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Title: Recovery of capsaicin from *Capsicum frutescens* by applying aqueous two-phase systems based on acetonitrile and cholinium-based ionic liquids

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Highlights

Cholinium and acetonitrile form a new aqueous two-phase system for purification

Capsaicin partitioning preferentially to acetonitrile-rich phase (top phase)

The best partitioning condition was acetonitrile-[Ch]Cl (30-35 wt%) at 318 K

Acetonitrile solution (60%) promote the best extraction of capsaicin

The purification factor in the best condition was 3.26 fold

Recovery of capsaicin from *Capsicum frutescens* by applying aqueous two-phase systems based on acetonitrile and cholinium-based ionic liquids

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Abstract

Peppers are the principal source of natural capsaicinoids, a class of bioactive compounds with different therapeutic properties (anticancer, antioxidant, anti-obesity) which make it interesting to the development of extraction and purification processes. This work aims at developing an integrated process of extraction and purification to recover capsaicin from pepper *Capsicum frutescens*. Thus, ATPS consisting of acetonitrile and some cholinium-based ionic liquids were investigated, characterized and the partition of capsaicin on these systems properly optimized. The main results showed that capsaicin is preferentially migrating for the acetonitrile (top) phase. With a simple technology, mild conditions and less solvents, the success of the capsaicin purification from the pepper biomass was achieved ($K_{CPS} = 60.95 \pm 1.29$; $EE_{CPS} = 90.57 \pm 0.48$ %, and $PF_{CPS} = 3.26 \pm 0.08$) using ATPS. Summing up, this work allowed the development of an integrated process of extraction and purification of capsaicin from the chili pepper biomass.

Keywords: capsaicin, aqueous two-phase systems, acetonitrile, cholinium-based ionic liquids, integrated process.

1. INTRODUCTION

The peppers of genus *Capsicum* belong to the *Solanaceae* family, as tomatoes, potatoes, eggplant and tobacco, and have more than 90 species already described (Ismail and Revathi, 2006). China, Mexico, Turkey and India are the main world producers of *Capsicum*, being responsible together, for the production of more than 30 million tons of *Capsicum* (fresh and dried products) in 2011 (FAOSTAT, 2015). This spice is widely consumed due to its flavor, color, content on vitamins C and E, provitamin A, carotenoids, and alkaloids (responsible for the spice pungency), those in particular designed as capsaicinoids, for example nordihydrocapsaicin, dihydrocapsaicin, homodihydrocapsaicin, homocapsaicin and, specially, capsaicin (Davis et al., 2007). They are a group of chemicals with important pharmacological effects, with capsaicin being one of the most popular capsaicinoids widely recognized by its biological and pharmacological activities. The ingestion of capsaicin, either through direct consumption of pepper or *via* oral or local drug administration, has shown a positive effect on the human health, as a pain relief chemical (Fraenkel et al., 2004), principally against the rheumatoid arthritis or fibromyalgia (Fraenkel et al., 2004). Moreover, this natural compound is also identified by its anti-inflammatory (Desai et al., 2013), anticancer (Luo et al., 2011), and antioxidant (Lee et al., 2011) properties, as well as by its benefits against cardiovascular and gastrointestinal diseases (Peng and Li, 2010).

Capsaicin (Figure A1 in Supporting Information) is a phenolic compound whose biosynthesis can be done by condensation of fatty acids and vanillyllamine (Thiele et al., 2008). It is a volatile, pungent, colorless and odorless chemical, considered as practically insoluble in water and easily soluble in various organic solvents, in particular, alcohols, ethers, benzene and chloroform (Chauhan et al. 2011; Sharma et al., 2013). Capsaicin can be extracted from its natural sources using conventional techniques such as the maceration (Kirschbaum-Titze et al., 2002, Contreras-Padilla and Yahia, 1998) or through unconventional techniques, in particular by supercritical fluid extraction (Barbero et al., 2006a; Aguiar et al., 2013), microwave-assisted extraction (Barbero et al., 2006b), micro-solid phase extraction (Peña-Alvarez et al., 2009; Chanthai et al., 2012) and ultrasonic-assisted extraction (Barbero et al., 2008; Boonkird et al., 2008). Regarding the maceration, solvents such as water, methanol, ethanol, ethyl acetate, hexane and acetonitrile were investigated (Barbero et al., 2006a,b; Barbero et al., 2008; Chanthai et al., 2012; Aguiar et al., 2013). However,

both maceration and other less conventional techniques have led to crude extracts of low purity. To enhance the purity of the final product, a purification step must complement the extraction, namely aqueous two-phase systems (ATPS), well described and deeply studied in different processes in the last decades. This particular case of liquid-liquid extraction systems are normally used as purification technologies due to their capacity to allow the fractionation of several compounds from the simplest to the most complex matrices. They are normally considered as biocompatible and more sustainable techniques of extraction and purification, due to the higher content of water constituting them, which makes these systems a favorable environment for the biomolecules, capable to keep their chemical structure and main activities (Wu et al., 2011; Reis et al., 2012; Cardoso et al., 2014a).

The application of ATPS as extraction/(re)concentration/purification techniques was firstly recognized in the 1950's (Albertsson, 1958) and, since then, these methods have been developed and recurrently applied in the fractionation of various bioactive compounds (Albertsson, 1958; Freire et al., 2012). However, there are still some constraints in the use of polymeric-based ATPS, namely their high viscosities and cost, which have been minimized by the use of alternative phase formers and, consequently ATPS, such as alcohol-salt (Reis et al., 2012; Reis et al., 2014), acetonitrile-carbohydrates (Cardoso et al., 2013), acetonitrile-polyols (Cardoso et al., 2014a), and ionic liquid-acetonitrile (Okano et al., 2013) systems.

Ionic liquids (ILs) are low temperature melting salts that, due to their "tunable" nature, have been established as "designer solvents", thus letting the possibility of to change their properties through specific anion/cation combinations, allowing them to be designed to meet the requirements of a particular process. They have some physico-chemical properties advantageous over conventional molecular organic solvents such as their high solvation ability, high chemical and thermal stability, high selectivity, excellent microwave-absorbing ability, broad liquid temperature range, and lower environmental impact (at least in the air compartment) that makes them good choices for the extraction and purification of biomolecules (Wasserscheid and Keim, 2000; Sheldon et al., 2002; Passos et al., 2014).

