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Isolation of bluish anthocyanin-derived pigments obtained from blueberry surplus using centrifugal partition chromatography

Ana N. Nunes[a,b,*], Viktoriya Ivasiv[a], Luís F. Gouveia[c], Naiara Fernández[a], Joana Oliveira[d], Maria Rosário Bronze[a,b,e], Ana A. Matias[a]

[a] iBET, Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2781-901 Oeiras, Portugal
[b] ITQB, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. Da República 2780-157 Oeiras, Portugal
[c] iMed.ULisboa, Instituto de investigação do medicamento, Faculdade de Farmácia, Universidade de Lisboa, Av. Professor Gama Pinto, 1649-003 Lisboa, Portugal
[d] REQUIMTE, Laboratório Associado para a Química Verde, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre, 687, 4169-007 Porto, Portugal
[e] FFULisboa, Faculdade de Farmácia, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003, Lisboa, Portugal

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ABSTRACT

Replacement of synthetic colorants with natural ones is a current marketing trend. Nevertheless, the naturally occurring blue color is rare compared to other colours. In this work, centrifugal partition chromatography (CPC) process was developed as a more efficient and sustainable alternative to reversed phase column chromatography (RP-CC) for the preparative-scale purification of portisins. The strategy began with the extraction of anthocyanins from blueberry surplus and hemi-synthesis of respective portisins. Then, the CPC method development started with the biphasic solvent system selection followed by the optimization of the operating parameters and ended up with a comparison with RP-CC. Aiming at maximizing the portisin content, process throughput, efficiency, and minimizing the environmental risk factor, the effect of sample load (100–500 mg/100 mL of column volume), mobile phase flow rate (10–20 mL/min), and rotation speed (1000–1600 rpm) was evaluated. The two-phase solvent system consisted of tert–butyl–methyl ether, n-butanol, acetonitrile, and water (volume ratio 2:2:1:5) acidified with 0.1 vol.% of HCl was selected. The best conditions were 464 mg of sample/100 mL of column volume, 20 mL/min of mobile phase flow rate, and 1600 rpm of rotation speed at reversed phase mode, allowing the purification of portisins by 5-fold. Compared to the RP-CC, the CPC process efficiency was 2.4 times higher, while the CPC process environmental risk factor was 5.5 times lower. Overall, this study suggests that CPC can be considered an effective, and sustainable alternative process for the preparative isolation of portisins. With this purification approach, the blueberry surplus has been valorized and a naturally derived product has been prepared, allowing its subsequent use as a natural blue colorant.

1. Introduction

The market of natural colors is growing at 10–15% annually and the global demand for natural colorants represents an important part of the total need for dyeing agents [1], with synthetic colorants being progressively substituted by natural ones. The reduced safety of synthetic dyes has been related to high levels of toxicity, allergic reactions, and behavioral and neurocognitive effects [2]. On the other hand, naturally derived pigments are associated with safety and health-promoting effects [2].

Among natural colors, blue is the biggest challenge since natural sources of blue are limited [3,4]. Portisins are a family of anthocyanin-derived pigments (also known as vinylpyranoanthocyanin–flavanols) found in aged Port red wines [5]. These compounds display unusual spectroscopic features, presenting a bluish color in acidic conditions with a visible λ_{max} at approximately 570 nm. This class of anthocyanin-derived pigments can be obtained through the reaction between anthocyanin-pyruvic acid adducts and flavanols in the presence of acetaldehyde (Fig. 1) [5]. The resulting structure presents an extended π electrons conjugation conferring high stability to these molecules as well as the uncommon bluish color [6]. In addition, the molar extinction coefficient of these bluish pigments is significantly higher when compared to their anthocyanin precursors [7].

* Corresponding author.
E-mail address: annunes@ibet.pt (A.N. Nunes).

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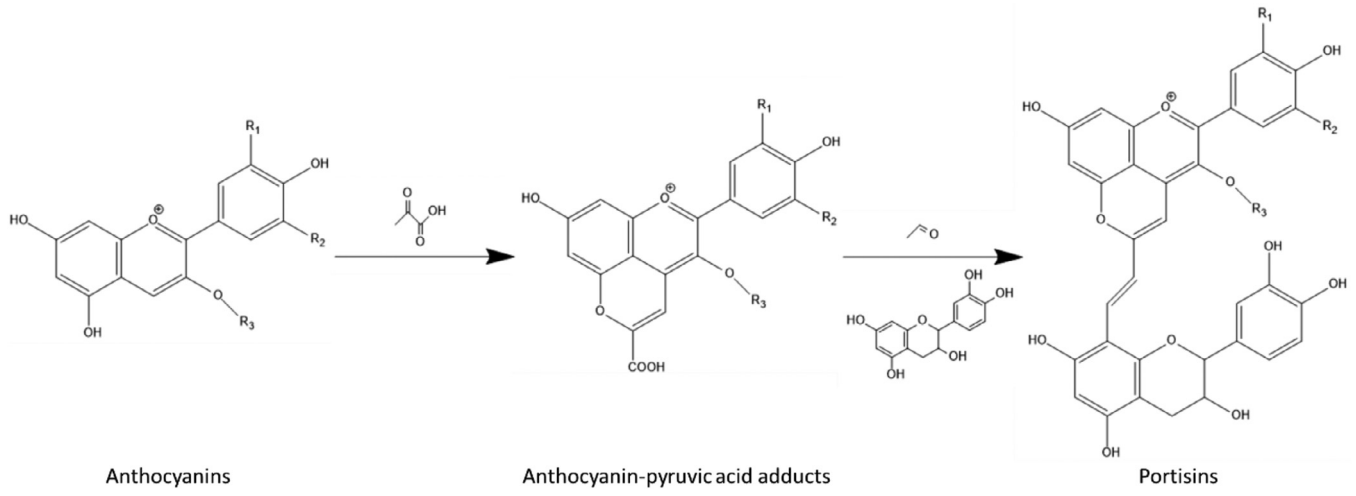

Fig. 1. General scheme for portisins hemi-synthesis.

The portisins chromatic properties make this pigment a potential candidate as a naturally occurring blue colorant with applications in the nutraceutical and food sectors. However, the concentration of these pigments in Port wines are very low. Mateus and co-workers, study the hemi-synthesis of these anthocyanin-derived pigments found in red wines. The authors used several red fruit extracts as anthocyanin natural sources to produce these bluish pigments. The different red fruit anthocyanins yielded the respective anthocyanin-derived pigments [6]. Within anthocyanin-rich fruits (elderberry, blueberry, black currant, blackberry, and sweet cherry), both the total anthocyanin content and the type of anthocyanins present are quite variable. Elderberries present the highest anthocyanin content but only cyanidin is reported in this fruit. On the other hand, blueberries present higher anthocyanin content and also a more diverse anthocyanin profile (fifteen different anthocyanins) [8,9].

