



## Purification of anthocyanins from grape pomace by centrifugal partition chromatography

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### ABSTRACT

The purpose of this work is to integrate the extraction of anthocyanins from grape pomace with a sequential purification process using an aqueous two-phase system (ATPS), and scaled-up in centrifugal partition chromatography (CPC) using as a common solvent the protic ionic liquids based on ethanolammonium and sulfuric acid. The choice of the best operational condition was performed through the selectivity (ratio between the concentration of anthocyanin and total phenolic compounds), which allowed the purification of anthocyanins of 16.36 fold (S/L = 55 mg.mL<sup>-1</sup>; 35 °C; [PIL] = 12.5%), and 2.21 fold (S/L = 55 mg.mL<sup>-1</sup>; 25 °C; [PIL] = 12.5%). ATPS based on protic ionic liquid and acetonitrile purified the anthocyanins between 19.30 and 23.88 fold at 25 °C and pressure atmospheric. At 25 °C (limitation of equipment), CPC increased the purification factor to 41.88, although with the decrease of anthocyanin recovery. However, the best operational condition depends on the anthocyanins application.

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### 1. Introduction

Polyphenols are a group of secondary plant metabolites, characterized by the presence of more than one phenol unit or building block per molecule. In general, they are classified into hydrolyzable tannins (gallic acid esters of glucose and other sugars) and phenylpropanoids such as lignins, flavonoids, and condensed tannins [1].

Anthocyanins are one type of water-soluble flavonoids, which are synthesized by the flavonoid branch of the phenylpropanoid pathway in higher plants [2,3]. There are more than 600 types of anthocyanins, and the majority of anthocyanin aglycones is based on anthocyanidins (sugar-free anthocyanins) such as cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin [4]. They share a 2-phenylbenzopyrilium (flavylium) skeleton hydroxylated in 4', 5, and 7 positions, with different substitutions at R<sub>1</sub> and R<sub>2</sub> (Fig. 1). The biomolecules have a positive charge in its C-ring, which leads to dependence color and stability in the function of pH [5], and represent major drawbacks in anthocyanins application as pigments. In acid media, anthocyanins exist in an equilibrium between red-colored flavylium cation (pH 1–3) and colorless hemiketal forms (pH 4–5). On the other hand, at alkaline pH the

flavylium cation is converted into blue-colored quinoidal bases (pH 6–7) due to an acid-base equilibrium, and the stability of quinoidal pigment is poor [6].

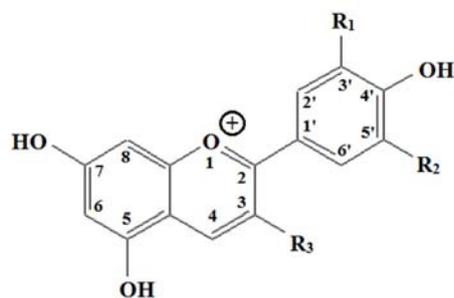
Anthocyanins are the most widely distributed pigment present in the plant and responsible for the red, blue, and purple colors in flowers [7], fruits [8], leaves [9], and seeds [10].

Grapes have a high content of anthocyanins, which can be found inside of vacuoles of grape skins cells. The variety of grape and ripeness degrees have a great influence on the composition of anthocyanins [11]. Worldwide was produced 77.2 million tonnes of grape in 2013 [12], of which 55% was used in the winery industry [13], generating 20% of waste (skins, seeds, and rachis) [14]. Consequently, approximately 8.49 million tonnes of waste is produced in the world, which characterizes as an important natural source of valuable phenolic compounds like flavonoids and anthocyanins [15].

These biomolecules are widely used in the food and beverage industry as nutraceuticals, in the pharmaceutical and cosmetic industry for their positive effects on human health. In pharmaceutical products, the anthocyanins are extremely applied as therapeutics biocompounds due to their high bioavailability, providing several health benefits such as reducing the risk of coronary heart diseases and stroke, ocular diseases, anti-carcinogenic and antioxidant activities, and anti-inflammatory effect [16,17]. The effects of anthocyanins in cosmetic products are associated with their capability as a colorant, antioxidant, anti-fatigue, and anti-aging active [18,19].

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Anthocyanidin	R <sub>1</sub>	R <sub>2</sub>	λ <sub>max</sub> (nm)	MW	Color
Cyanidin (Cy)	OH	H	545	287	Red
Delphinidin (Dp)	OH	OH	557	303	Purple
Malvidin (Mw)	OCH <sub>3</sub>	OCH <sub>3</sub>	546	331	Red
Pelargonidin (Pg)	H	H	530	271	Orange
Peonidin (Pn)	OCH <sub>3</sub>	H	542	301	Red
Petunidin (Pt)	OCH <sub>3</sub>	OH	558	317	Purple

Anthocyanins - R<sub>3</sub> = glucose, galactose, rhamnase, xylose or arabinose

λ<sub>max</sub> = wavelenght of maximum absorption

MW = molecular weight

Fig. 1. Chemical structure and characteristics of anthocyanins and its corresponding anthocyanidins.

The different anthocyanins applications require efficient extraction methods of target biomolecules from biomass. Therefore, traditional solvent extractions were performed and extraction assisted by ultrasound [20,21] or by microwaves [22,23], and supercritical extraction [24,25] have an important role in the recovery of anthocyanins from raw material.

The literature has studied the application of several solvents (methanol and ethanol), acidified or not with mineral or organic acid, to enhance the extraction because the acids denature the cellular membranes and facilitate the solubilization of anthocyanins [26,27]. On the other hand, Revilla et al. [28] suggested avoiding acids (example: 1% of 12 N hydrochloric acid) in anthocyanins extraction from grapes containing acetylated anthocyanins. A new generation of solvents namely ionic liquids is also used, despite literature do not describe this possibility until now.