Among the various IL families, the cholinium based have recently been the focus of attention as ATPS phase formers, since they can form biphasic systems with both salts (Pereira et al., 2013; Pereira et al., 2014; Taha et al., 2015) and polymers (Shahriari et al., 2013). Cholinium chloride (2-hydroxyethyltrimethylammonium chloride) in one of

the most recent families of ILs being utilized in the extraction and purification of biomolecules, due to its claimed biocompatibility, which was firstly attributed due to the fact that this cation derives from an important nutrient for the structural integrity of cell membranes, methyl metabolism, cholinergic neurotransmission, transmembrane signaling, and lipid and cholesterol transport and metabolism (Institute of Medicine, 1998; Zeisel and Costa, 2009). Associated with its biocompatibility, it is a cheap raw material, widely used as food additive, and thus a safer and more environmentally friendly salt when compared with some of the most common ILs' cations (Petkovic et al., 2010; Santos et al., 2015; Silva et al., 2014; Sintra et al., 2015; Ventura et al., 2014). Acetonitrile (ACN) or methyl cyanide is well known as a medium-polarity solvent, miscible with water at ambient temperature and widely used in organic synthesis. This solvent belongs to the class of dipolar aprotic solvents and it is also one of the most preferred organic solvents or mobile phase in various separations techniques, due to its physicochemical properties such as low viscosity, high resolution and low boiling point (Nemati-KandeandShekaari, 2013). The extraction of biomolecules using acetonitrile–water systems was focused on the partition of antibiotics, peptides and amino-acids under the effect of negative temperatures (Gu and Zhang, 2007), but they were also used for the extraction of metals at room temperature using ATPS based in acetonitrile (Zhang, et al., 2012). More recently, the use of acetonitrile + carbohydrates-, acetonitrile + poly(vinyl alcohol)- and acetonitrile + polyols-based ATPS was proposed for the extraction of vanillin (Cardoso et al., 2015; Cardoso et al., 2014a; Cardoso et al., 2013; Cardoso et al., 2014b). As discussed, the number of works dealing with aqueous solutions of acetonitrile as the main solvent for the extraction of biomolecules is very limited, and not previously attempted for the extraction of capsaicin. In fact, the use of common or alternative solvents to extract or purify capsaicin from its natural source are scarce or practically inexistent, as proved by checking the last complete revisions made about the development of techniques/processes with ILs aqueous solutions or with ILs-based ATPS, respectively to extract and to purify capsaicin (Freire et al., 2012; Passos et al., 2014).

Considering the industrial potential of capsaicin and the continuous need for the development of more efficient and sustainable purification techniques, this work proposes the development of new ATPS by combining cholinium compounds and acetonitrile to purify capsaicin from its natural source, the pepper *Capsicum frutescens* var. malagueta. The binodal curves of the ternary systems composed

of cholinium + acetonitrile + water were established at (298 ± 1) K and atmospheric pressure. Then, these systems were applied in the optimization of the APTS regarding their partition and purification performances experimentally determined with a commercial standard of capsaicin. In the optimization step, the capsaicin partition between both aqueous phases was assessed considering the effect of several processing conditions, namely the cholinium structure by assessing the effect of different anions, the mixture point selected (allowing the study of several cholinium and acetonitrile concentrations), and the temperature of the partition process. Through the optimization study the best conditions were identified and these systems used in the purification of capsaicin from an acetonitrile crude extract, obtained from the solid-liquid extraction of capsaicin from the pepper *Capsicum frutescens* var. malagueta, by applying the best APTS selected.

2. MATERIALS AND METHODS

2.1 Materials

The acetonitrile, HPLC grade with purity of 99.9 wt% was purchased from Tedia. The cholinium ionic structures were acquired at Sigma-Aldrich: cholinium chloride [Ch]Cl, cholinium bitartrate [Ch][Bit], and cholinium dihydrogen citrate [Ch][DHCit]. The purity of each cholinium-based structure is ≥ 98 wt%. The water used in all the experiments is ultrapure and distilled. A commercial sample of capsaicin (CPS) was acquired at Sigma-Aldrich with high purity ($\geq 97\%$). All the chemical structures are represented in Figure A1 in the Supporting Information.

The peppers *Capsicum frutescens* var. malagueta (*C. frutescens*) used in this work were locally acquired in the city of Lagarto, Sergipe - Brazil, in their ripestage. The peppers were selected, sanitized with a sodium hypochlorite solution (10 mg.L^{-1}), dried in an oven at (338 ± 1) K until constant weight (or free of water), macerated in a blender, packed in polypropylene bags and stored for next tasks.

2.2 Phase diagrams and tie-lines

The APTS were formed using aqueous solutions of acetonitrile at 80 wt% and aqueous solutions of three cholinium-based ILs, in particular, the [Ch][Bit], [Ch][DHCit], [Ch]Cl, at 50, 60 and 65 wt% of maximum concentration, respectively. The phase diagrams were determined at 298 ± 1 K and at atmospheric pressure, by the cloud point titration method (Sintra et al., 2014) and the tie-lines (TLs) were determined according

to the gravimetric method well reported in literature (Merchuk et al., 1998). Briefly, the mixing points located in the biphasic region of the diagram were chosen and the solutions prepared, vigorously stirred and centrifuged at 3000 g for 10 min. After the equilibrium time reached (at 298 K for at least 18h), the top and bottom phases were separated and weighed. Each experimental binodal curve was correlated using Equation (1) (Merchuk et al., 1998).

$$[\text{ACN}] = \mathbf{A} \times \exp \left\{ \left(\mathbf{B} \times [\text{Ch}X]^0, 5 \right) - \left(\mathbf{C} \times [\text{Ch}X]^3 \right) \right\} \quad (1)$$

where [ACN] and [[Ch]X] refer, respectively, to the acetonitrile and cholinium-based IL weight fraction percentages (X represents the anion species which could be one of the three anions tested, Cl, [DHCit] or [Bit]) and A, B and C are constant parameters obtained by the regression.

The TLs were determined using Equations (2) to (5) for unknown values of [ACN]_T, [ACN]_B, [[Ch]X]_T and [[Ch]X]_B.

$$[\text{ACN}]_T = \mathbf{A} \times \exp \left\{ \left(\mathbf{B} \times [\text{Ch}X]_T^0, 5 \right) - \left(\mathbf{C} \times [\text{Ch}X]_T^3 \right) \right\} \quad (2)$$

$$[\text{ACN}]_B = \mathbf{A} \times \exp \left\{ \left(\mathbf{B} \times [\text{Ch}X]_B^0, 5 \right) - \left(\mathbf{C} \times [\text{Ch}X]_B^3 \right) \right\}$$

(3)

$$[\text{ACN}]_T = \left(\frac{[\text{ACN}]_M}{\alpha} \right) - \left(\frac{1 - \alpha}{\alpha} \right) \times [\text{ACN}]_B \quad (4)$$

$$[\text{Ch}]_T = \left(\frac{[[\text{Ch}X]]_M}{\alpha} \right) - \left(\frac{1 - \alpha}{\alpha} \right) \times [[\text{Ch}X]]_B \quad (5)$$

where the subscripts M, T and B refer, respectively, to the initial mixture, top and bottom phase. The value of α corresponds to the ratio between the mass of the top phase and the total mass of the mixture.