Blueberry fruits (*Vaccinium* spp.) present an attractive blue color, unique taste, special fragrance, and high nutritional value [10–12]. The health-related benefits associated with the increasing health awareness and consumption trends have significantly increased the blueberry production. [12,13]. At the same time, a considerable amount of agricultural waste is generated during crop production and harvesting, including blueberry leaves and branches, broken blueberry fruits debris, and blueberry fruit that display unsatisfactory size and ripeness (blueberry surplus) [14]. Therefore, the production of portisins at a laboratory scale using blueberry surplus anthocyanins as precursors was identified as a promising alternative to obtain bluish colors.

The produced anthocyanin-derived extracts are complex systems comprising not only the portisins but also the remaining anthocyanins and anthocyanin-pyruvic acid adducts. Traditionally, portisins isolation from Port red wine or the red fruit extracts after hemi-synthesis has been performed by RP-CC. Mateus and coworkers described the purification of portisins using a TSK Toyopearl HW-40 (S) gel column chromatography followed by semipreparative HPLC [5,7,9,15,16]. However, there is a lack of information regarding the throughput and efficiency of the process, as well as the solvent consumed to obtain the purified portisins. The main goal of these works was to characterize the new compounds rather than to evaluate the performance of the separation process. Additionally, column chromatography-based processes suffer from significant drawbacks when it comes to scalability in industrial settings. These drawbacks include prolonged separation time, extensive utilization of organic solvents, and diminished recovery rate due to irreversible adsorption of the solid support [17,18]. Therefore, there exists a pressing need to develop efficient and sustainable methodologies for the isolation of these bluish pigments from a complex mixture.

Centrifugal partition chromatography (CPC) is a viable alternative to conventional column chromatography. CPC is a liquid-liquid chromatography which does not require the use of a solid stationary phase. The separation principle is based on the fast-partitioning effects of the compounds between two immiscible liquid phases of a solvent system. During the separation process, centrifugal force keeps the stationary phase in the column, while the mobile phase, which is not miscible with the stationary phase, is pumped through the column. The partition coefficient (K) determines the elution order of the components from the column [19].

As a liquid-liquid chromatographic technique, CPC avoids the sample loss attributed to irreversible adsorption on the solid support, allowing excellent sample recovery without chemical modifications or loss of bioactivity [20]. Also, CPC offers a larger surface area of the stationary phase for solute interaction compared to solid-liquid chromatography [20,21]. Apart from the initial investment, CPC provides the advantage of lower operational and maintenance costs. This is achieved by streamlining the processing steps, reducing solvent usage or even enabling the in-line eluent recycling, and dispensing expensive and pollutant solid-support materials [22–26]. As a result, CPC can significantly contribute to cost-effective and sustainable operations.

CPC has already demonstrated its efficacy in the isolation of anthocyanins from various sources, such as berry fruits [27–30], wine by-products [28,31,32], and flowers [33]. Du et al. [34], described the preparative isolation of anthocyanins by high-speed countercurrent chromatography (HSCCC) from a crude extract of blueberry (*Vaccinium myrtillus*). The authors explored the biphasic solvent system composed by tert–butyl–methyl ether, n-butanol, acetonitrile, and water (volume ratio 1:4:1:5) acidified with 1 vol.% of trifluoroacetic acid. The results demonstrated that preparative HSCCC separation was able to yield pure anthocyanins from a complex matrix of natural products. However, the performance of HSCCC was not evaluated. To date, there is no information in the existing literature regarding the utilization of CPC as a tool for the separation of portisins.

The objective of the present study was to establish a scalable and efficient process for selectively recovering portisins from the blueberry surplus, which was used as a source of anthocyanin for the portisin synthesis. For the first time, the feasibility of employing CPC for portisins purification was investigated. The proposed technique was compared to RP-CC. The portisin content in the ob-

tained fractions, process throughput, efficiency, and environmental risk factor were investigated to optimize the CPC operating conditions. The proposed approach represents a significant advancement in the challenging task of producing novel natural blue colorants for the nutraceutical and food industries, contributing to the valorization of this blueberry surplus.

2. Materials and methods

2.1. Raw material

Blueberry (*Vaccinium myrtillus*) surplus was kindly provided by Delícias do Tojal, CultiBaga, and Mirtilsul, Portugal. The raw material underwent dehydration using a Coolsafe Superior Touch 55−80 freeze-dryer (Labogene, Lillerød, Denmark) at a temperature of −55 °C. Subsequently, the dried material was milled using a cutter-emulsifier CKE-8 (Sammic, Azkoitia, Spain). The processed biomass was protected from light and stored at a temperature of −20 °C until subsequent analyses.

2.2. Chemicals

The chemicals employed for the extraction of anthocyanins and the hemi-synthesis of portisins included the following: methanol 99.8% from Fisher Scientific (Loughborough, UK), pyruvic acid 98% from Acros Organics (Geel, Belgium), ethanol absolute 99.9% from Carlo Herba (Val-de Reuil, France), hydrochloric acid ≥37%, (+)-catechin hydrate ≥96.0% and acetaldehyde ≥99.0% from Sigma-Aldrich (St. Louis, Missouri, USA).

The specific chemicals used for various fractionation methodologies were as follows: methanol from 99.8% from Fisher Scientific (Loughborough, UK), hydrochloric acid ≥37% from Sigma-Aldrich (St. Louis, Missouri, USA), tert–butyl–methyl ether 99.8% and acetonitrile 99.9% from Honeywell (Muskegon, USA), 1-butanol 99.8% and ethyl acetate 99.9% from Carlo Herba (Val-de Reuil, France), Flash pure EcoFlex C18 12 g columns from Büchi Labortechnik (Flawil, Switzerland), LiChroprep RP-18 40–63 µm from Millipore (Massachusetts, USA).

The chemicals utilized for extract chemical characterization included the following: acetonitrile >99.9% from Chem Lab (Zedelgem, Bélgium), formic acid 99% from Carlo Herba (Val-de Reuil, France), malvidin-3-glucoside for HPLC ≥95% from Extrasynthese (Genay, France) and vinylpyranomalvidin-3-O-glucoside-catechin used as standard for portisins quantification obtained as described by Mateus et al. [35].

2.3. Anthocyanins extraction from blueberry surplus

Anthocyanins were extracted and purified following the protocol described previously by Faria et al. [16] with slight modifications. The blueberry surplus was extracted with 50 vol.% aqueous methanol at pH 1.5 (adjusted with HCl) for 2 h at room temperature. Anthocyanins were exhaustively recovered from the residues by two extraction cycles of 10 mL per g of dry residue (ratio 10:1). The resulting extract was filtered, and the methanol was subsequently removed using a rotary evaporator at a temperature of 38 °C under reduced pressure. The anthocyanin crude extract was then applied to a C-18 silica gel reversed phase previously conditioned with methanol and water. Firstly, deionized water was used to sugars and phenolic acids present in the extract. Then, anthocyanins were eluted with methanol at pH 1.5 (adjusted with HCl). The solvent was evaporated under reduced pressure at a temperature of 38 °C and the anthocyanin-rich extract was stored at a temperature of −20 °C in the absence of light, until further use.