Ionic liquids (IL) are defined as molten salts with a melting point of less than 100 °C, due to reduced electrostatic forces between cations and anions, and their asymmetric, making it difficult to form a regular crystalline structure [29]. This class of solvents can be classified as aprotic ionic liquids (AIL) and protic ionic liquid (PIL). The protic ionic liquid is a subset of ionic liquid synthesized through a neutralization reaction between acid- and base-Brønsted. The transference of proton from the acid to the base is the main property that distinguishes PIL from AIL [30]. The structure of PIL has a proton donor available for hydrogen bonding and leads to usually non-negligible vapor pressure and some are distilled media [31]. The main cations are ammonium, 1-alkyl imidazolium, 1-alkyl-2-alkyl imidazolium, and 1,1,3,3 tetraethylguanidine, whilst the main anions are organic (carboxylates), inorganic (nitrate or hydrogen sulfate), or fluorinated (bis(trifluoromethanesulfonyl)imide (TFSI), trifluoroacetic acid (TFA), and bis(perfluoroethylsulfonyl)imide (BETI), tetrafluoroborate, or hexafluorophosphate). A large number of combinations of cations and anions allows the formation of various ionic liquids. Thus, a wide range of properties such as viscosity, density, and solvation power can be modulated [32]. In this sense the ILs are called designer solvents.

Besides target molecules, the conventional extraction processes remove from biomass other compounds such as phenolic compounds, flavonoids, organic acids, carbohydrates, and proteins, which act as contaminants and accelerate the anthocyanin degradation during the storage. Therefore, the process is not selective [33]. Moreover, a suitable and efficient purification procedure should be applied, since it is known that 80% of the process costs are associated with the purification methods. Some protocols for purification of anthocyanins are reported in the literature like chromatography [34–36], and adsorption [37,38].

Another method, which has been highlighted in the purification of anthocyanins, is the aqueous two-phase system (ATPS). This system is formed as the result of mutually incompatible between two aqueous solutions of different compounds above a certain critical concentration and a spontaneous phase separation takes place [39,40]. ATPSs are traditionally formed of two polymers or polymer-salt [41]; however, the limited polarity window and the high viscosity of polymer affect the phase formation, partitioning, and purification of molecules. As an alternative, other systems have also been reported such as organic solvents-salts [42] or -carbohydrates [43]; and since 2003 ionic liquids have used [44] as a constituent in ATPS in different combinations such as IL-salt [45]; IL-carbohydrates [46]; IL-polymers [47,48]; and IL-organic solvents [49–51]. Despite ATPS has been well described and deeply studied in a single-step, few data using ATPS based on protic ionic liquids and organic solvents such as acetonitrile, and scale-up using centrifugal partition chromatography (CPC).

CPC is a support free preparative chromatographic method, in which a biphasic system is used to partition compounds between two immiscible liquid phases (one is the stationary phase and the other one is the mobile phase) according to their partition coefficient; and the phases are maintained separated in the column by centrifugal forces [52]. This procedure differs from counter-current chromatography because there is no counter-current between the phases (one is mobile, and the other is stationary) and from conventional solid-support chromatography in several ways [53]. Scaling up is a concern in the separation and purification processes of biomolecules. According to Delaunay et al. [54], preparative high-pressure liquid chromatography (HPLC) is used to purify phenolic compounds from grapes. However, there are sample loss and deterioration of the column with the extract and above 50% material weight never eluted because sticks strongly onto the solid support. Higher selectivity is reached using a specific design for the system, which decreases the amount of solvent. According to Bouju et al. [52] after the initial optimization procedures in ATPS (simple step) and in CPC using small volume (multi-stage), it is necessary to expand the initial cell volumes. In the scale-up method used to separate aspirin and coumarin, the increase in the separation cell volume was increased from 35 to 239 mL (6.8 times), which allowed an increase in productivity from 867 to 4550 mg.h<sup>-1</sup>. CPC suppliers such as Kromaton Rousselet-Robatel (equipment used in this work) provide equipment with an analytical scale of 25 and 50 mL, and a preparative scale of 50, 200 and 1000 mL, allowing an increase between 40 and 50 times between the preparative and analytical scale. Thus, the scale-up is easily achieved and there is no irreversible adsorption onto a solid support [55].

This work addressed the development of a platform for the purification of anthocyanins from grape pomace using protic ionic liquid, named bis(2-hydroxyethyl)ammonium hydrogen sulfate – [2HEA]HSO<sub>4</sub>. In this sense, the protic ionic liquid based on diethanolamine and sulfuric acid was synthesized and used in the extraction of anthocyanins, which were studied the effect of solid-liquid ratio, temperature, and concentration of the ionic liquid. Then, phase diagrams reported in the literature using [2HEA]HSO<sub>4</sub> and acetonitrile supported the purification of anthocyanins using aqueous two-phase systems. It was studied the effect of the tie-line length and temperature. Finally, the scale-up of anthocyanins purification process was carried out using a centrifugal partition chromatography.

## 2. Material and methods

### 2.1. Material

Grape pomace samples from Portuguese caste were kindly provided by winemakers from Lower Austria. The samples were dried at 50 °C in a vacuum oven (Binder VD53) until constant weight to prevent degradation of anthocyanins [56]. The dried samples were frozen using liquid nitrogen, ground in an ultra-centrifugal mill (Retsch ZM 100), packed in polypropylene bags, and finally stored [50].

The ionic liquid used in this work bis(2-hydroxyethyl)ammonium hydrogen sulfate – [2HEA]HSO<sub>4</sub> was synthesized according to the protocol established by George et al. [57] in neutralization reaction (Fig. S1 of Supporting Information), and already presented in the literature by our research group [58]. The ionic liquid was chosen because it presents an intermediate biphasic area among those used in previous works of the group, and in our point of view, it brings more assertive responses of the studied effects. The details of the synthesis and purity of IL (Fig. S2) are presented in the Supporting Information. The phase diagram (Fig. S3), mass fraction composition and tie-line length (Table S1) also present in the Supporting Information.