The length of each tie-line (TLL) was calculated from Equation (6).

$$TLL = \sqrt{([\text{Ch}]X_T - [\text{Ch}]X_B)^2 + ([\text{ACN}]_T - [\text{ACN}]_B)^2} \quad (6)$$

2.3 Optimization study - Capsaicin partition in the ATPS

The partition systems were prepared using graduated centrifuge tubes (50 mL) by weighting the appropriate amount of acetonitrile, [Ch]X and a capsaicin aqueous solution (60 mg.L^{-1}) in a total mass of 15 g. The mixtures were then gently stirred and centrifuged at 3000 rpm for 10 minutes. The graduated tubes were placed at different temperatures, ranging from 278 to 318K and at atmospheric pressure, for at least 18 hours, using a thermostatic bath MARCONI MA-127, to reach the equilibrium and to promote the complete capsaicin migration. The two phases were then carefully collected for the determination of their volume and weight, and the capsaicin was properly quantified in triplicate in both phases, using a Varian Cary-50 Bio UV-visible Spectrophotometer, at 280 nm. In this task, to evaluate the capsaicin partition, different parameters were calculated, namely the partition coefficient (K_{CPS}), the extraction efficiency (EE_{CPS}) and the volume ratio (R_V) for each ATPS under study - Equations (7) to (9).

$$K_{\text{CPS}} = \frac{[\text{CPS}]_T}{[\text{CPS}]_B} \quad (7)$$

$$R_V = \frac{V_T}{V_B} \quad (8)$$

$$EE_{\text{CPS}}(\%) = \frac{K_{\text{CPS}} \times R_V}{1 + (K_{\text{CPS}} \times R_V)} \times 100 \quad (9)$$

It should be remarked that for all ATPS studied, the top phase was the acetonitrile-rich phase while the bottom phase corresponds to the cholinium-rich phase.

2.4 Thermodynamic functions

The thermodynamic parameters of phase transfer, such as the standard molar Gibbs energy of transfer ($\Delta_{tr}G_m^o - \text{J.mol}^{-1}$), the standard molar enthalpy of transfer ($\Delta_{tr}H_m^o - \text{J.mol}^{-1}$) and the standard molar entropy of transfer ($\Delta_{tr}S_m^o - \text{J.mol}^{-1}.\text{K}^{-1}$) were determined through the van't Hoff methodology and calculated according to Equations (10) and (11):

$$\ln(K_{CPS}) = -\left(\left(\Delta_{tr}H_m^0/R\right) \times \left(1/T_{ref}\right)\right) + \left(\Delta_{tr}S_m^0/R\right) \quad (10)$$

$$\Delta_{tr}G_m^0 = \Delta_{tr}H_m^0 - T_{ref} \times \Delta_{tr}S_m^0 \quad (11)$$

where T_{ref} represents the temperature (Kelvin), K_{CPS} is the partition coefficient of capsaicin, and R is the universal gas constant ($8.314 \text{ J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$).

2.5 Extraction and purification of CPS from pepper *Capsicum frutescens* var. *malagueta*

The extraction of capsaicin was carried following the steps of the conventional methodology described by Chinn and co-workers (2011). The first step was the solid-liquid extraction of capsaicin from the pepper biomass through the use of aqueous solutions of acetonitrile, obtaining thus a crude acetonitrile aqueous extracts rich in capsaicin. Briefly, 1 g of pepper previously prepared as described in Section 2.1 was weighed and 25 mL of an acetonitrile aqueous solution were added. To evaluate the best acetonitrile:water mixture to carry this extraction, acetonitrile aqueous solutions with concentrations ranging from 0-100% were tested. Then, the samples were placed in a bath at $(298 \pm 1) \text{ K}$, under constant stirring, for 5 hours, using a Marconi MA-095 shaker. The water solutions and acetonitrile aqueous extracts were filtered through a Millipore filter $0.45 \mu\text{m}$ and analyzed by High Performance Liquid Chromatography (HPLC). To the crude acetonitrile extracts rich in capsaicin obtained, distinct amounts of the cholinium-based ILs and water were added to prepare the ATPS up to a final weight of 15 g. The selected ATPS were prepared considering the conditions previously selected in the optimization section regarding the systems and conditions of maximum partition and extraction performances. The mixtures were stirred, centrifuged at $3000 \times g$ for 10 minutes, placed in equilibrium in the optimum temperature condition, for at least 18 hours. Then, both phases were separated, collected and their volume and weight measured as well as the CPS content quantified by HPLC (model Prominence, brand Shimadzu system with UV-VIS detector, at 280 nm, C18 column type, mobile phase consisted of acetonitrile:water (60:40) at a flow rate of $1.0 \text{ mL}\cdot\text{min}^{-1}$, isocratic mode, column temperature $30 \text{ }^\circ\text{C}$, $20 \mu\text{L}$ of injection volume). The phenolic content (PC) in the crude extracts obtained in the solid-liquid (or solvent) extraction step and in both aqueous phases was assessed by colorimetric spectrophotometry using the Folin-

Ciocalteau method, using the gallic acid as standard (Swain and Hillis, 1959). The purification factor of capsaicin (PF_{CPS}) was determined by the ratio between the specific concentration of capsaicin (SC_{CPS}) present in the acetonitrile crude extract rich in capsaicin (obtained from the solid-liquid extraction) and in each phase of the ATPS according to Equations (12) and (13), respectively.

$$SC_{CPS} = \frac{C_{CPS}}{C_{PC}} \quad (12)$$

$$PF_{CPS} = \frac{(SC_{CPS})_T}{(SC_{CPS})_E} \quad (13)$$

where, C_{CPS} is the concentration of capsaicin, C_{PC} represents the concentration of phenolic compounds simultaneously extracted in the solid-liquid step and the subscripts T and E are indicative of the top and acetonitrile crude extract rich in capsaicin, respectively.

