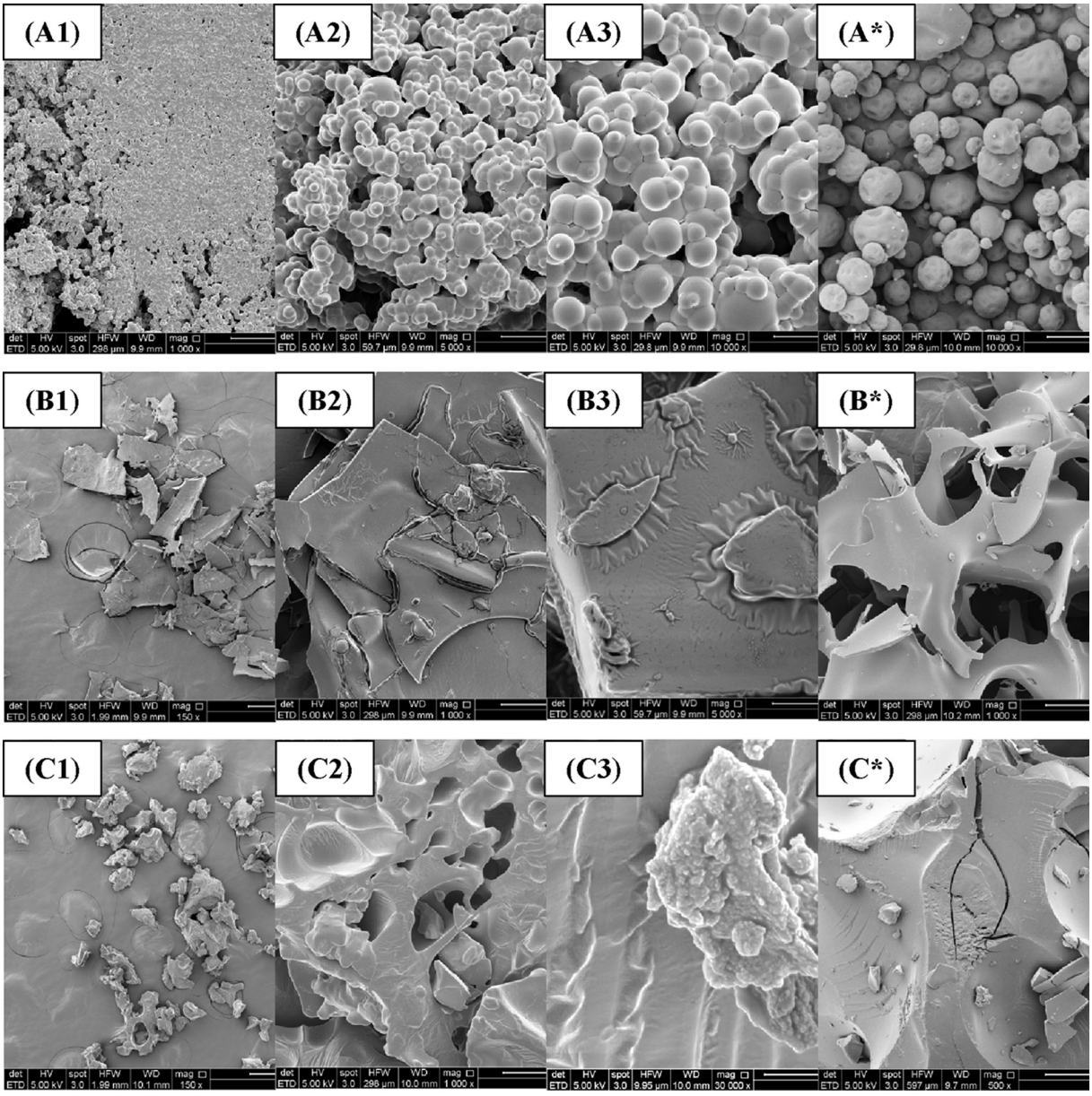

Fig. 2. FESEM images of the dry particles obtained by SD (A), FD (B)and SAS (C)with different magnifications. (*) refers to PVP particles obtained from solution without extract.

particles formed by the SAS technique [12,42]. According to Machado et al. [42] and Sosa et al. [12] higher temperatures and flow rates of the organic solution tend to increase the residual ethanol content in the particles and to form more agglomerated particles. Considering this, it can be noted that lower values of these operational parameters are required for the successful production of encapsulating systems by the SAS technique. Similar flow rate effect is also notable for the SD process [43], but in the case of the inlet temperature, researches have shown that its increase produces drier particles [9]. However, high temperatures tend to decrease the concentration of the encapsulated bioactive compounds, especially when they are thermosensitive [9,43]. In general terms, the results found in SD and FD, both well-known and founded processes, are in accord to those reported in literature for processing conditions similar to those applied in the present work [9,30].