2.4. Anthocyanin-pyruvic acid adducts synthesis

The synthesis of anthocyanin-pyruvic acid adducts was achieved through the reaction between the previously isolated anthocyanins with pyruvic acid (molar ratio pyruvic acid/anthocyanin of 400:1) in water at pH 2.6 (adjusted with NaOH) and at a temperature of 37 °C following to the method outlined by Mateus et al. [36]. The reaction progress was monitored by high-performance liquid chromatography–diode array detector (HPLC-DAD) analysis at a wavelength 511 nm. The reaction was stopped by cooling the mixture after a maximum yield of anthocyanin-pyruvic acid adducts was reached (usually 7 days). Subsequently, the reaction mixture was applied to a C-18 silica gel reversed phase, and the anthocyanin-pyruvic acid adducts fraction was eluted using a mixture of 30 vol.% aqueous methanol pH 1.5 (adjusted with HCl). The methanol was evaporated at a temperature of 38 °C under reduced pressure, and the resulting reaction mixture (RM1) was stored at a temperature of −20 °C in the absence of light, until further use.

2.5. Portisins hemi-synthesis

Portisins were synthesized by subjecting the previously obtained anthocyanin-pyruvic acid adducts (RM1) to a reaction with (+)-catechin in the presence of acetaldehyde according to the methodology described by Mateus et al. [35]. The anthocyanin-pyruvic acid adducts were incubated with a mixture of 20 vol.% aqueous ethanol pH 1.5 (adjusted with HCl). The reaction took place at a temperature of 37 °C with (+)-catechin (molar ratio anthocyanin-pyruvic acid adducts/catechin 1:29) in the presence of acetaldehyde (molar ratio catechin/acetaldehyde 2:1). The reaction progress was monitored by HPLC-DAD analysis at a wavelength 570 nm. The reaction was stopped by cooling after a maximum yield of portisins was obtained (usually 8 days). The excess acetaldehyde was removed on the C-18 silica gel reversed phase. The bluish anthocyanin-derived compounds were eluted with methanol pH 1.5 (adjusted with HCl). The solvent was evaporated at a temperature of 38 °C under reduced pressure and the reaction mixture (RM2) was stored at a temperature of −20 °C in the absence of light, until further use.

2.6. Portisins separation procedures

2.6.1. Conventional reversed-phase liquid chromatography

Preparative RP-LC separations were conducted on a Sepacore® chromatography system X50 (Büchi Labortechnik, Flawil, Switzerland), which comprised of two C-605 pump modules, a C-620 control unit, a C-640 UV detector, and a C-660 fraction collector. This method was developed based on the purification procedure previously described for portisins isolation by Toyopearl gel column chromatography [16]. To prepare the sample solution, 100 mg of RM2 was dissolved in 20 mL of a 0.5 vol.% aqueous ethanol pH 1.5 (adjusted with HCl). The solution was filtered through a 0.45 µm membrane filter. The injection of the sample was carried out through a 6-way valve with a 20 mL loop. The sample was loaded on the flash C-18 cartridges (Flash pure EcoFlex C18 12 g) and a gradient elution with water/methanol pH 1.5 (adjusted with HCl) at a flow rate of 15 mL/min was performed. The eluent was monitored at 570 nm (portisins), 520 nm (anthocyanins), 511 nm (anthocyanin-pyruvic acid adducts) and 280 nm (phenolic compounds). Fractions were collected every 50 mL automatically and subsequently grouped according to the color and the maximum absorption wavelength detected. The entire process, including method development, pumping, detection, fraction collection, equipment control, and data handling, was managed by Sepacore-Control® 1.3 software.

2.6.2. Centrifugal partition chromatography

2.6.2.1. Apparatus. Preparative separations were carried out on a Fast Centrifugal Partition Chromatography (FCPC®) system A200 (Kromaton Rousselet Robatel, Annonay, France). The system's rotor had twin cells, giving a total column capacity of 265 mL, with 200 mL assigned to the cell volume. The solvent was pumped through the system using an analytical HPLC pump, while the sample was manually injected through a 20 mL sample loop. The FCPC® system was connected to a DAD detector capable of operating with four wavelengths simultaneously and performing continuous scans (200 - 800 nm). The fractions were easily collected with an automatic fraction collector. The whole process, including method development, pumping, detection, fraction collection, automated control of FCPC®, and data handling was managed by the Kromaflash integrated CPC-peripheral system, using the InterSoft® X software (version 10).

2.6.2.2. Biphasic solvent system selection. To assess the suitability of different two-phase solvent systems, HPLC-DAD analysis was conducted at a wavelength of 570 nm to determine the partition coefficient (K) value. The K was calculated as the ratio of the HPLC peak relative area percentage of individual portisins in the upper phase to that in the lower phase (Eq. (1)).

$$K = \frac{peak\ area\ upper\ phase}{peak\ area\ lower\ phase} \quad (1)$$

Each biphasic solvent system was mixed with the RM2 (4 g/L) in a stoppered test tube. After the mixing, an equilibration step was implemented to facilitate the complete partition of anthocyanin derivatives. Both phases were collected to determine of the area of individual portisins. The K values were expressed as a mean of triplicates.

2.6.2.3. Biphasic solvent system and sample solution preparation. The CPC experiments were carried out using the biphasic system tert-butyl-methyl ether/n-butanol/acetonitrile/water (volume ratio 2:2:1:5). These solvents were gently mixed and settled, forming two distinct layers, the upper and lower phases. After phase separation, both phases were acidified by adding 0.1 vol.% of HCl. The preparation of the sample solution consisted of dissolving the dried RM2 in a mixture of the lower phase and upper phase, with a volume ratio of 9:1 [37].

2.6.2.4. Experimental procedure.

2.6.2.4.1. Equilibration step. A homogeneous solvent equilibration on the rotor as achieved by loading the column with the organic stationary phase at a flow rate of 30 mL/min and a rotation speed of 600 rpm. The rotation speed was then tuned to the higher speed required for the retention of stationary phase. After establishing the working rotational speed, the aqueous mobile phase was pumped through the organic stationary phase until the hydrostatic equilibrium.

2.6.2.4.2. Injection step. The prepared sample solution was filtered through a 0.45 µm membrane filter to remove any precipitate. The filtered solution was then injected into the system via loop (100–500 mg/100 mL of column volume) after the hydrostatic equilibrium was reached.

2.6.2.4.3. Elution step. During the elution step, different rotation speed (1000–1600 rpm) and mobile phase flow rate (10–20 mL/min) were tested. These operating parameters were adjusted to enhance the stationary phase retention within the column while simultaneously reducing the separation time. The elution was performed in reversed phase mode (also known as descending mode), where the lower phase was used as the mobile phase. Compounds with lower K were eluted closer to the solvent front, while compounds with higher K were eluted later. The eluent was monitored at 570 nm (portisins), 520 nm (anthocyanins), 511 nm (anthocyanin-pyruvic acid adducts), and 280 nm (phenolic compounds). The fractions were collected every 25 mL automatically and subsequently grouped according to the color and the maximum absorption wavelength detected.