3. RESULTS

3.1 Phase diagrams and tie-lines

The formation of ATPS of acetonitrile with three cholinium-based ILs was assessed, through the experimental determination of the respective phase diagrams at $298(\pm 1)K$, depicted in Figure 1. The composition values were expressed in molality units to avoid discrepancies in the evaluation of the ILs potential to induce the liquid-liquid demixing related with the different molecular weights of the ILs involved and the acetonitrile as well. The experimental weight fraction data of the binodal curves are provided in the Supporting Information (Table A1). By analyzing Figure 1, it is observed that the potential of the cholinium compounds to form ATPS increases as follows: $[Ch][DHCit] \approx [Ch][Bit] > [Ch]Cl$. This sequence is related to the lipophilic/hydrophilic nature of the cholinium salts, as derived from their octanol–water partition coefficients ($\log K_{ow}$) (Chemspider, 2015). The tendency observed for the ATPS formation is related to the decrease in their $\log K_{ow}$, namely $\log K_{ow} ([Ch][DHCit]) = -1.32$, $\log K_{ow} ([Ch][Bit]) = -1.43$ and $\log K_{ow} ([Ch]Cl) = -3.70$.

The binodal curve data were fitted using Equation 1, firstly applied by Asenjo and collaborators (Merchuk et al., 1998). The parameters A, B and C (estimated by least-

squares regression), and the corresponding standard deviations (std) and regression coefficients (R^2) are reported in Table A2 (Supporting Information). At the same time, Figure A2 presents the complete phase diagrams with the TLs and respective TLLs calculated (the numerical results are reported in Table A3 of Supporting Information).

Finally, the ATPS here developed were also defined in terms of the pH of each aqueous phase formed, as shown in Table A4 (Supporting Information). In general terms, the pH is in its acidic form, in the range of 3.58 to 6.22. According to the speciation curves shown in the Supporting Information (Figure A3) and the results of Table A4, it is observed that the effect of pH on the capsaicin partition can be explained by its effect on the electrical charge, which influenced its solubility in the top phase. The system composed of [Ch]Cl presented pH values for the top phase in the range of 6.22-5.66, and in this range of pH, the capsaicin is more negatively charged, thus favoring the occurrence of electrostatic interactions favorable to the migration of capsaicin for this phase. Regarding the systems composed of [Ch][Bit] and [Ch][DHCit], the top phase presented pH values between 4.62-5.21, which was not much influenced by electrostatic forces because in this range, the capsaicin is mostly in its neutral form, which explains the poorest affinity of capsaicin for the top-phase, and the smaller value of K_{CPS} . The pH values found in the bottom phase are included in the same range found elsewhere (Souza et al., 2015). In this work, ATPS composed of tetrahydrofuran and cholinium-based compounds were applied on the purification of a lipase and observed that the pH also influenced the partition of the enzyme by the electrostatic interactions formed.

3.2 Optimization study - application of ATPS on the capsaicin partition

In this work, different ATPS based in acetonitrile and three cholinium salts were used to investigate the partition of capsaicin, as an alternative method to purify it from the pepper *Capsicum frutescens* var. malagueta. After the adequate definition of the phase diagrams, and consequently, of the biphasic region for each system, the optimization of the partition of capsaicin using a commercial sample with high purity ($\geq 97\%$ of purity) as a model compound was investigated. Different parameters were evaluated to optimize and identify the best extraction/partition conditions, not only regarding the most appropriate ATPS, but also the most adequate processing conditions. Examples of those conditions are the cholinium anion, the composition of the system (regarding the mixture point used to prepare each ATPS) and the temperature of extraction, whose results are depicted in Figure 2.

3.2.1. Effect of the cholinium anion

The results presented in Figure 2(A) show the partition coefficients (squares) and the extraction efficiency results (grey bars) obtained for capsaicin (numerical data is reported in Supporting Information- Table A5) when applied the three ATPS, namely 40 wt% of [Ch]X (X is representing Cl, [DHCit] or [Bit]) + 35 wt% of acetonitrile + 25 wt% of water. The experiments show that the capsaicin partitions preferentially for the top acetonitrile-rich phase, with values of K_{CPS} of 22.92, 20.55 and 5.79, for [Ch]Cl, [Ch][Bit] and [Ch][DHCit], respectively. Moreover, this trend is in close agreement with the extraction efficiencies (EE_{CPS}) results ranging from 78.85 to 91.29 %. These results demonstrated the higher affinity of capsaicin for the acetonitrile phase [$\log K_{ow} = -0.17$ (Chemspider, 2015)], due to its hydrophobic nature, as demonstrated by the octanol-water partition coefficient ($\log K_{ow} = 3.75$), being the biomolecule affinity decreased when the cholinium-based ILs with more hydrophobic anions. In this sense, [Ch]Cl is the most efficient regarding the concentration of capsaicin in the acetonitrile-rich phase. In addition to the most favorable results found for [Ch]Cl regarding the concentration of capsaicin in the top phase, [Ch]Cl is much cheaper, widely available in industrial scale and a harmless compound (that is actually an essential nutrient for human nutrition and widely used as supplement in animal feed), and thus, the ATPS based on this cholinium compound will be used in the next optimization steps.

3.2.2. Composition of the system

Considering the ATPS based in [Ch]Cl + acetonitrile + water, identified as the best system to partition capsaicin into the acetonitrile-rich phase, the effect of different mixture points was assessed. The results regarding the various extraction points are represented in Figure 2(B) (numerical data in Supporting Information, Table A6). In this case, the ATPS was composed of 35 wt% of acetonitrile and various concentrations of [Ch]Cl ranging from 30 to 50 wt%. The results indicate that the effect of different mixture points is not very relevant for the partition of capsaicin, since the data of K_{CPS} (ranging from 22.93 - 30.35) and EE_{CPS} (included in the range of 91.14 - 93.06%) are similar between the various ILs compositions. The effect of different concentrations of acetonitrile was also tested, as depicted in Figure 2(C), for systems composed of 35 wt% of [Ch]Cl and the concentration of acetonitrile varying from 30 to 50 wt% (numerical results reported in Table A7 of Supporting Information). In this case, the

migration of capsaicin was influenced by the variations in the acetonitrile concentration, *i.e.* the partition coefficient decreases with the increase in the acetonitrile content (ranging from 17.58 – 37.29). However, due to the change in the relative phase volumes, the extraction efficiencies change in the opposite way, but only marginally, between 90.74 and 94.93%.