3.2.3. Anthocyanins: content, profile and precipitation yield

As observed in Table 3, the SAS and SD processes did not differ significantly in terms of MA and APY. However, remarkable losses of these compounds initially contained in the feed solution are noted in both techniques. Anthocyanins are very sensible to adverse ambient and process conditions, such as light, oxygen, metal ions, high pH, high temperatures and processing times [1], which affect their stability.

The APY achieved in FD was higher than those of SD and SAS, as expected, since freeze-drying does not have steps with large material loss and operates at mild temperatures and pressures (room temperature and vacuum). SD was performed at 105 °C and SAS operated at 40 °C and 10 MPa. Patras et al. [44] report high temperatures among the most powerful degradation factors of anthocyanins. Nevertheless, SD and SAS were efficient to concentrate these pigments, as noted in their higher anthocyanin contents per dry particle (1.42 and 1.36 mg ECy3Gl/g dp, respectively) than the dry extract containing PVP (see Table 2–1.05 mg ECy3Gl/g de). Indeed, in SAS it was clear that SC-CO_2, besides removing ethanol, extracted lipophilic compounds from the organic solution, leading to precipitates more concentrated in anthocyanins. Fang and Bhandari [45], Marqués et al. [46], Osorio-Tobón et al. [36] and Visentin et al. [11] had also found SD and SAS as effective processes to concentrate phenolics, with little impact to their degradation. SAS must be highlighted, since it operates at milder drying conditions

Table 4
Concentrations of the anthocyanins identified and quantified by UHPLC in the particles produced by SD, FD and SAS.

Method	Anthocyanin				C_{Total} (mg/g dp)
	Cy3Gl (mg/g dp)	Cy3Ru (mg/g dp)	Cy3MG (mg/g dp)	Cy3DG (mg/g dp)	
SD	$0.79 \pm 0.11^{A,B}$	0.04 ± 0.01^{A}	0.03 ± 0.01^{A}	0.04 ± 0.01^{A}	$0.90 \pm 0.11^{A,B}$
FD	0.57 ± 0.04^{A}	0.03 ± 0.01^{B}	0.02 ± 0.01^{A}	0.03 ± 0.01^{A}	0.65 ± 0.03^{A}
SAS	1.04 ± 0.06^{B}	0.05 ± 0.02^{C}	0.03 ± 0.01^{A}	0.05 ± 0.01^{A}	1.17 ± 0.07^{B}

- Cy3Gl: cyanidin-3-*O*-glucoside; Cy3Ru: cyanidin-3-*O*-rutinoside; Cy3MG: cyanidin-3-*O*-malonyl-glucoside; Cy3DG: cyanidin-3-*O*-dioxalyl-glucoside; C_{Total}: total content of anthocyanins determined by UHPLC (sum of Cy3Gl, Cy3Ru, Cy3MG and Cy3DG); dp: dry particle.
- Results expressed as mean ± standard deviation.
- Uppercase letters in the same column indicate no significant difference at 5% level according to Tukey's test.

and is selective for anthocyanin precipitation, due to the low affinity of these compounds to CO_2. The small difference in MA between SD and SAS is possibly due to the effect of temperature.

As the extracts obtained by mechanical stirring, the particles produced by SD,FD and SAS contained cyanidin-3-*O*-glucoside, representing almost 90% of their total anthocyanin content (see Table 4). The three other identified anthocyanins are found in much smaller amounts. As for MA, the anthocyanins contents quantified by UHPLC were the highest in SAS, followed by SD and FD.

3.2.4. Antioxidant capacity

Both methods applied to determine AC presented similar trends, as observed in Table 3. FD produced particles with the highest AC, followed by SD and SAS. This suggests that FD processing conditions are less aggressive to the antioxidants contained in the extract from blackberry residue. The lower AC of SAS particles can also be due to the removal of lipophilic compounds by SC-CO_2, since compounds from oily extracts also have antioxidant potential [47,48]. Once in FD there is not removal of oily fraction, higher AC could be expected.