### 2.2. Solid – Liquid extraction

The extraction of anthocyanins was performed on a magnetic stirrer with temperature control for 60 min using [2HEA]HSO<sub>4</sub> and several condition of solid-liquid ratio (10, 25, 40, 55 and 70 mg.mL<sup>-1</sup>), temperature (25, 35, 45 and 55 °C) and ionic liquid concentration (5, 10, 12.5, 15, 20 and 25%, m/v). Glass vial were loaded with different amount of dried residues and ionic liquid solution was added (6 mL). After the extraction, the samples were centrifuged at 10,000 rpm for 5 min (VWR Micros tart 12) to remove the finely suspended particles.

The total anthocyanin concentration ([ANT]) was determined via the pH-differential method [59]. Briefly, appropriate amount of samples was diluted with potassium chloride buffer (0.025 M, pH 1.0) or sodium acetate buffer (0.4 M, pH 4.5). Absorbances were determined at 534 and 700 nm on a Shimadzu UV-1800 UV-Vis spectrophotometer employing the following eqs. (1–2):

$$[ANT] = \frac{A \times M_w \times DF}{\epsilon} \times L \quad (1)$$

$$A = (A_{534} - A_{700})_{pH\ 1.0} - (A_{534} - A_{700})_{pH\ 4.5} \quad (2)$$

where A is the absorbance, M<sub>w</sub> is the molecular weight for the main anthocyanin present in grape (malvidin 3-glucoside – 493.2 g.mol<sup>-1</sup>), DF is the dilution factor, ε is the molar absorptivity (29,500 L.cm<sup>-1</sup>) and L is the path length (1 cm). The data were expressed as milligram total anthocyanins.g<sup>-1</sup> dry grape pomace (solid-liquid extraction) or milligram total anthocyanins.mL<sup>-1</sup> (ATPS).

Total phenolic concentration ([TPH]) were determined by the Folin-Ciocalteu method [60]. Briefly, aliquots of 0.1 mL were mixed with 2.8 mL of deionized water, 2 mL of 2% sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>),

and 0.1 mL of 50% (v/v) Folin-Ciocalteu reagent. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 750 nm against a deionized water blank on a UV-Vis Spectrophotometer (Shimadzu UV-1800). Gallic acid – GA (0–200 mg.L<sup>-1</sup>) was chosen as standard. The data were expressed as milligram gallic acid equivalents (GAE).g<sup>-1</sup> dry grape pomace (solid-liquid extraction) or milligram quercetin equivalents (QE).mL<sup>-1</sup> (ATPS).

### 2.3. Anthocyanins purification

The purification of anthocyanins was carried out using aqueous two-phase system based on acetonitrile and the same protic ionic liquid used in solid-liquid extraction. The partition systems were prepared using the anthocyanin extraction biomass-free, in which was added the appropriate amounts of the acetonitrile, ionic liquid (considering the quantity present in the extract solution) in 2.5 mL screw-cap vials to a total mass of 2.0 g. Then, the mixtures were gently stirred and the graduated tubes were equilibrated at the chosen 25 °C and atmospheric pressure for 1 h using a thermostatic bath Haake K15 to reach the equilibrium and to promote the complete partition process of anthocyanins. The two phases were then cautiously collected for the determination of their volume and weight, before the total anthocyanins (target biomolecule) and total phenolic compounds (contaminant biomolecule) were quantified in both top and bottom phases. The quantification was performed in triplicate using as a blank consisting of top or bottom phase. The final partition coefficients and extraction efficiencies was reported as average of the three assays (accompanied by the respective standard deviations). It should be remarked that for all studied ATPS, the top phase was the acetonitrile-rich phase while the bottom phase corresponded to the ionic liquid-rich phase.

The partition coefficient (K) was defined as the ratio between the concentrations of total anthocyanins (K<sub>ANT</sub>) and total phenolic compounds (K<sub>THP</sub>) in the top and bottom phases (Eq. (3)). In the process, it was also evaluated the volume ratio (R<sub>v</sub>) and the recovery percentages of total anthocyanin in the top (R<sub>T</sub>) and bottom (R<sub>B</sub>) phases according to the Eqs. 4–6.

$$K = \frac{C_T}{C_B} \quad (3)$$

$$R_v = \frac{V_T}{V_B} \quad (4)$$

$$R_T = \frac{100}{1 + \left(\frac{1}{R_v \times K_{ANT}}\right)} \quad (5)$$

$$R_B = \frac{100}{1 + R_v \times K_{ANT}} \quad (6)$$

where C is the concentration of total anthocyanin concentration or total phenolic compounds, V is phase volume and T and B correspond to the top and bottom phases, respectively.

The purification factor (PF) was determined by the ratio between the selectivity (S) in top or bottom phase and in the crude ionic liquid extract according to Eqs. 7 and 8.

$$S = \frac{[ANT]}{[TPH]} \quad (7)$$

$$PF_T \text{ or } B = \frac{S_T \text{ or } B}{S_E} \quad (8)$$

where, [ANT] is the anthocyanin concentration, [TPH] is the concentration of total phenolic compound, the subscripts T and B correspond to top and bottom phase, respectively; and E is indicative of the ionic liquid crude extract.

### 2.4. Scale-up using CPC

The scale-up experiments were performed using a Fast Centrifugal Partition Chromatography (FCPC)<sup>®</sup> system; model FCPC-C, from Kromaton Rousselet-Robatel (Annonay, France). The design comprises a pattern of cells interconnected by ducts and dug in a stainless steel disk. The cell design was called as “twin” cells and contains a restriction in the middle ducts of the canal creating two superimposed chambers. The rotor was made associating 13 disks, each one containing 64 “twin” cells and being a total of 832 twin cells. The total cell volume was about 50 mL with 10 mL or 20% of the column volume corresponding to the connecting ducts. The maximum theoretical liquid stationary phase retention factor  $S_f (= V_s/V_C)$  is 80% since the 20% of connecting duct volume can only contain mobile phase. The maximum rotor rotation is 3000 rpm generating a maximal centrifugal field of  $\approx 1500$  G. Two rotating seals are needed at the rotor entrance and exit (also called column top or “head” and column bottom or “tail”). They can withstand a maximum pressure of 70 bar (7 MPa or 1000 psi). The CPC system was connected to an ECOM ECB2004 Gradient box with degasser, an ECOM ECP2010 Analytical HPLC pump, and an ECOM Flash 14 DAD Detector with four wavelengths simultaneously and continuous scan (ECOM spol. S.r.o., Czech Republic). Fractions were collected with an ADVANTEC<sup>®</sup> Super Fraction Collector CHF122SC (Advantec Toyo Kaisha, Ltd., Tokyo, Japan). The sample was injected manually using a Rheodyne valve model 3055–023 through a 10 mL sample loop. Analogical detector signals were processed using the ECOMAC software (ECOM spol. S.r.o., Czech Republic).