3.2.3. Effect of temperature

In order to study the effect of temperature in the partition of capsaicin, the range between 278 and 318 (± 1) K was evaluated and the experimental results depicted in Figure 2(D) (detailed data at Table A8 in Supporting Information). The selected system was based on the best conditions found to maximise the capsaicin migration to the top phase rich in acetonitrile, namely composed of 30 wt% of acetonitrile + 35 wt% of [Ch]Cl + 35 wt% water. From the results presented in Figure 2(D), it can be seen that the K_{CPS} increases with temperature, until the maximum of 50.68, achieved at 318 (± 1) K. This behavior is justified by the increase in the solubility of capsaicin into the top phase, promoted by entropic factors. Meanwhile, the EE_{CPS} is not significantly affected by temperature, ranging from 89.75 to 93.07%. Following the study of the temperature effect on the capsaicin migration, the thermodynamic parameters were also evaluated, aiming at achieving a better understanding of the partition process of capsaicin between the aqueous phases. The thermodynamic parameters of transfer, namely the standard molar Gibbs energy ($\Delta_{tr}G_m^o$), enthalpy ($\Delta_{tr}H_m^o$) and entropy ($\Delta_{tr}S_m^o$) were calculated considering Equations (10) and (11) and the results presented in Figure 3 and Table A9. It is possible to conclude that the capsaicin migration for the acetonitrile-rich phase using these specific ATPS is a spontaneous process ($\Delta_{tr}G_m^o$ is negative, equal to $-18.17 \text{ kJ}\cdot\text{mol}^{-1}$). Moreover, the $\Delta_{tr}H_m^o$ is positive ($9.13 \text{ kJ}\cdot\text{mol}^{-1}$), suggesting that the transference of capsaicin from the [Ch]Cl to the acetonitrile-rich phase is an endothermic process ($\Delta_{tr}H_m^o > 0 \text{ kJ}\cdot\text{mol}^{-1}$). In this case, it is possible to recognize the important role of the entropic effects, since the absolute value of $T\Delta_{tr}S_m^o$ ($18.18 \text{ kJ}\cdot\text{mol}^{-1}$) is higher than $\Delta_{tr}H_m^o$ ($9.13 \text{ kJ}\cdot\text{mol}^{-1}$).

3.3 Purification of Capsaicin from Pepper *Capsicum Frutescens* var. *Malagueta*

Having evaluated and optimized the partition coefficients of capsaicin on the ATPS using the commercial standard of capsaicin (high level of purity), they were further

applied in the purification process considering the removal of capsaicin from the pepper *Capsicum frutescens* var. malagueta. The partition of the target capsaicinoid and the main contaminants (normally phenolic compounds) present in the pepper biomass were considered in the analysis. The integrated process proposed is depicted in Figure 4, which represents the process diagram for the extraction of capsaicin from the *Capsicum frutescens*.

The samples of *Capsicum frutescens* are subjected to a solid-liquid extraction starting with a mechanical maceration of the pepper biomass, assisted by solvent extraction with aqueous solutions of acetonitrile (different concentrations of the organic solvent were tested), aiming at the complete extraction of capsaicin from the biomass. The acetonitrile aqueous extract rich in capsaicin obtained was then used in the implementation of the final step of purification. In this step, the acetonitrile aqueous solution rich in the capsaicinoid was used to prepare the ATPS with the best performance composed of 35 wt% of [Ch]Cl + 30 wt% of acetonitrile + 35 wt% of acetonitrile (conditions previously optimized, Section 3.2), at temperatures of 298 and 318 (± 1) K.

3.3.1. Solid-liquid extraction of capsaicin from *Capsicum frutescens* var. malagueta

To recover any bioactive compound from the natural biomass, the first step to be conducted must be a solid-liquid extraction. The simplest and industrially most used approach is the maceration in which the solvent contacts directly with the grounded biomass at temperatures not far removed from the ambient. To carry this extraction, it is necessary to start with the selection of the adequate solvent or a combination of solvents, taking into account the affinity of the target bioactive chemical to be recovered from this solvent or mixture of solvents. In this work, the extraction of capsaicin from the solid biomass was evaluated in aqueous solutions of acetonitrile with concentrations ranging from 0 to 100% (Figure 5). These results seem to indicate that the pure solvents are not the best choices to extract capsaicin from the biomass, actually the data demonstrated that the mixture of water/acetonitrile improves the extraction of capsaicin from the pepper biomass, which is agreement with other studies (Barbero et al., 2006b). The best solvent mixture to extract capsaicin from the pepper was found to be the mixture of 40% of water and 60% of acetonitrile, represented by a concentration of capsaicin extracted of 0.146 ± 0.001 mg of capsaicin.g⁻¹ of pepper (Figure 5), conditions

used in the preparation and implementation of ATPS as purification systems to capsaicin.

3.3.2. Purification of capsaicin from *Capsicum frutescens* var. *malagueta* using ATPS

After the optimization carried out using the capsaicin commercial standard, the ATPS presenting the higher K_{CPS} parameters were considered in the purification of the natural capsaicin from the acetonitrile aqueous crude extract. This extract obtained from the maceration was used to prepare the ATPS composed of 30 wt% of acetonitrile + 35 wt% of [Ch]Cl + 35 wt% of water selected in the Optimization Study (Section 3.2.). The purification was conducted at 298 and 318 (± 1) K, to evaluate the effect of temperature when the capsaicin is purified from a natural source. The partition coefficient and extraction efficiency data were determined to conclude about the purification performance achieved by the selected ATPS when a much more complex matrix is investigated. In this case, the purification factor (Table 1), described by Equation (13) is also relevant to measure the ATPS performance, since it is directly measuring the separation of capsaicin from the phenolic compounds, the main contaminants present in the extract.

Regarding the partition behavior of the natural capsaicin, the results for the best ATPS, at 318 (± 1) K, evidenced the preferential partition of the biomolecule for the acetonitrile (top)-rich phase ($K_{CPS} \gg 1$), and the EE_{CPS} values remained constant at circa 90%. By the application of these ATPS, it was found a good purification factor ($PF_{CPS} = 3.20$ or 3.26), meaning that the capsaicin concentrated in the acetonitrile phase is free of some of the contaminants. The standard and samples chromatograms [commercial capsaicin, the natural pepper extract obtained in Section 3.3.1 and the top phases obtained after the purification of capsaicin at 298 and 318 (± 1) K], obtained by HPLC analysis were reported in Supporting Information (Figure A4). The chromatograms confirm that the use of ATPS was a successful step on the development of the capsaicin' purification process, due to the significant increase in the specific capsaicin concentration in the top phase (Figure A3). Regarding the temperature effect, it was observed an increase in the value of K_{CPS} (from 60.9 to 67.7) however, its impact on the extraction efficiency and purification factor was not very significant. In this case, the lower temperature was selected as the most adequate, since it allows the minimization of the energy costs enhancing the process economic viability while maintaining the system purification capacity. Furthermore, the recovery of capsaicin from the solid biomass is practically

the same obtained from a liquid chromatography - electrospray ionization (86% of capsaicin recovery) by Álvarez-Fernández and co-workers (Garcés-Claver et al., 2006), but in our case, using a simpler and low-cost methodology.