2.6.2.4.4. Extrusion step. The remaining sample components that were significantly retained on the column were rapidly eluted by pumping the organic stationary phase at a flow rate of 30 mL/min with the rotation speed set at 600 rpm. Each experiment resulted in four main fractions that were dried at a temperature of 38 °C under reduced pressure and stored at a temperature of −20 °C in the absence of light, for further analysis.

2.6.2.9. Stationary phase retention determination. The stationary phase retention Sf was calculated from Eq. (2):

$$Sf = \frac{V_c - \left(V_{mp} - V_x\right)}{V_c} \quad (2)$$

where Vc is the column volume, Vmp is the mobile phase volume retained in the column and Vx is the extra column volume (injection loop, connecting tubing, and detection cell).

2.6.3. Anthocyanins and anthocyanin derivatives identification and quantification

2.6.3.10. High-Performance liquid chromatography-diode array detector (HPLC-DAD). The anthocyanin-rich extract and anthocyanin-pyruvic acid adducts fractions were analyzed in a Dionex UltiMate 3000 HPLC system equipped with a quaternary pump, autosampler, column compartment and coupled to an UltiMate 3000 Diode Array Detector (Thermo Scientific, Waltham, MA, USA) according to the method described by Oliveira et al. [7]. Chromatographic separation was performed on a Macherey-Nagel® RP-18 column (250 mm length, 4 mm width, and 5 μm particle size) maintained at 25 °C. The eluents were A: H_2O/HCOOH (volume ratio 9:1), and B: CH_3CN/H_2O/HCOOH (volume ratio 3:6:1). The gradient consisted of 20–85% B for 70 min at a flow rate of 1.0 mL/min. The column was washed with 100% B for 10 min and then stabilized with the initial conditions for another 10 min [38]. The injection volume was 100 µL and the autosampler was held at 8 °C. The photodiode array detector was programmed for scanning between 190 and 580 nm at a speed of 5 Hz with a bandwidth of 2 nm. The detection was monitored using four individual channels, 570 nm (portisins), 520 nm (anthocyanins), 511 nm (anthocyanin-pyruvic acid adducts), and 280 nm (phenolic compounds). For the acquisition and processing of data Chromeleon® software version 7.2 SR4 was used. The calibration curve for malvidin-3-glucoside was linear within the range of 0–100 ppm using acidified water pH 1.5 (adjusted with HCl) as solvent. Results for total anthocyanin content were presented as mg of malvidin-3-glucoside equivalents per g of extract and expressed as a mean of triplicates. The portisins fractions were also analyzed by HPLC-DAD using the same conditions but with a different eluent B: CH_3CN/H_2O/HCOOH (volume ratio 8:1.95:0.05). The calibration curve for vinylpyranomalvidin-3-O-glucoside-catechin was linear within the range of 0–80 ppm using 30 vol.% aqueous ethanol 0.1 M HCl as solvent. Results for portisin content were presented as mg of vinylpyranomalvidin-3-O-glucoside-catechin equivalents per g of extract and expressed as a mean of triplicates.

2.6.3.11. High-Performance liquid chromatography-diode array detector – mass spectrometry (HPLC-DAD–MS). The identity of anthocyanins and anthocyanin-derived compounds was determined by HPLC-DAD-MS analysis as previously described by Oliveira et al. [38]. A Finnigan Surveyor series liquid chromatograph, equipped with a LicroCART® reversed phase C18 column (150 mm length,

4.6 mm width, and 5 μm particle size) thermostatted at 25 °C was used. Detection was carried out between 200 and 700 nm using a Finnigan Surveyor PDA Plus detector. The mass detection was carried out by a Finnigan LCQ DECA XP MAX (Finnigan Corp., San José, Calif., USA) mass detector with an API (Atmospheric Pressure Ionization) source of ionization and an ESI (ElectroSpray Ionization) interface. Eluents were A: H_2O/HCOOH (volume ratio 99:1), and B: CH_3CN/H_2O/HCOOH (volume ratio 30:69:1) for anthocyanins and anthocyanin-pyruvic acid adducts. For portisins, eluent B was: CH_3CN/HCOOH/H_2O (volume ratio 80:0.5:19.5). The HPLC gradient used was the same as reported above for the HPLC analysis except for the flow rate which was 0.5 ml/min. The capillary voltage was 4 V, and the capillary temperature was 325 °C. Spectra were recorded in positive ion mode between *m/z* 150 and 2000. The mass spectrometer was programmed to do a series of three scans: a full mass, a zoom scan of the most intense ion in the first scan, and a MS-MS of the most intense ion using a relative collision energy of 30 and 60 V.

2.6.4. Process throughput, process efficiency and environmental risk factor estimation

Process throughput (PT) was evaluated as a measure of mass processed per unit time:

$$PT = \frac{m_{processed\ RM2}}{t_{separation}},\ mg\ processed\ RM2/h \quad (3)$$

where $m_{processed\ RM2}$ represents the mass of RM2 processed per run and $t_{separation}$ denotes the time taken for a single run.

Process efficiency (PE) was assessed as a metric for evaluating the production rate of one unit mass of the target compound:

$$PE = \frac{m_{P\ recovered}}{t_{separation}},\ mg\ portisins/h \quad (4)$$

where $m_{P\ recovered}$ represents the mass of portisins recovered and $t_{separation}$ denotes the time consumed during the separation.

Process environmental risk factor (Er) was determined to assess the volume of waste solvent generated in the production of one unit mass of the target compound:

$$Er = \frac{V_{total\ solvent}}{m_{P\ recovered}},\ L\ solvent/g\ portisins \quad (5)$$

where, $V_{total\ solvent}$ represents the total volume of solvent used and $m_{P\ recovered}$ denotes the mass of portisins recovered.

In the optimization of the CPC process, PT and PE were used as key indices, along together with the portisin content, to evaluate the performance of CPC. Additionally, the Er was determined to estimate of the environmental impact of the CPC process. These parameters (PT, PE and Er) were also determined for the RP-LC to compare and evaluate the performance and environmental impact of both methodologies.

2.6.5. Experimental design and statistical analysis

The impact of process factors on CPC fractionation was determined using a response surface technique. The program Modde® v.12 (Umetrics, Umea, Sweden) was used to evaluate the portisin content, process throughput, process efficiency, and environmental risk factor data obtained from tests conducted under experimental settings specified by a central composite face design. Adjustments to the design model and impacts of the variables were statistically significant whenever the resulting p-value was less than the predetermined = 0.05 (ANOVA and *t*-test).

3. Results and discussion

The objective of the present study was to establish a practical procedure to produce bluish anthocyanin-derived pigments known as portisins. The developed methodology involves three steps, namely (1) production of an extract rich in anthocyanins, (2) hemi-synthesis of anthocyanin-derived pigments, and (3) isolation of portisins using CPC.