The FCPC operations were carry out using two TLL (TLL = 46.45 – [ACN] = 49.97 wt%, [PIL] = 10.00 wt% and [H<sub>2</sub>O] = 40.03 wt%; and TLL = 88.17 – [ACN] = 49.70 wt%, [PIL] = 18.54 wt% and [H<sub>2</sub>O] = 31.76 wt%). This system was used on descending and ascending modes. The rotor was entirely filled with the ACN-rich phase – top phase for the descending mode and with the ionic liquid-rich phase – bottom phase for the ascending mode at 600 rpm to have a homogeneous solvent reequilibration on the rotor. Then the rotation was set up at the higher speed (2500 rpm) needed for appropriate stationary phase retention. After the working rotational speed was set up, the ionic liquid-rich phase – bottom phase or the ACN-rich phase – top phase in the descending mode or ascending mode, respectively, was pumped through the stationary phase until the equilibrium was reached, i.e. when only the mobile phase came out of the column and the signal baseline stabilized. The mobile phase flow rate was 1.5 mL min<sup>-1</sup> in order to increase the stationary phase retention ratio and at the same time decrease the purification time. The stationary phase retention  $S_f$  is calculated by the ratio of the stationary phase volume ( $V_s$ ) and the column volume ( $V_C$ ):  $S_f = V_s/V_C$ . For ascending mode was obtained a  $S_f$  value of 0.46 and for descending mode was achieved a  $S_f$  value of 0.36. The sample loop was filled with the ionic-liquid rich-phase in descending mode and the completed ATPS in ascending mode. Fractions of 5 mL were collected after the mobile phase pump was started; this gave an accurate record of the displaced volumes of upper and lower phases from the column for the duration of the run.

### 3. Results and discussion

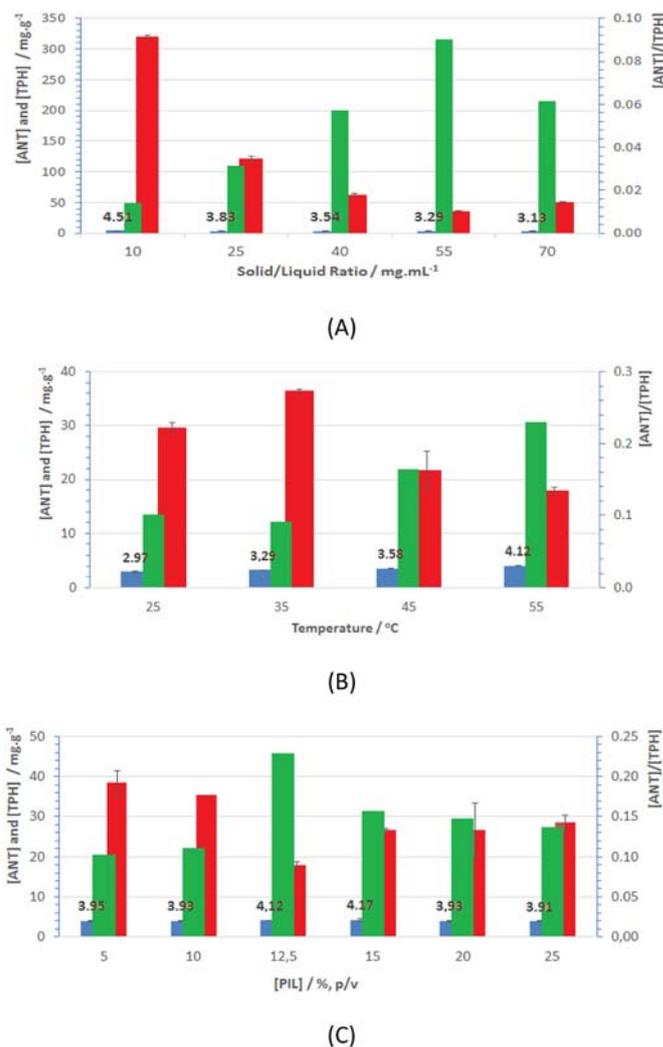
Nowadays, the great interest in improving the life quality has led to replacement of synthetic compounds by their corresponding natural, and encouraged a large number of research into developing extraction and purification methods of biomolecules. The food, pharmaceutical and cosmetics industry given the behavioral changes also have interest in these compounds, but with different purity levels, which are directly correlated to their application. In the case of anthocyanins, the purification is necessary in their application as coloring agents and antioxidants, but the physical and economic efforts of separation into individual antioxidants are questionable. For this reason, the results presented in this section are based on the content of total anthocyanins and their

separation and purification from phenolic compounds, which we use as standard contaminant. All assays were performed with spectroscopic method and we did not use high-pressure liquid chromatography (HPLC), according Lapornik et al. [26] the total anthocyanins concentration measured in extract with 70% of ethanol using spectrophotometer ( $995.1 \pm 9.2$  mg.L<sup>-1</sup>) were similar to that found in HPLC ( $1043.4 \pm 8.9$  mg.L<sup>-1</sup>), this mean a difference between 2.9 and 6.3%. Therefore, the implementation of a sophisticated and time-consuming technique does not justify for a determination of total anthocyanins content.