For the process proposed to be of industrial relevance, the isolation of capsaicin from the acetonitrile-rich phase (also known as a polishing step) and the recycling of the phase components must be addressed. It is here proposed the recycling of both phases by the evaporation of acetonitrile and its reuse in the extraction process (more details in the flowchart of the integrated process proposed, Figure 4), the [Ch]Cl is then washed out with water and capsaicin is precipitated due to its lower solubility in water. The precipitation of capsaicin will be promoted by the addition of high amounts of water as anti-solvent (at low temperature, if needed), due to the limited solubility of capsaicin in water (Turgut et al., 2004). The acidification of the [Ch]Cl-rich phase is here proposed aiming at the removal of the phenolic compounds by precipitation at very low pH values (Li et al., 2014). After the removal of the contaminants and capsaicin from both aqueous phases, the acetonitrile phase will be directly reintroduced in the ATPS preparation and the IL-rich phase will be neutralized with a base, and then reintroduced in the purification system (in the step of ATPS preparation).

4. CONCLUSIONS

Cholinium and acetonitrile based ATPS were successfully developed and applied in the purification of capsaicin from crude extracts obtained from the pepper *Capsicum frutescens* var. malagueta. From the optimization study carried out to previously select the best ATPS and processing conditions, high partition coefficients and extraction efficiencies at the acetonitrile-rich phase were achieved, and the best ATPS, regarding its capacity to concentrate capsaicin in the acetonitrile-rich phase was selected: 30 wt% of acetonitrile, 35 wt% of [Ch]Cl and 35 wt% of water, at 318 (± 1) K to further perform the purification of the natural capsaicin from the pepper. With a simple technology like ATPS, with mild conditions and requiring less solvents, the success of the capsaicin purification from pepper was achieved ($K_{CPS} = 60.95 \pm 1.29$; $EE_{CPS} = 90.57 \pm 0.48$ %, and $PF_{CPS} = 3.26 \pm 0.08$), with lower environmental impacts and costs when compared with the conventional methodologies already applied. Summing up, with the integrated purification process here developed, it would be possible to purify capsaicin from the chili pepper using aqueous solutions of acetonitrile, by means of an effective and simple purification process integrating ATPS.

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Figure Caption

Figure 1: Phase diagrams for the ternary systems composed of acetonitrile + [Ch]X + water at 25°C: (◆) [Ch]Cl, (■) [Ch][Bit], and (▲) [Ch][DHCit].

Figure 2: Partition Coefficient, K_{CPS} (■) and Extraction Efficiency, EE_{CPS} (grey bars) of capsaicin determined for different conditions: **A)** ATPS composed by 40 wt.% [Ch]X + 35 wt.% acetonitrile, at 298 (\pm 1) K; **B)** effect of the initial concentration of [Ch]Cl: ATPS composed by 30 to 50 wt% [Ch]Cl + 35 wt% acetonitrile, at 298 (\pm 1) K; **C)** effect of the acetonitrile initial concentration: ATPS composed by 35 wt% [Ch]Cl + 30 to 50 wt% of acetonitrile, at 298 (\pm 1) K; **D)** effect of temperature of extraction: ATPS composed by 35 wt% [Ch]Cl + 30 wt% of acetonitrile, at 278 to 318 (\pm 1) K.

Figure 3: Correlation between the logarithmic function of K_{CPS} - $\ln(K_{CPS})$ - versus T^{-1} (K^{-1}) for the capsaicin partitioning considering the system 30 wt% of acetonitrile + 35 wt% of [Ch]Cl + water at different temperatures, namely 318, 308, 298, 288, 283, 278 (\pm 1) K.

Figure 4: Flowchart of the integrated process for the extraction and purification of capsaicin from *Capsicum frutescens* var. malagueta. The process starts with the solvent extraction of capsaicin from the biomass through the use of water or aqueous solutions of acetonitrile, followed by the purification of capsaicin from the acetonitrile aqueous extract rich in capsaicin by applying the ATPS based on the [Ch]Cl under the optimized conditions of composition and temperature (the phenolic compounds are the main contaminants considered). The polishing of capsaicin and the recycle of the phase components are steps also included in the purification process.

Figure 5: Effect of the acetonitrile concentration on the extraction of capsaicin from the *Capsicum frutescens* biomass.