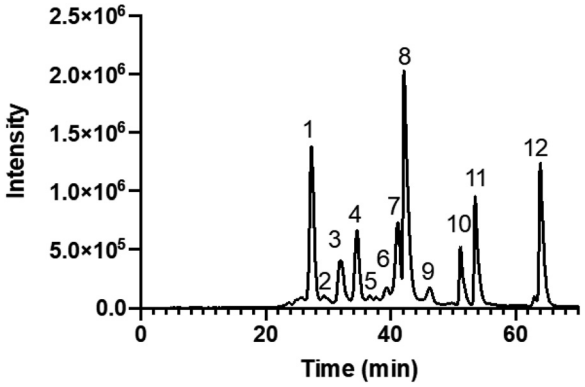

Fig. 2. Chromatogram of the blueberry extract recorded at 520 nm. 1, delphinidin-3-galactoside; 2, delphinidin-3-glucoside; 3, cyanidin-3-galactoside and delphinidin-3-arabinoside; 4, petunidin-3-galactoside; 5, petunidin-3-glucoside; 6, petunidin-3-arabinoside; 7, malvidin-3-galactoside; 8, malvidin-3-glucoside; 9, malvidin-3-arabinoside; 10, cyanidin; 11, petunidin; 12, malvidin.

3.1. Anthocyanins extraction from blueberry surplus

Conventional solid-liquid extraction with acidified water and methanol was performed to recover anthocyanins from the blueberry surplus. The blueberry surplus extract used to produce the blue pigments was analyzed in terms of the anthocyanin content by HPLC-DAD. The anthocyanin content was found to be 60 mg malvidin-3-glucoside equivalents/g extract. The identity of each anthocyanin present in the blueberry surplus extract was determined by HPLC-DAD-MS analyses, and the respective liquid chromatography chromatogram at 520 nm is presented in Fig. 2. In a previous study, fifteen different anthocyanins corresponding to the galactoside, glucoside, and arabinoside derivatives of delphinidin, cyanidin, petunidin, peonidin, and malvidin have been identified in a blueberry (*Vaccinium myrtillus)* extract [9]. It should be noticed that in the prepared extract, the peonidin-derivatives were not identified and that the presence of cyanidin, petunidin and malvidin aglycones was observed which could indicate a partial decomposition of anthocyanins present in the extract during the extraction and concentration process yielding to the hydrolysis of the ether bond.

3.2. Portisins hemi-synthesis

Portisins have been previously reported to result from the reaction between anthocyanin-pyruvic acid adducts and other compounds, namely flavanols, in the presence of acetaldehyde [6]. Considering this, the anthocyanin-pyruvic acid adducts were previously obtained through the reaction between blueberry anthocyanins with pyruvic acid as reported elsewhere [39]. To achieve a higher reaction yield and minimize the thermal degradation of anthocyanins and anthocyanin-pyruvic acid adducts, the reaction progress was monitored by HPLC-DAD. It was found that a mixture containing a high anthocyanin concentration (~500 mg/L) and a great excess of pyruvic acid (molar ratio 400:1 regarding anthocyanins) for 7 days yielded the highest conversion to anthocyanin-pyruvic acid adducts in aqueous solution at pH 2.6 and 37 °C. Then, the production of portisin was assayed in a model solution of ethanol 20 vol.% pH 1.5 and 37 °C, starting from anthocyanin-pyruvic acid adducts and (+)-catechin. From the results, the reaction of a high concentration (~150 mg/L)

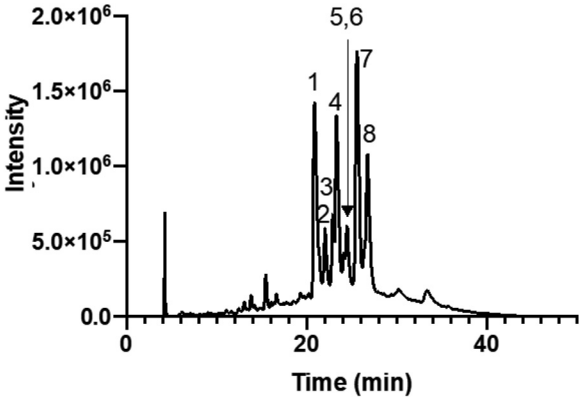

Fig. 3. Chromatogram of the reaction mixture after 8 days showing the formation of portisins (peak 1–8) and the anthocyanin-pyruvic acid adducts precursors recorded at 570 nm. 1, vinylpyrano-delphinidin-3-galactose (or glucose)-catechin; 2, vinylpyrano-delphinidin-3-arabinose-Catechin; 3, vinylpyrano-cyanidin-3-galactose (or glucose)-catechin; 4, vinylpyrano-petunidin-3-galactose (or glucose)-catechin; 5, vinylpyrano-cyanidin-3-galactose (or glucose)-catechin; 6, vinylpyrano-malvidin-3-galactose (or glucose)-catechin; 7, vinylpyrano-malvidin-3-galactose (or glucose)-catechin; 8, vinylpyrano-malvidin-3-arabinose-catechin.

anthocyanin-pyruvic acid adducts with a great excess of (+)-catechin (molar ratio 1:29) in the presence of acetaldehyde (molar ratio catechin/acetaldehyde 2:1) for 8 days showed the highest reaction yield of portisin. The calculated reaction yield for portisins was 99.2 wt.%. The chromatogram of the final reaction solution recorded at 570 nm (Fig. 3) clearly showed the presence of several new peaks: vinylpyrano-delphinidin-3-galactose (or glucose)-catechin (peak 1), vinylpyrano-delphinidin-3-arabinose-catechin (peak 2), vinylpyrano-cyanidin-3-galactose (or glucose)-catechin (peak 3), vinylpyrano-petunidin-3-galactose (or glucose)-catechin (peak 4), vinylpyrano-cyanidin-3-galactose (or glucose)-catechin (peak 5), vinylpyrano-malvidin-3-galactose (or glucose)-catechin (peak 6), vinylpyrano-malvidin-3-galactose (or glucose)-catechin (peak 7), vinylpyrano-malvidin-3-arabinose-catechin (peak 8) (the chromatogram of the initial reaction solution recorded at 570 nm is provided in the SI).

The identity of the portisins that were formed during the synthesis was performed by HPLC coupled with a mass detector. The most important peaks identified correspond to vinylpyrano-delphinidin-3-galactose (or glucose)-catechin (peak 1), vinylpyrano-petunidin-3-galactose (or glucose)-catechin (peak 4), vinylpyrano-malvidin-3-galactose (or glucose)-catechin (peak 7) and vinylpyrano-malvidin-3-arabinose-catechin (peak 8). By mass spectrometry, it was not possible to undoubtedly identify if the sugar moiety corresponds to glucose or galactose (as they are isomers) but according to the relative abundance of the respective anthocyanins in the precursor extract (Fig. 2) it is possible to assume that peaks 1 and 4 should correspond to a galactoside derivative and peak 7 to a glucoside.