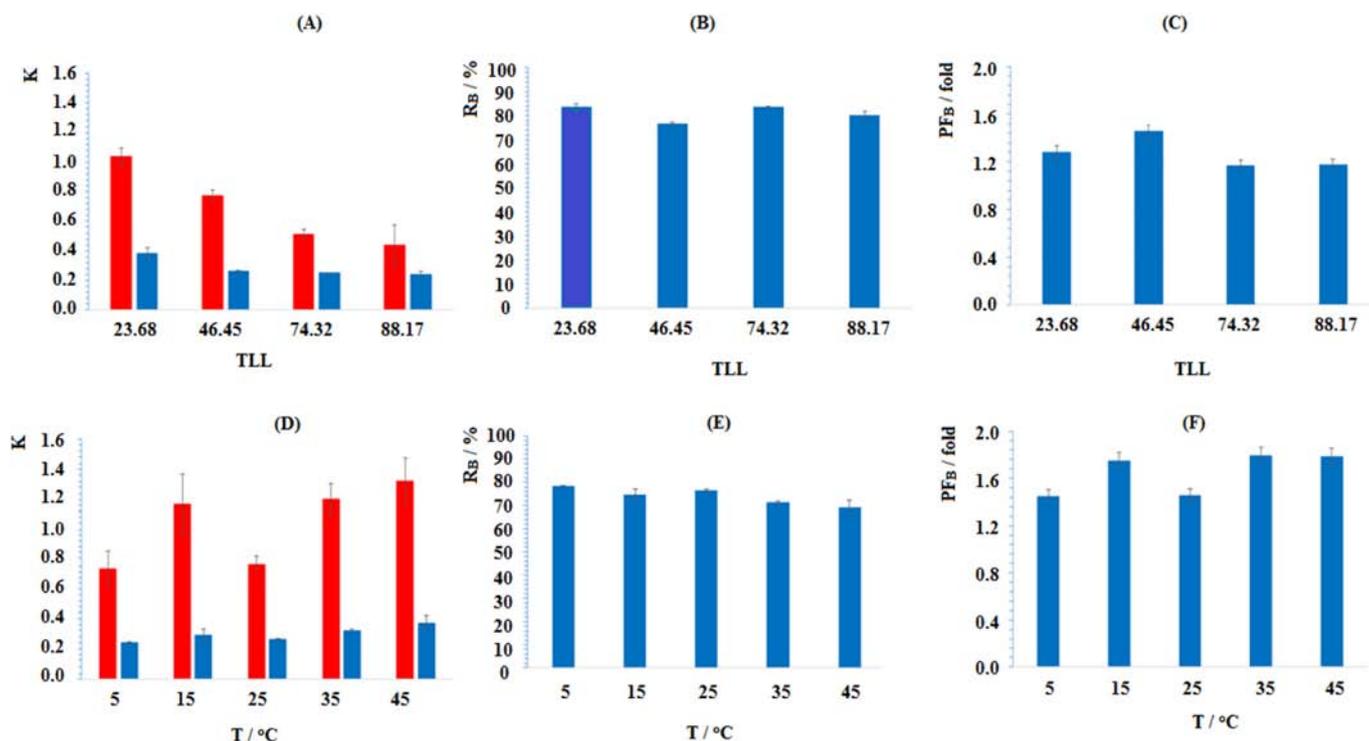
#### 3.1. Solid-liquid extraction

Initially, it was compare the effect of traditional solvent such as water and ethanol (Fig. S4), as well as the ionic liquid [2HEA]HSO<sub>4</sub> in anthocyanin extraction. [2HEA]HSO<sub>4</sub> was used because had a larger bi-phasic area. The data of this preliminary study are shown in Fig. S5 of Supporting Information and refers to anthocyanin extraction with 25 mg.mL<sup>-1</sup> of solid-liquid ratio at 35 °C, ranging the ethanol concentration from 0 to 100% (v/v).

Anthocyanin extraction with aqueous solution of ethanol (75%) reached the maximum of  $141.44 \pm 4.09$  mg.L<sup>-1</sup>. Water and pure ethanol gave extracts with  $45.68 \pm 1.32$  mg.L<sup>-1</sup> and  $57.65 \pm 1.67$  mg.L<sup>-1</sup>,



**Fig. 2.** Effect of solid-liquid ratio (A), temperature (B) and protic ionic liquid (C) in anthocyanin extraction. ■ - anthocyanin concentration; ■ - total phenol concentration; ■ - selectivity (ANT/TPH).



**Fig. 3.** Effect of tie-line length (A–C): [2HEA]HSO<sub>4</sub> at 25 °C, and temperature (D–F): [2HEA]HSO<sub>4</sub> in partition coefficient of anthocyanins - K<sub>ANT</sub> (blue) and phenolic compounds - K<sub>TPH</sub> (red), recovery (R<sub>b</sub>) and purification factor (PF<sub>b</sub>) of anthocyanins in bottom phase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

respectively. The dielectric constant of alcohol decreases with increasing of alkyl chain, and they are lower than in water [61]. Thus, solvents with low dielectric constant enhancing the solubility and diffusion of anthocyanins. On the other hand, in presence of pure organic solvents, the vegetable cell dehydrates and collapses, leaving to denaturation of cell wall proteins and difficult the diffusion of anthocyanins from biomass to solvent [62]. The anthocyanin extraction using the protic ionic liquid [2HEA]HSO<sub>4</sub> (12.5%, p/v) at 35 °C in the solid-liquid solvent of 25 mg.mL<sup>-1</sup> (141.51 ± 1.17 mg.L<sup>-1</sup>) was similar when comparing with those using the ethanol (traditional solvent).

In order to understand the effect of each variable in anthocyanins from grape pomace extraction, the range of solid-liquid ratio, temperature and ionic liquid concentration were studied, and the results are shown in Fig. 2 (Tables S2 - S4 of Supporting Information).