The Pearson's correlation coefficients (r), presented in Table 5, showed that AC is moderate to strongly related to MA($r = 0.8022$ for MA*vs* DPPH; $r = 0.7342$ for MA*vs* FRAP; $r = 0{,}9700$ for C_{Total}*vs*DPPH and $r = 0.9386$ for C_{Total}*vs* FRAP) indicating that the particles' high antioxidant capacity is also due to compounds other than anthocyanins, such as flavonols, phenolic acids and tannins, which can also be found in blackberries [32] and their residues. Also in Table 5, it is possible to note the correlation between the other different parameters analyzed until then to characterize the SD,FD and SAS processes and the particles produced by them. As can be seen, in general, the other parameters correlate moderately well.

3.3. Surface morphology

Fig. 2 presents FESEM images of the particles produced by SD (A), FD (B)and SAS (C), obtained at different magnifications. Different morphologies are observed depending on the preparation process, with characteristics similar to those reported in literature [30,36,49]. The particles produced by SD have spherical shape, with an almost smooth surface and variable sizes (Fig. 2A2, A3 and A*). Those obtained by FD and SAS present irregular agglomerates. FD particles present a morphology of thin sheets with rough and porous continuous surface, typical of lyophilized materials (Fig. 2B3 and B*), whereas the SAS particles show continuous surfaces (Fig. 2C2 and C*). Some works in SAS [50–53] have achieved micrometer scale particles with more homogeneous and spherical shapes, but using toxic solvents like dichloromethane, acetone, chloroform, tetrahydrofuran, methanol and dimethyl sulfoxide in the organic solution. These solvents are usually chosen for their great affinity to SC-CO_2, which enhances their removal and leads to fast and effective precipitation of the target compounds. Moreover, the mentioned works used much simpler solutions (such as β-carotene, cefonicid, resveratrol, 10-hydroxycamptothecin, lycopene) than the extract applied here. Vegetable extracts contain many compounds with complex structures, such as complexes of carbohydrates, proteins and lipids, which hamper the production of micro- and nanoparticles with well-defined shape. The SAS particles produced from these solutions tend to form agglomerates and complex molecular structures right after precipitation [36].

The FESEM image obtained with large magnification (Fig. 2-C3) and the picture taken inside the precipitation cell (Fig. 3) suggest that, once the extract droplets containing PVP meet SC-CO_2, they achieve rapid saturation, leading to the production of smaller and spherical particles.

Table 5
Correlations between the different parameters analyzed to characterize the techniques SD, FD and SAS and the produced particles.

Parameters	Pearson correlation coefficients (r)							
	GPY (%)	RE (%)	Moisture (%)	MA (mg Cy3GlE/g dp)	APY (%)	C_{Total} (mg/g dp)	DPPH (μmol TE/g dp)	FRAP (μmol TE/g dp)
GPY (%)	–	0.4047	0.4452	0.9181	0.9826	0.6956	0.5000	0.4051
RE (%)	–	–	0.9989	0.7339	0.5673	0.9384	0.9943	1.000
Moisture (%)	–	–	–	0.7635	0.6036	0.9530	0.9980	1.000
MA (mg Cy3GlE/g dp)	–	–	–	–	0.9757	0.9233	0.8022	0.7342
APY (%)	–	–	–	–	–	0.8168	0.6520	0.5676
C_{Total} (mg/g dry dp)	–	–	–	–	–	–	0.9700	0.9386
DPPH (μmol TE/g dp)	–	–	–	–	–	–	–	0.9943
FRAP (μmol TE/g dp)	–	–	–	–	–	–	–	–

- GPY: global particle yield; RE: residual ethanol; MA: monomeric anthocyanin content determined spectrophotometrically; APY: anthocyanin precipitation yield; C_{Total}: total content of anthocyanins determined by UHPLC; Cy3GlE: cyanidin-3-O-glucoside equivalent; TE: trolox equivalent; dp: dry particle.

Fig. 3. Photography of the inner SAS precipitation cell after decompression.

The choice of SAS operation conditions (pressure, temperature and ethanol:CO_2 ratio), in which the system ethanol + CO_2 + PVP is in the two-phase region of the liquid-vapor equilibrium diagram also favors particle aggregation. In this region, mass transfer rate and droplet supersaturation are lower than in the one-phase region. As the mixture ethanol + CO_2 coexists in two phases (liquid and vapor) [10,39], products with different aggregation states can be formed in the precipitation cell.

The FESEM images of the SD particles (Fig. 2A) suggest that the extract acts as a plasticizer for PVP, thus enhancing their chain mobility through the reduction of intra- and intermolecular forces [30]. It is clear, in the particles containing extract (Fig. 2A2 and A3), that spherical particles tend to coalesce, whereas PVP particles without extract (Fig. 2A*) remain perfectly separated.