3.3. Centrifugal partition chromatography process optimization

The successful separation by CPC largely relies on various parameters, including the properties of the sample in the selected solvent system, physical properties of the solvent system itself, and operating parameters [40]. In fact, a crucial aspect for achieving an effective isolation in CPC is the ability to retain the stationary phase within the column chambers. Therefore, it is essential to optimize both the biphasic solvent system and the operating parameters to ensure efficient and reliable separation in CPC.

Table 1
The K value of portisins for different biphasic solvent systems.

Solvent system	Volume ratio	K range
EtOAc/BuOH/W	1:4:5	3.9 – 8.8
	2:3:5	4.0 – 11.1
	4:1:5	0.2 – 0.9
MtBE/BuOH/ACN/W	1:3:1:5	3.5 – 9.6
	2:2:1:5	1.5 – 6.3
	3:1:1:5	0.3 – 0.7

EtOAc: ethyl acetate, BuOH: n-butanol, W: water, MtBE: tert–butyl–methyl ether, ACN: acetonitrile.

3.3.1. Biphasic solvent system selection

The initial optimization step in CPC separation involves the selection of suitable biphasic solvent system. This can be achieved by careful selection of solvents and their ratios to form a biphasic solvent system which, consequently, would adjust the K values of portisins. The K value is the ratio of the target compound concentration between the two solvent phases (Eq. (1)). Ideally, the compound concentration should be equilibrated in the two immiscible solvent phases ($0.4 < K < 2.5$, ideally $K = 1$) [41]. A compound with a smaller K value tends to elute closer to the solvent front with lower resolution, while compounds with higher K values exhibit higher resolution but longer elution time [42]. To achieve optimal separation, the K value should be adjusted to 1 by modifying the ratio of the components in the biphasic solvent system.

The RM2 contains a mixture of anthocyanin derivatives in a great range of quantities and polarities. To achieve efficient separation of portisins, which are medium polar compounds, organic-aqueous solvent systems were used. According to previous studies, several solvent systems have been commonly employed for the isolation of anthocyanin and anthocyanin-derivatives by counter-current chromatography. These solvent systems include (1) ethyl acetate, n-butanol, and water; and (2) tert–butyl–methyl ether, n-butanol, acetonitrile, and water [43]. Hence, different compositions of both biphasic solvent systems were investigated for the isolation of portisins. All the phases were acidified with HCl (pH 1.5) to preserve the pH-sensitive structure of anthocyanin-derived pigments. Also, adding the acid to the solvent system often substantially shortens the settling time, improving the retention of the stationary phase during the CPC separation [37].

Three different compositions for each biphasic solvent system were used and the respective range of K value of the target compounds are summarized in Table 1. The results demonstrated that the presence of a larger amount of n-butanol in the solvent system resulted in a greater K value of portisins. The observed phenomenon can be attributed to the increase in polarity of the organic phase in the solvent system. As the polarity of the organic phase increases, thereby improving the portisins dissolution. Among all the solvent systems, MtBE/BuOH/ACN/W in the volume ratio of 2:2:1:5 acidified with HCl (pH 1.5) exhibited a range of K value (1.5 – 4.1) closer to the sweet spot of K for optimal performance. Different K were obtained for each portisin. These observed differences may be associated with the different affinity between each portisin and mobile phase (e.g., hydrogen bonds). In this specific case, the elution of the first portisin from the CPC column will occur at a retention volume equal to the sum of the volume of mobile phase inside the column plus 1.5 times the column volume [37]. This ensures that the separation process of portisins might be performed by this solvent system within an acceptable timeframe, optimizing the efficiency and productivity of the CPC system. Consequently, MtBE/BuOH/ACN/W (volume ratio 2:2:1:5) solvent system acidified with HCl (pH 1.5) was selected for portisins isolation by CPC.

3.3.2. Process parameters optimization

In CPC, once a suitable biphasic solvent system is adjusted for a specific separation, the adjustable operating parameters must be optimized. Considering the objectives and characteristics of the CPC process, a logical methodology capable of addressing the anticipated process parameters and complicated interactions. Also, the required high selectivity was necessary. The knowledge acquired from previous works [28–31,33], as well as some preliminary experimental results, simplified the task by keeping constant some factors (biphasic solvent system composition, sample solution preparation, injection mode, and operation mode),hence lowering the dimensions of the explorable space.

The preparation of the sample solution was fixed according to the K value of portisins. According to Ito [37], if $K > 0.5$, the sample solution should be prepared with the mobile phase. When $K \leq 0.5$, the stationary phase should be used as solvent in the preparation of the sample solution. In both cases, a small amount of the other phase should be added until two phases are formed. So, the sample solution was dissolved in a mixture of the mobile phase and stationary phase in a volume ratio of 9:1. The equilibrium mode was fixed, i.e., the sample was injected after the hydrostatic equilibrium was reached. This samples' injection mode is known to provide clear tracing of the elution curve and minimum carryover of the stationary phase [37].

The operating parameters and their ranges, sample load (100–500 mg/100 mL of column volume), mobile phase flow rate (10–20 mL/min), and rotation speed (1000–1600 rpm), were chosen under consideration of each effect and the maximum allowable pressure drop of 80 bar. A suitable balance was established between the number of needed experimental runs and the amount of acquired information. Considering the number of factors (3) and the predetermined objectives, a central composite face design consisting of a three-level full factorial design and star points put on the faces of the sides was chosen as an appropriate method for completing this task. To test the experimental variability and subsequently the uncertainty of the results as well as the adequacy of the underlying mathematical model, central duplicate points were added. Using response surface approach, the optimum conditions for portisins isolation by CPC were determined. Portisin content, process throughput, process efficiency, and environmental risk factor were examined as responses.

In this study, the elution-extrusion mode was fixed since this operation mode is known to extend the polarity range, being suitable for complex samples which contain solutes with a large range of K values. The extrusion step was used to collect all compounds that were retained on the CPC column after the portisins eluted, shortening the separation time.

The RM2 was fractionated by CPC yielding four main fractions (detailed information about each pool is provided in the SI). After being automatically collected every 25 mL, the fractions were subsequently grouped according to the color and the λ_{max} detected. In this sense, the initial fractions had an orange coloration and a λ_{max}~511 nm (which is a characteristic λ of anthocyanin-pyruvic acid adducts) and were therefore grouped into fraction 1 (F1). F1 contained the compounds that had the lowest K (0–$0.9 \leq K \leq 1.0$–3.3). The following fractions showed a blue/purple coloration and a λ_{max}~570 nm and were therefore grouped into fraction 2 (F2). F2 comprised the compounds that had values of K in the range $1.0–3.3 \leq K \leq 4.9–11$. Fraction 3 (F3) is composed of weakly blue/purple-colored fractions, showing a λ_{max}~220 nm. These fractions were separated from F2 because other compounds in higher concentration started to elute with the portisins. The K of these fraction pool ranged from 4.9–11 to 5.1–19.5. Fraction 4 (F4) contained the highly retained compounds with λ_{max}~220 nm. These fractions were grouped separately from F3 since they had a dark reddish coloration. F4 contained the compounds that had the highest partition coefficient ($4.2–12.6 \leq K \leq 6.2–23.6$).