Five solid-liquid ratios (mg.mL<sup>-1</sup>) were used for each part, all in 6 mL of solvent: 10 (60 mg), 25 (150 mg), 40 (240 mg), 55 (330 mg) and 70 (420 mg) at 35 °C and 12.5% (v/v) of [2HEA]HSO<sub>4</sub>. The anthocyanins and phenolic compounds concentration decreased with the increase of solid-liquid ratio (Fig. 2A). These results suggest a saturation of solvent extractor with increasing biomass content, which are more drastic for phenolic compounds (reduces by 88.6%, from 320 to 36.4 mg.g<sup>-1</sup>) than for anthocyanins (reduces by 30.6%, from 4.51 to 3.13 mg.g<sup>-1</sup>). Mané et al. [63] extracting polyphenol compounds from grape (Pinot Noir variety) and using water:acetone (40:60 v/v) at 22 °C during 2 h found similar results. In this case, the concentration of anthocyanins keep constant (~ 6 mg.g<sup>-1</sup> skin) and phenolic acid decreased significantly, 82.6% (from 2.3 to 0.4 mg.g<sup>-1</sup> skin).

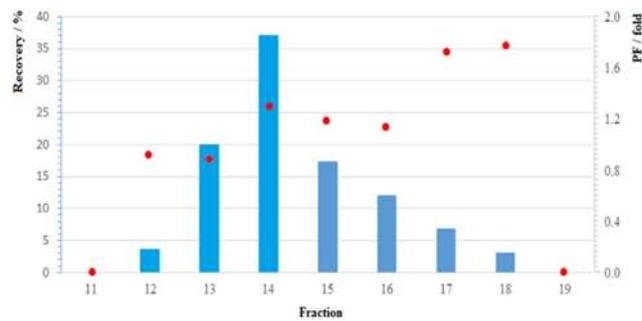
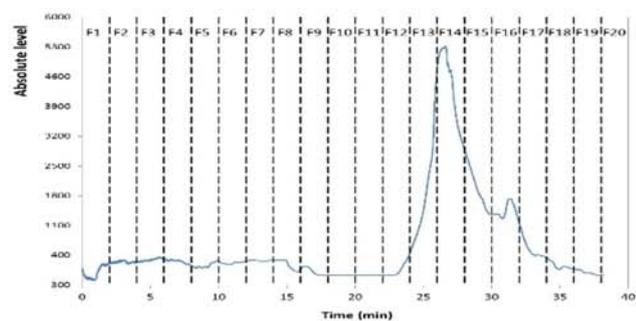
Traditionally, the best condition in extraction process are chosen according to highest concentration of target biomolecule. Nevertheless, in this study we inserted the approach of separation engineering, in which concern for the purification is thought from the extraction [64]. In this sense, it determined the selectivity of extraction (S) by the ratio between the anthocyanins and phenolic compounds concentration. Thus,

the best extraction occurred at 55 mg.mL<sup>-1</sup> ( $S = [ANT]/[TPH] = 0.090 \text{ mg ANT. mg}^{-1} \text{ TPH}$ ), this mean that anthocyanins and present in most quantitative in the extract than the contaminants (total phenolic compounds). This condition (55 mg.mL<sup>-1</sup> of solid-liquid ratio) was used in the further experiments.

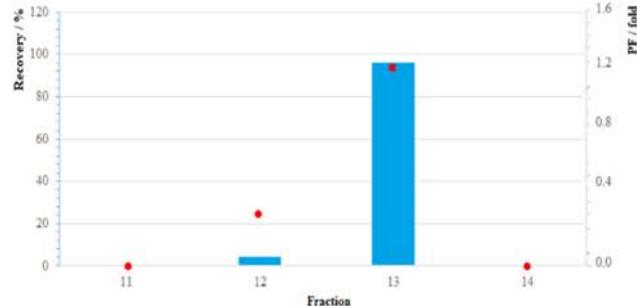
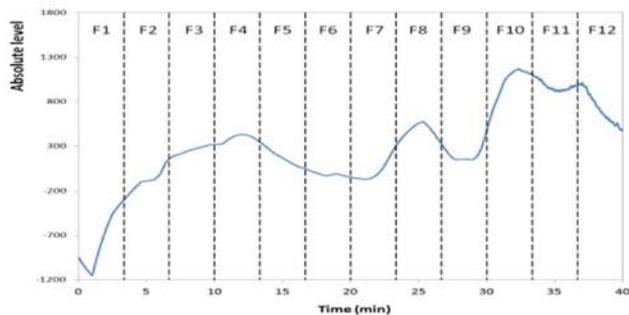
The effect of temperature in anthocyanin extraction was carried out using 55 mg.mL<sup>-1</sup> of solid-liquid ratio and 12.5% of [2HEA]HSO<sub>4</sub> (Fig. 2B). Anthocyanin extraction was favored by increasing extraction temperature (25 to 55 °C), that according to Pinelo et al. [65] is due to enhancing of anthocyanin solubility and diffusion coefficient. Anthocyanins are thermolabile; hence, the temperature can not be increased indefinitely. Vatai et al. [66] also reported that the extraction temperature usually increases the amount of anthocyanins and phenolic compound. Nevertheless, this argument was not valid for phenolic compounds, which reached a maximum concentration at 35 °C, probably due to saturation of solvent. The selectivity ([ANT]/[TPH]) increased continually and reached 0.229 mg ANT. mg<sup>-1</sup> TPH.

The influence of protic ionic liquid concentration (5 to 25%, v/v) in anthocyanins extraction was investigated (Fig. 2C). The increase or decrease of previous best condition of PIL concentration (12.5% of PIL, condition 55 mg.mL<sup>-1</sup> of solid-liquid ratio and 35 °C) did not enhance the extraction of anthocyanins, but significantly affected the extraction phenolic compounds. The maximum selectivity remained at 0.229 mg ANT. mg<sup>-1</sup> TPH. The literature reports increasing of phenolic compounds extraction with increase of AIL concentration. Wu et al. [67] verified increase of quercetin and rutin from Chinese herbal medicine with increase of [C<sub>4</sub>mim]Cl from 0.1 to 1.0 mol.L<sup>-1</sup> followed by steady state to 1.5 mol.L<sup>-1</sup>. Moreover, Lou et al. [68] also reported similar results in quercetin, chlorogenic and caffeic acid from burdock leaves between 0.4 and 1.6 mol.L<sup>-1</sup> of [C<sub>4</sub>mim]Br and steady state to 2.2 mol.L<sup>-1</sup>.