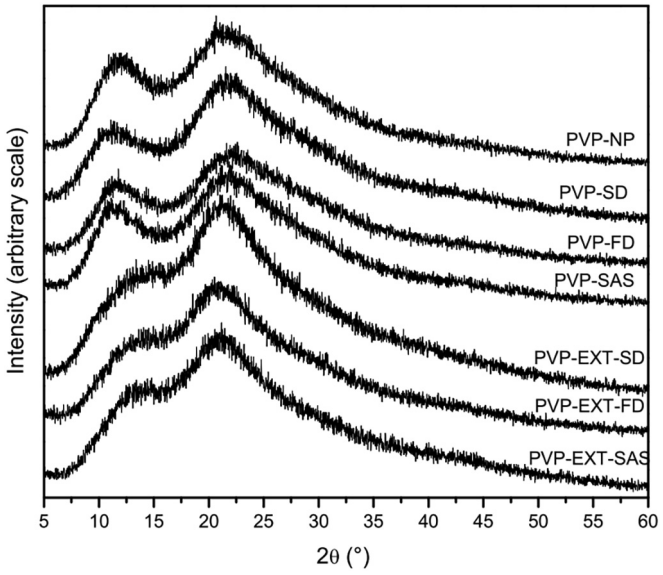

Fig. 4. DRX images of non-processed PVP (PVP-NP), PVP particles obtained by SD (PVP-SD), FD (PVP-FD) and SAS (PVP-SAS) and PVP + extract particles obtained by SD (PVP-EXT-SD), FD (PVP-EXT-FD) and SAS (PVP-EXT-SAS).

Table 6
Glass transition temperatures of non-processed PVP (PVP-NP), PVP processed by SD without extract (PVP-SD) and with extract (PVP-EXT-SD) in different proportions.

Sample	*Tg* (°C)
PVP-NP	135.45
PVP-SD	133.07
PVP-EXT-SD (1:3.5)	122.89
PVP-EXT-SD (1:2.5)	110.17

3.4. Physical-chemical properties of the particles

3.4.1. Crystallinity

Fig. 4 shows the diffractograms obtained from non-processedPVP (PVP-NP) and PVP after SD,FD and SAS with and without extract from blackberry residue. Two diffraction peaks with low intensity are observed, indicating that the samples are predominantly amorphous. This characteristic can be proven by the TGA analysis, which did not detect the existence of fusion peaks in the samples, but only peaks referring to the glass transition temperature (Table 6). It can also be noticed that the encapsulation did not affect the polymer solid state, since the amorphous structure remains even after SD,FD or SAS. The same observation was made by Liparoti et al. (2015) [18].