Typical chromatograms of the collected fractions by CPC are shown in Fig. 4. From the results, it was possible to observed that the remaining anthocyanin-pyruvic acid adducts (λ_{max}~511 nm) were eluted in F1. These compounds have higher polarity compared to portisins and therefore have lower affinity for the stationary phase. As a result, they eluted earlier during the separation process. The separation of the more polar anthocyanin derivatives in the earlier fractions allows for a more selective isolation of the desired portisins in subsequent fractions.

The produced portisins (λ_{max}~570 nm) were subsequently eluted in F2 and F3. Among these fractions, F2 was found to be the richest fraction in portisins. However, it should be noted that F3 also contained some undesirable compounds (λ_{max}~220 nm) that co-eluted with remaining portisins. Based on HPLC analysis at 570 nm, the content of portisins in F1, F2, F3, and F4 of this representative CPC experiment was 0.1, 600.4, 40.7 and 1.4 mg/g, respectively (Fig. 4). These results provide insights into the distribution and concentration of portisins among the different fractions, with F2 being the most enriched fraction in terms of portisin content.

Table 2 presents the details of the experimental conditions and outcomes for each CPC experiment. The results indicated that the tested CPC conditions were suitable for preserving the stationary phase retention (over 50%) throughout the separation procedure, allowing for efficient separation of the compounds. The fraction with the highest portisin content was obtained with a sample load of 100 mg/100 mL of column volume at 10 mL/min of mobile phase flow rate and 1600 rpm of rotation speed, which was 600 mg portisin/g (Table 2 - experiment N5). Under these conditions, the process throughput, and the process efficiency of the experiment N5 were the lowest ones, 57 mg processed RM2/h and 4 mg portisin/h, respectively. Also, the environmental risk factor was higher (221 L solvent/g portisin). Whereas the highest process throughput and efficiency were 545 mg processed RM2/h and 37 mg portisin/h, respectively. These values were observed with a sample load of 500 mg RM2/100 mL at 15 mL/min of mobile phase flow rate and 1300 rpm of rotation speed (Table 2 - experiment N10.2), corresponding to a portisin content of 436 mg portisin/g, a lower value compared with experiment N5. Under these conditions, the environmental risk factor was lower (25 L solvent/g portisin). To obtain a selective separation (higher portisin content), the separation process takes more time. As a result, the process throughput and process efficiency decrease, and the environmental risk factor increase. The improvement in the CPC process might be in all four evaluated responses (portisin content, process throughput, process efficiency, and environmental risk factor).

In this study, the sample load and mobile phase flow rate had significant effects on two of the four evaluated responses: portisin content and process throughput. The process efficiency and environmental risk factor were only significantly affected by the sample load. The application of different rotation speed (1000–1600 rpm) resulted in no significant improvement in all the evaluated responses (model fit is provided in the SI). The goal was to optimize the isolation of portisins, maximizing the portisin content, process throughput, and process efficiency, minimizing the process environmental risk. To achieve that, a multiple linear regression approach with an automated optimizer (Modde® v.12) was used to predict one set of optimal conditions for all response variables. Twelve sets of optimal CPC conditions were proposed by the software. The output with the lowest overall distance to target ($\log(D) = 1.16$) was the following parameter-setting combination: sample load of 499 mg/100 mL of column volume, 20 mL/min of mobile phase flow rate, and 1600 rpm of rotation speed. Under these conditions, the fractionation of RM2 by CPC can result in optimal portisins content (460 mg portisin/g), process throughput

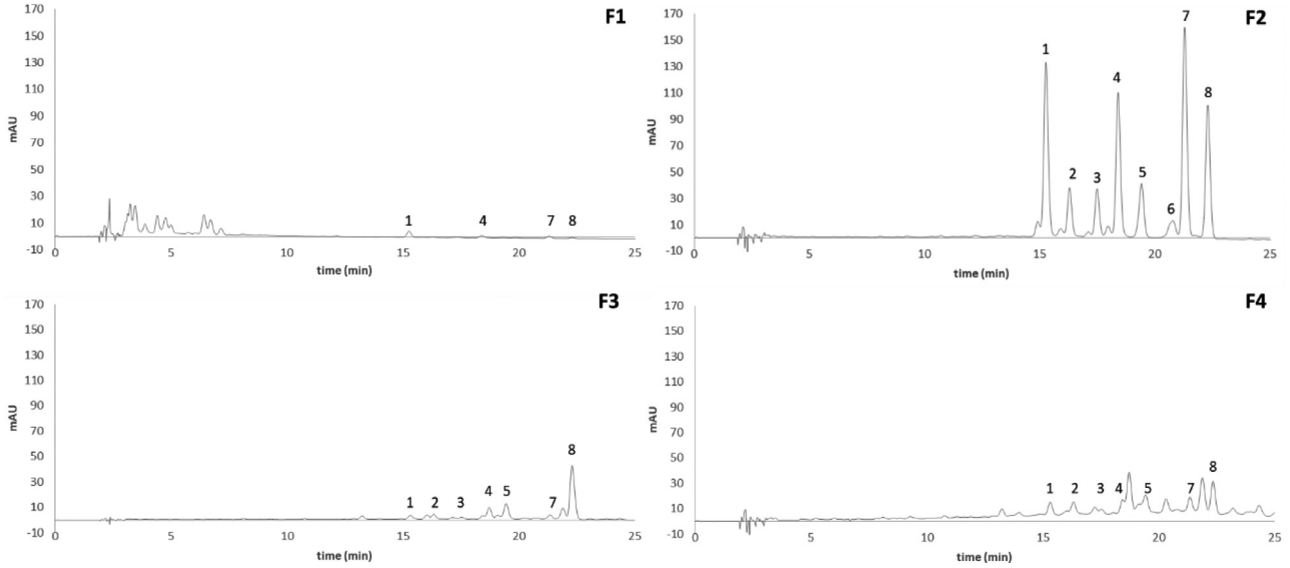

Fig. 4. Chromatograms of portisins detected at 570 nm corresponding to (F1) fraction 1, (F2) fraction 2, (F3) fraction 3 and (F4) fraction 4. Fractions collected during the CPC experiment N5 (2 mg RM2/100 mL of column, 10 mL/min of mobile phase flow rate, and 1600 rpm of rotation speed).

Table 2
CPC conditions applied and respective stationary phase retention (Sf), portisin content (P content) in the obtained portisin-enriched fraction (F2), process throughput (PT), process efficiency (PE), and environmental risk factor (Er).