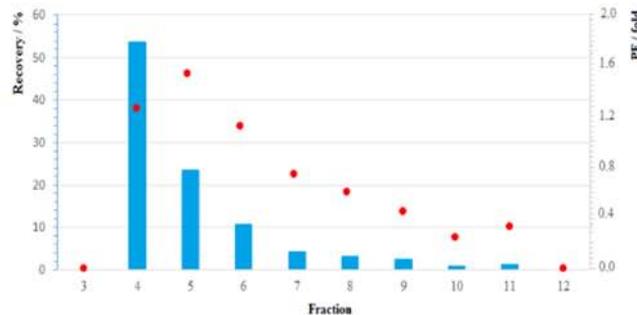
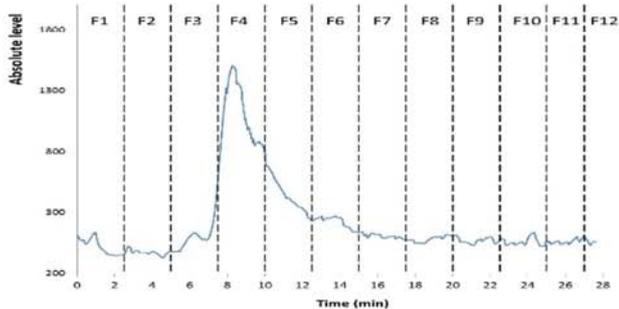
It is worth highlighting that the variables have little influence on the anthocyanins extraction, however they greatly affect the phenolic compounds extraction, and consequently modifies the selectivity of



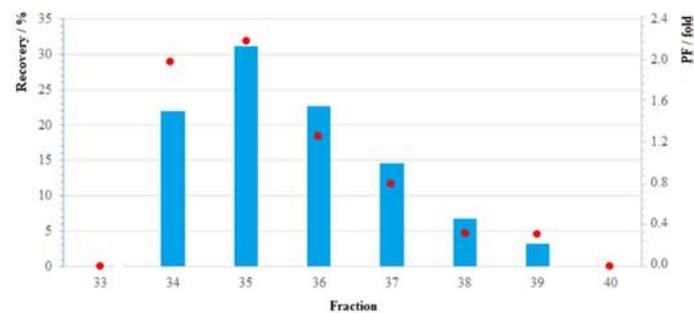
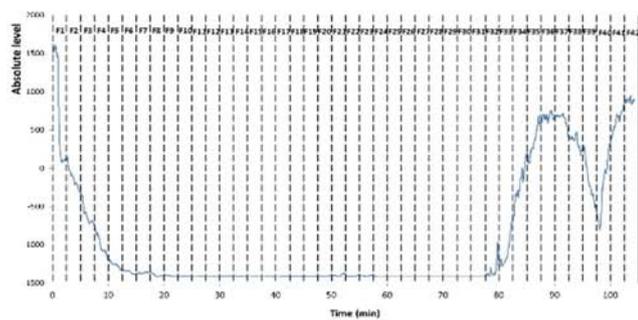
(A)



(B)



(C)



(D)

Fig. 4. CPC chromatogram, recovery (■) and purification factor (●) of anthocyanins using ATPS based on [2HEA]HSO<sub>4</sub> and acetonitrile at 25 °C in different conditions. A: TLL = 46.45 and descending mode; B: TLL = 46.45 and ascending mode; C: TLL = 88.17 and descending mode; D: TLL = 88.17 and ascending mode.

**Table 1**  
Integral extraction and purification process of anthocyanins.

Condition	Recovery (%)	PF (fold)
Solid - Liquid extraction		
S/L = 10 mg.mL <sup>-1</sup> ; 25 °C; [PIL] = 12.5%	100	1.00
S/L = 55 mg.mL <sup>-1</sup> ; 25 °C; [PIL] = 12.5%	100	2.21
S/L = 55 mg.mL <sup>-1</sup> ; 35 °C; [PIL] = 12.5%	100	16.24
Single-Step purification - Aqueous two-phase system (25 °C)		
TLL = 46.45	76.68	23.88
TLL = 88.17	80.17	19.30
Scale-up purification - Centrifugal partition chromatography (25 °C)		
TLL = 46.45; descending mode	28.37	29.13
TLL = 46.45; ascending mode	73.61	29.61
TLL = 88.17; descending mode	43.29	23.55
TLL = 88.17; ascending mode	24.85	41.88

extraction. The use of separation engineering concept improves the selectivity from 0.0141 (10 mg.mL<sup>-1</sup> solid-liquid ratio, 35 °C and 12.5% of PIL) to 0.229 (55 mg.mL<sup>-1</sup> solid-liquid ratio, and 55 °C and 12.5% of PIL), representing a purification factor of 16.24 fold.

### 3.2. Single step purification on ATPS

The effect of TLL and temperature in anthocyanins purification were performed using the systems based on [2HEA][HSO<sub>4</sub>] and acetonitrile, as shown the Fig. 3. Initially, the influence of PIL and ACN was investigated through range the tie-line length (23.68–88.17) at 25 °C (Fig. 3A-C). The target biomolecule (anthocyanins) and the contaminant (phenolic compounds) preferentially migrated to ionic liquid-rich phase ( $K < 1$ ) owing to stronger biomolecules-PIL interaction. With the increase of TLL, the partition coefficient of phenolic compounds ( $K_{TPH}$ ) decreased whereas the partition coefficient of anthocyanins remained constant (TLL > 46.45). The same partition behavior was observed in ATPS based on PEG 4000 and magnesium sulfate to purify anthocyanins from red cabbage having sugars as contaminants [69].

The different partitioning of biomolecules allowed the increase of purification factor that reached 1.46 fold and 76.68% of recovery in bottom phase (TLL = 46.45). Chethana et al. [70] reported that the simultaneous increase of partition coefficient of target biomolecule (C-phycocyanin) and contaminant (total protein) result in a decreased in purity of C-phycocyanin when used ATPS based on PEG 4000 and potassium phosphate.