Figures

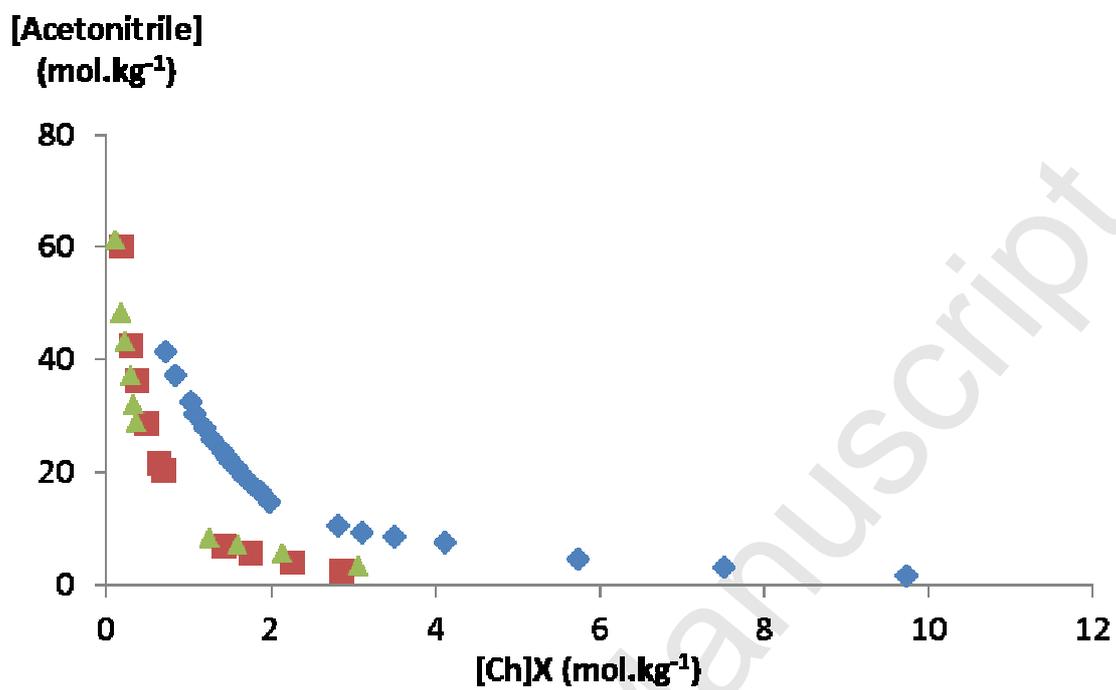


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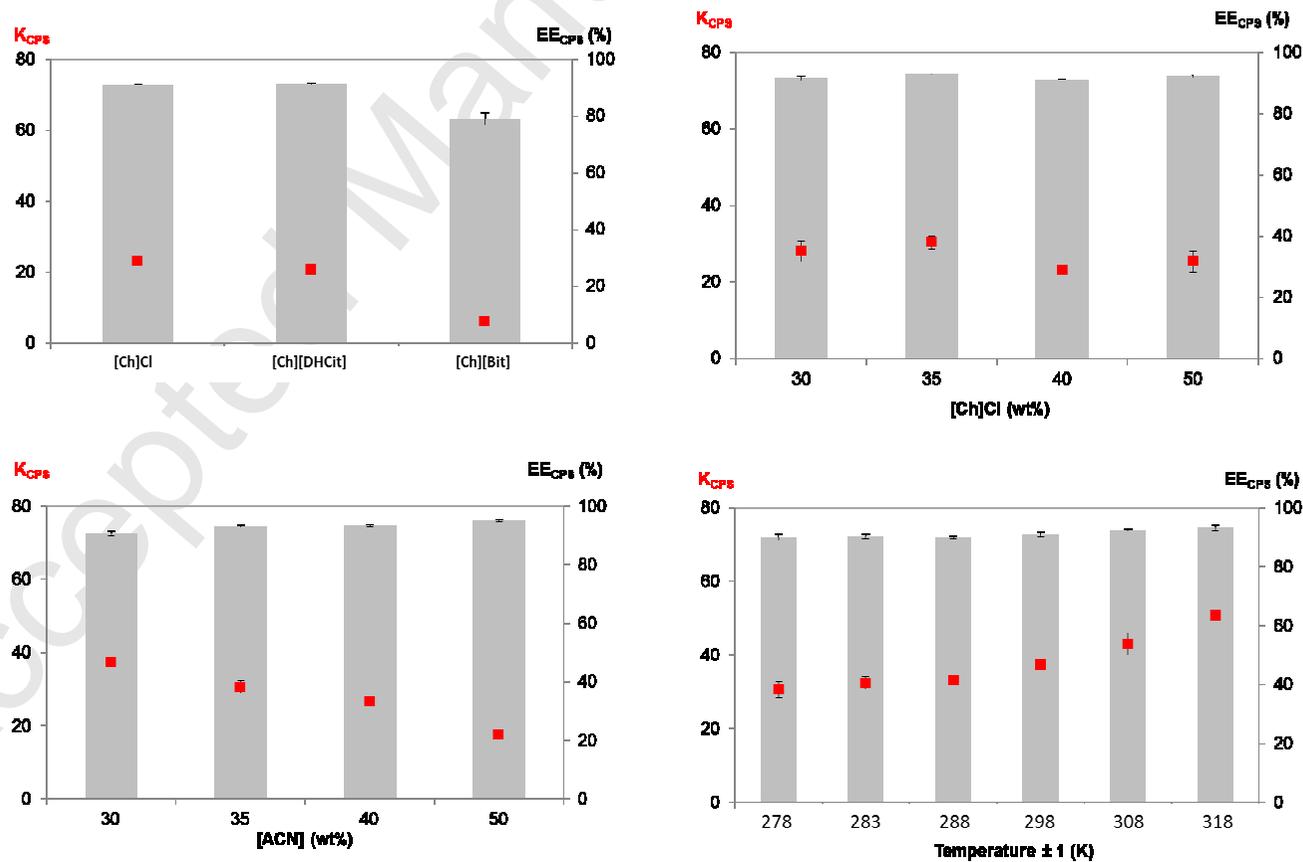


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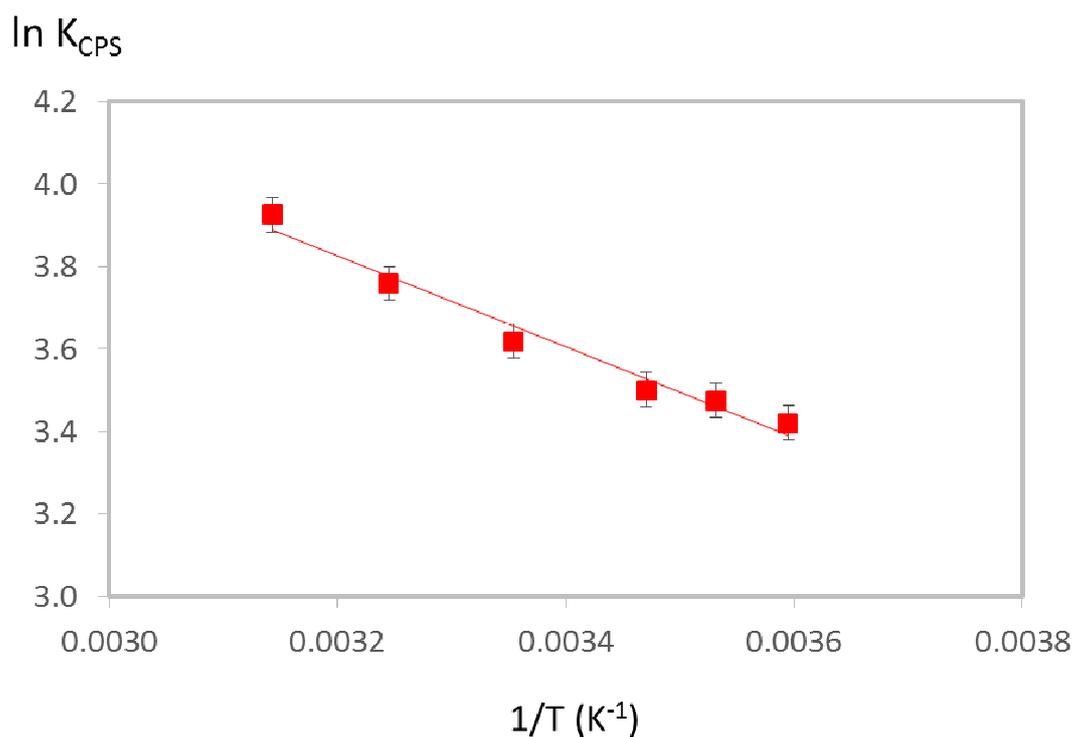