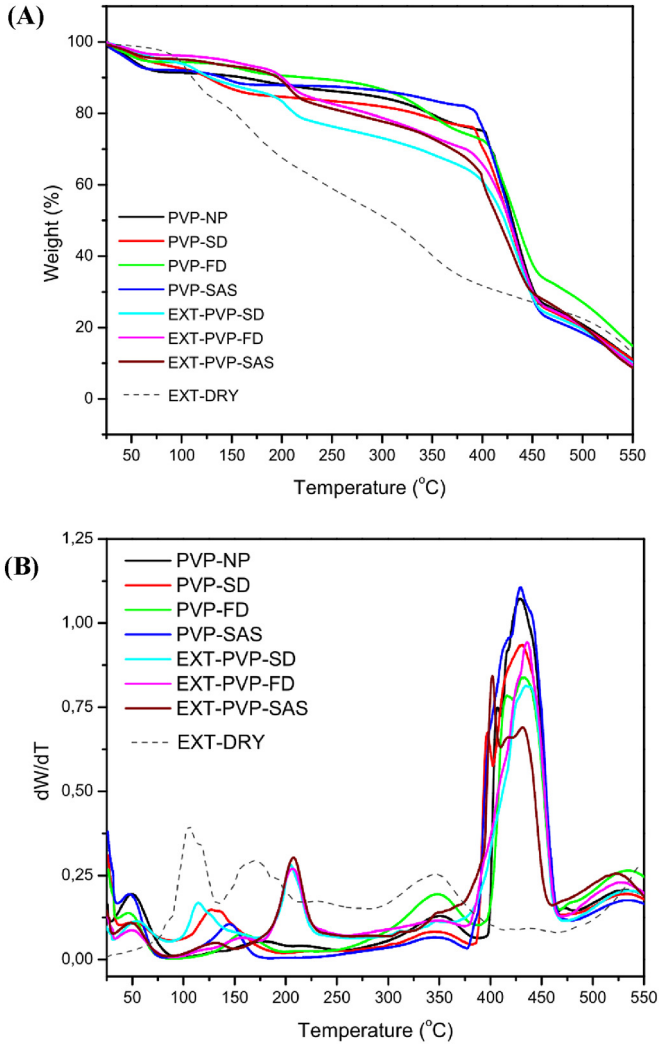

Fig. 5. TGA (A)and DTG (B)curves of non-processed PVP (PVP-NP), PVP particles obtained by SD (PVP-SD), FD (PVP-FD) and SAS (PVP-SAS), PVP + extract particles obtained by SD (PVP-EXT-SD), FD (PVP-EXT-FD) and SAS (PVP-EXT-SAS), and dry extract without PVP (EXT-DRY).

3.4.2. Glass transition temperature

The glass transition temperatures (T_g) of non-processedPVP and the particles obtained by SD are presented in Table 6. In order to assess the effect of the extract on the thermal behavior of PVP, two extract:PVP proportions were evaluated (1:2.5 e 1:3.5 *w*/w). All samples presented T_g above 110 °C, suggesting strong molecular bonds between the PVP chains [30]. SD did not affect PVP's glass transition temperature, as noted by the close T_gs of non-processed and processed PVP. Similar results are reported by Dereymaker and Mooter [54] and Nair et al. [55]. Even so, the extract had some influence on T_g, which decreased in the presence of extract at both concentrations. As already mentioned in Section 3.3, this change can be attributed to the plasticizer action of the extract. It is also noted that *Tg* is lower in the particles produced from the solution with higher extract proportion (1:2.5), thus reinforcing the negative effect of the extract on *Tg*.

3.4.3. Thermal stability

Fig. 5 shows the TGA(Fig. 5A) and DTG (Derivative Thermogravimetry) (Fig. 5B) curves of non-processedPVP and the particles obtained by SD,FD and SAS. Three to four thermal decomposition steps are better noted in the DTG curves. The first and second (around 68 and 100 °C, respectively, for samples containing PVP) may be related to the evaporations of ethanol and water. The third step, which occurs between 180 and 220 °C, can be due to the decomposition of groups containing oxygen. This step is observed only in the samples containing extract + PVP. This decomposition event occurs at a lower temperature for the dry extract without PVP (between 140 and 180 °C), thus indicating that the presence of the polymer increases the thermal stability of the anthocyanic extract. This is possibly due to the strong interactions between the components of the extract and the polymer chains. The abrupt loss mass in the fourth step, from 400 to 450 °C, is related to the combustion of the main PVP portions.

It is worth noticing that the precipitates containing extract had lower relative mass loss at 400–450 °C than those without extract. Once part of these samples had been already oxidized from 180 to 220 °C, less content remained to be decomposed. Finally, the complete oxidation of the particles is observed around 550 °C.

It can be seen (Fig. 5) that the mass losses related to extract and PVP did not vary with the encapsulation method. Therefore, it can be concluded that the SD,FD and SAS processes are equivalent in terms of thermal protection of the extracts.

4. Conclusions

Spray-drying, freeze-drying and supercritical antisolvent precipitation produced particles with high antioxidant capacity from blackberry residue extracts, with no great degradation of the target compounds. These techniques were efficient in the concentration of anthocyanins with PVP, with FD overcoming SAS and SD. Cy3Gl was the major anthocyanin found in the dry particles, and Cy3Ru, Cy3MG and Cy3DG were the minor. High residual ethanol and moisture contents were observed in SAS particles, unlike those of SD and FD. Different morphologies were obtained at each process. SD particles presented spherical and smooth particles with different sizes. FD and SAS particles had irregular agglomerates, being the former rough and the latter with continuous surface. The processes are equivalent in terms of thermal protection of the extracts and they did not affect the crystallinity and thermal behavior of PVP. The presence of the polymer increases the thermal stability of the anthocyanic extract.

The SAS precipitation of anthocyanins in polymeric matrices appears as a promising strategy to eliminate the residual ethanol, which is a GRAS solvent, of the extract and produce a dry product richer in anthocyanins and with enhanced purity, since SC-CO_2 does not have any chemical affinity to anthocyanins. The results obtained by SAS are, in general, comparable to SD and FD. Even so, additional investigations on phase equilibria and the behavior of the ethanolic extract in the system are recommended, in order to find conditions to produce smaller and homogeneous particles with higher solid yield and lower residual ethanol.

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