Exp Name	Load (mg RM2/100 mL column)	Mobile phase flow rate (mL/min)	Rotation speed (rpm)	Separation time (h)	Sf (vol.%)	P content (mg P/g)	PT (mg RM2/h)	PE (mg P/h)	Er (L/g P)
N1	100	10	1000	3.3	79	595	60	4	223
N2	500	10	1000	4.0	79	478	248	17	55
N3	100	20	1000	2.7	68	503	75	6	278
N4	500	20	1000	2.5	66	373	391	19	83
N5	100	10	1600	3.5	79	600	57	4	221
N6	10	10	1600	3.7	80	465	265	20	46
N7	100	20	1600	3.1	67	591	64	4	417
N8.1	500	20	1600	2.2	69	483	458	29	48
N8.2	500	20	1600	2.1	72	459	480	35	38
N9	100	15	1300	2.8	73	559	71	4	310
N10.1	500	15	1300	1.9	72	404	522	26	38
N10.2	500	15	1300	1.8	75	436	545	37	25
N11	300	10	1300	3.5	80	587	170	13	70
N12	300	20	1300	2.5	72	468	234	16	95
N13	300	15	1000	3.0	72	539	195	12	108
N14	300	15	1600	3.0	75	425	197	13	96
N15	300	15	1300	3.4	71	494	176	11	120
N16	300	15	1300	3.0	74	496	197	15	83
N17	300	15	1300	3.4	69	585	176	12	114

Table 3
Predicted and experimental values (n = 2) of response variables under optimum CPC conditions and experimental values of response variables obtained by column chromatography (CC) experiment.

	Portisin content (mg P/g)	Process throughput (mg processed RM2/h/Vc*)	Process efficiency (mg P/h/Vc)	Environmental risk factor (L/g P)
CPC				
Predicted	460	2.40	0.15	52
Experimental	469 ± 46	2.06 ± 0.14	0.19 ± 0.03	30 ± 6
CC				
Experimental	396 ± 52	2.09 ± 0.02	0.08 ± 0.01	164 ± 38

* CPC column volume = 200 mL; RP-LC column volume = 12 mL.

(474 mg processed RM2/h), process efficiency (30 mg portisin/h) and environmental risk factor (52 L/g portisin). The comparison between the predicted results and the experimental results obtained using optimum isolation conditions is presented in Table 3. These findings reveal a strong agreement between the predicted and experimental outcomes, indicating that the response surface methodology model was successfully validated with a good correlation level. Also, it was possible to verified that the range of K calculated for F2 of the CPC experiment under the optimized conditions - K = 1.6–6.5 (more details in the SI) was close to the range of K determined in the shake flask test - K = 1.5–4.1 (Table 1).

3.4. Reversed-phase liquid chromatography

RM2 was fractionated by gradient preparative RP-LC to compare with the separation performance of CPC. The operating parame-

ters, namely sample load and mobile phase flow rate were selected considering the column manufacturer's specifications. The elution was carried out with aqueous ethanol with increasing percentage of ethanol up to 100 vol.% as described by Mateus and coworkers [5]. During the elution, 65 fractions were collected automatically and subsequently grouped into F1 and F2 according to the color and the maximum absorption wavelength detected. F1 contained the compounds that were eluted during the first 3 h of the separation. This fraction presented an orange color (λ_{max}∼511 nm), which is characteristic of anthocyanin-pyruvic acid adducts. Portisins were subsequently collected in F2 during the last 43 min of the separation process. Due to the presence of portisins, F2 exhibited a bluish/purple color (λ_{max}∼570 nm). Based on HPLC analysis at 570 nm, the portisins content in F1 and F2 of RP-LC experiments was 14.2 ± 0.1 mg/g, and 395.6 ± 51.9 mg/g, respectively.

There are two reports regarding the production portisins from a blueberry (*Vaccinium myrtillus*) extract [9,16]. The authors isolated portisins by Toyopearl gel column chromatography by elution with water/ethanol, following the same procedure previously described by Mateus and coworkers [5]. However, these works focused on the antioxidant properties [16] and MPP+ intestinal uptake [9] of the blueberry extract and two respective anthocyanin-derived reaction mixtures (anthocyanin pyruvic acid adducts and portisins). There is not enough data in the literature to allow comparison of separation process performance.

3.5. Comparison of different purification methods

The use of CPC for portisins isolation from RM2 demonstrated significant advantages when compared to RP-LC, as evident from the results presented in Table 3. The comparison of portisin content in the fractions obtained by the two chromatographic techniques reveled similar selectivity toward portisins. Both methods yielded comparable values of portisin content and process throughput, indicating similar overall performance in terms of product concentration and processing capacity. However, notable differences were observed in terms of process efficiency and environmental risk factor. The process efficiency was significantly higher in CPC (2.4 times) compared to RP-LC. This suggests that CPC offers a more efficient and rapid process for the isolation of portisins. Furthermore, the environmental risk factor, was found to be lower in the CPC technique (5.5 times). These results indicate that CPC has a lower environmental impact, as it generates less waste solvent compared to RP-LC. Other studies available in the literature shown the same promising effect when comparing countercurrent chromatography with RP-HPLC [44,45]. Overall, the results highlight the irrefutable advantages of CPC over RP-LC for the isolation of portisins from the RM2 in terms of process efficiency and environmental sustainability.

4. Conclusions

The study reported emphasizes the potential of CPC for the separation of bluish anthocyanin derived pigments. The optimum CPC conditions for the isolation of portisins were identified using a graphical optimization technique that incorporate response surface methodology with a desirability function, and the predicted values for all the analyzed responses were excellent agreement with the experimental ones. The optimum conditions include 464 mg of sample/100 mL of column volume, 20 mL/min of mobile phase flow rate, and 1600 rpm of rotation speed for the portisin content, process throughput, efficiency, and environmental risk factor of 469 mg portisin/g, 2.06 mg processed RM2/h/Vc, 0.19 mg portisin/h/Vc, and 30 L/g portisin, respectively. These conditions allowed the purification of portisins of 5-fold. The process efficiency was 2.4 times higher than the RP-LC process. The CPC process environmental risk factor was 5.5 times lower than in the conventional process. The key benefits of CPC technology include a substantial decrease in separation time and solvent usage, which improved the efficiency and environmental risk factor of the process in comparison to RP-LC. In conclusion, the results showed that the proposed protocol was an efficient and appropriate method for the production and isolation of blue portisin pigments with various application in the nutraceutical and food industries, thereby aiding in the sustainable use of these blueberry residues. As future work, it would be interesting to explore this technique for the separation of anthocyanin-pyruvic acid adducts prior to the portisins synthesis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ana N. Nunes: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Viktoriya Ivasiv:** Validation, Investigation, Writing – review & editing. **Luís F. Gouveia:** Methodology, Validation, Formal analysis, Writing – review & editing. **Naiara Fernández:** Resources, Writing – review & editing. **Joana Oliveira:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Supervision, Funding acquisition. **Maria Rosário Bronze:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition. **Ana A. Matias:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2023.464150.

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