In order to understand the results, the purification factor was correlated with the water content in bottom phase (Fig. S1 in Supporting information). The maximal water content it was observed in TLL = 46.45 (55.26%), and in this condition it was also observed the highest purification factor (1.46 fold). Beside the anthocyanins-IL interaction, the anthocyanins-water also has an important role in migration and consequently in purification of anthocyanins, due to stronger hydrophilic nature of anthocyanins than phenolic compounds. These results corroborates with those reported by Ventura et al. [71] for isolation of natural red colorant from fermented broth using ATPS formed by ionic liquid and citrate buffer (pH 7.0).

In the best condition ([2HEA]HSO<sub>4</sub>, PF = 1.46 fold), the temperature was ranged between 5 and 45 °C (Fig. 3C-E). The temperature had a strong effect in  $K_{THP}$  ( $0.75 < K_{THP} < 1.32$ ), in this case the phenolic compound migrated from bottom to top phase, whereas the effect in partition of anthocyanins was subtle ( $0.24 < K_{ANT} < 0.37$ ) when the temperature range between 5 and 45 °C. The opposite migration of anthocyanins and phenolic compounds led to an increase of the purification factor of 1.69 fold with 70% of recovery in bottom phase at 35 °C. Wu et al. [72] also reported a migration of anthocyanins from mulberry to ammonium sulfate-rich phase (more hydrophilic), however the range was very small (2–2.3) and the recovery ranged between 73 and 76%, like in this work.

### 3.3. Scale-up of Anthocyanins purification on CPC

The preparative CPC was performed to purify anthocyanins from grape pomace, previously purify in a single step aqueous two-phase system based on [2HEA]HSO<sub>4</sub> and acetonitrile at 25 °C (equipment limit), due to the high difference of partition coefficient between anthocyanins and total phenolic compounds [73].

The experiments were carried out using two TLL (46.45 and 88.17) and different operational mode (descending and ascending). It should be emphasize that in the aqueous two-phase system the anthocyanins are present in greater proportion in the ionic liquid-rich phase. In descending mode the mobile phase is the ionic liquid-rich phase, while acetonitrile-rich phase act as mobile phase in ascending mode is the. Moreover, the ionic liquid-rich phase and entire aqueous two-phase system was injected in descending and ascending mode, respectively. Fig. 4 depicts the monitoring of the chromatographic run at 521 nm (maximum absorbance of anthocyanins), and the recovery and purification factor of anthocyanins.

The peaks occurred at different retention times and depended on the length TLL used, i.e. differences in properties between the phases and the CPC operational mode. The base peak was broad, denoting the co-elution of the different kind of anthocyanins. Vidal et al. [74] and Renault et al. [53] reported a separation of different anthocyanins from grape using a gradient elution centrifugal partition chromatography; in order to enhance the separation a second CPC run was performed.

For descending mode, the phenolic compound migrated to stationary phase (ACN-rich phase), and the fraction is more rich in anthocyanins at short times; and for ascending mode the anthocyanins remained in stationary phase (ionic liquid-rich phase), for this reason more stationary phase was pumped, in sequence, the fractions were richer in anthocyanins.

The purification factor depended on the selectivity (increase target molecule - anthocyanin over the contaminant molecule - total phenolic compound) in the fraction collected in comparison with the feed sample (injected into the equipment). It can be seen that the maximum PF coincide with maximum anthocyanin recovery except for short TLL in descending order.

The values of purification factors discussed so far in this study refer only to the increase in the purification of anthocyanin at each stage of the process (Table 1). If we take as the standard of comparison the extraction conditions S/L = 10 mg.mL<sup>-1</sup>, 25 °C, and [PIL] = 12.5%, it can be seen that even during extraction the purification factor increases 2.21 fold (S/L = 55 mg.mL<sup>-1</sup>; 25 °C; [PIL] = 12.5%), and 16.36 fold (S/L = 55 mg.mL<sup>-1</sup>; 35 °C and [PIL] = 12.5%). In these cases, recovery is considered 100% maximum. In order to determine the recoveries and PF, the value of these parameters were multiplied for the corresponding of the previous stage at 25 °C (limitation of equipment). Comparing the effect of two TLL - 46.46 and long - 88.17), it was observed that while the anthocyanin recovery increases (long TLL - 80.17%) with increasing TLL, PF decreases (short TLL - 23.88 fold).

The choice of the best operational condition in CPC depends on the application of anthocyanin. In applications requiring high purity highlight the long TLL in ascending mode (PF = 41.88 fold and recovery of 24.85%), but when necessary large recovery and moderate purification can be used short TLL and ascending mode (PF = 29.61 fold and recovery of 73.61%).

## 4. Conclusions

The extraction and purification process of anthocyanin from grape pomace was performed using protic ionic liquid. In this study, a solid-liquid extraction has been proven that the choice of best process condition based on selectivity (ratio between concentration of anthocyanins and total phenolic compounds) allowed the increase of purification factor to 2.21 fold (S/L = 55 mg.mL<sup>-1</sup>; 25 °C; [PIL] = 12.5%), and 16.36 fold

(S/L = 55 mg.mL<sup>-1</sup>; 35 °C; [PIL] = 12.5%). In the ATPS based on [2HEA] [HSO<sub>4</sub>] and acetonitrile, the top phase is rich in acetonitrile, whereas the bottom phase corresponding to ionic liquid-rich phase. The best condition of purification in single step was observed using TLL = 46.45, at 35 °C and 25 °C, corresponding to PF = 29.28 fold, and PF = 23.88 fold, respectively. The multistep purification using CPC was carried out at 25 °C (equipment limitation). In this case the choice what best operational condition depend of anthocyanin application, however the best purification factor was 41.88 fold (TLL = 88.17 in ascending mode). These results suggest the potential use of CPC to purify molecules.

### 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.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molliq.2021.115324>.

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