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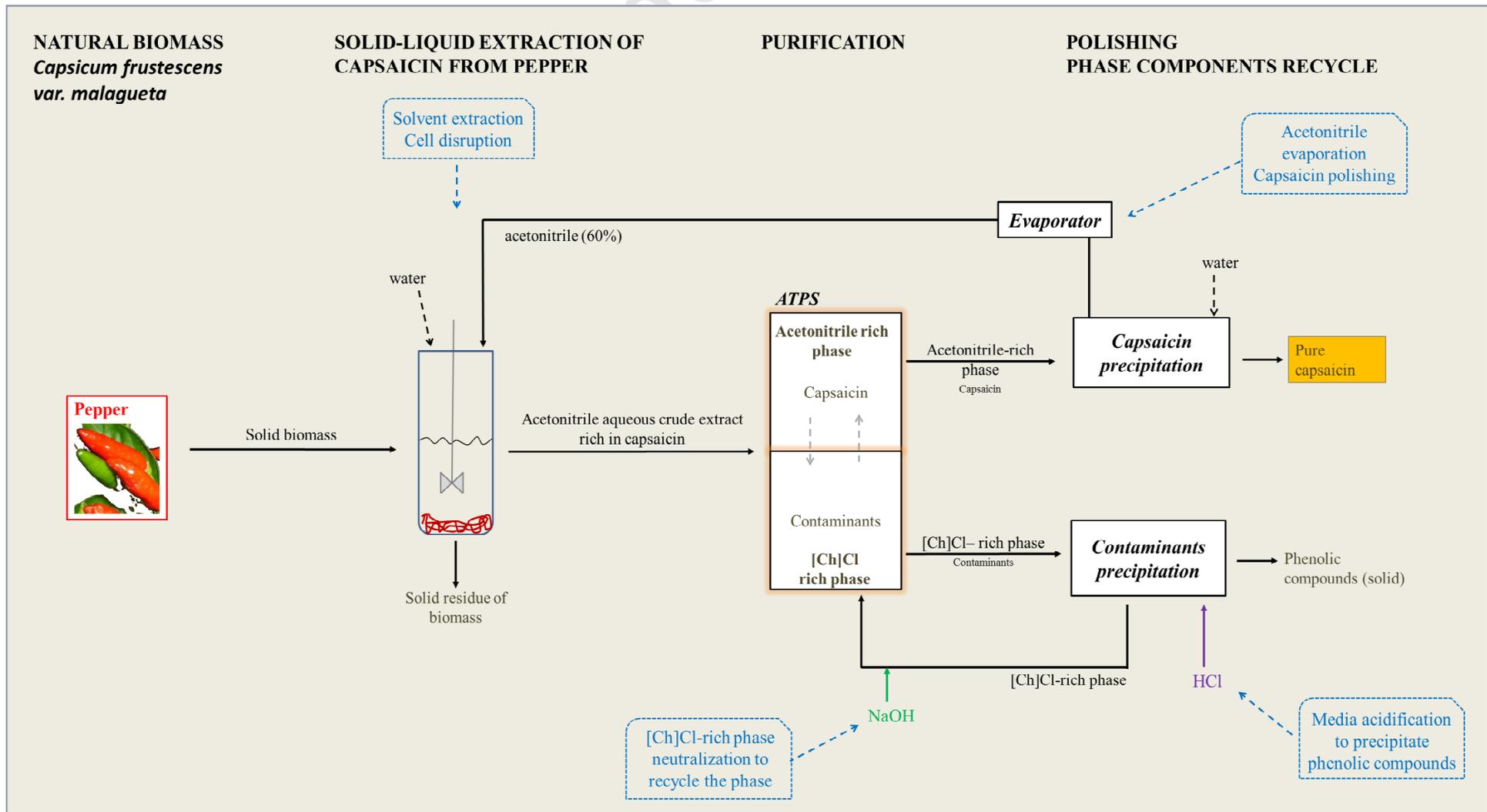
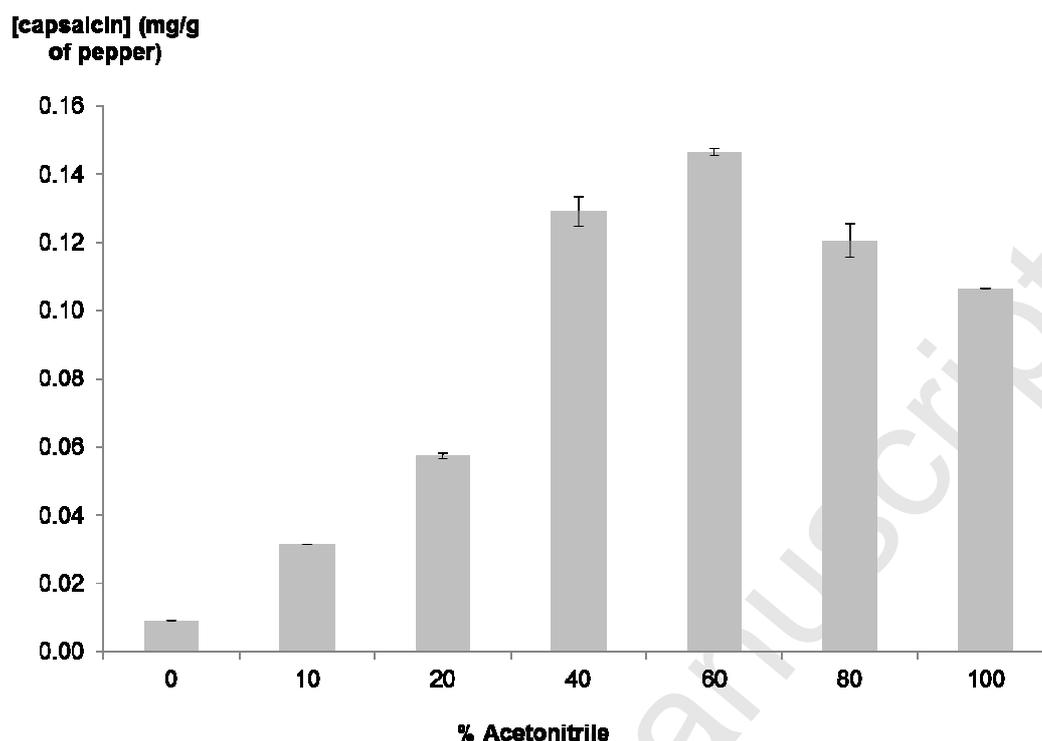


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3 **Figure 5:** Effect of the acetonitrile concentration on the extraction of capsaicin from the
4 *Capsicum frutescens* biomass.

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13 **Table 1:** Partition Coefficient (K_{CPS}), Extraction Efficiency (EE_{CPS}) and Purification
14 Factor (PF_{CPS}) of capsaicin from the pepper *Capsicum frutescens* var. malagueta obtained
15 using the optimized ATPS: 35 wt% of [Ch]Cl+ 30 wt% of acetonitrile at 298 or 318
16 (± 1)K.

Temperature ± 1 (K)	K_{CPS}	EE_{CPS} (%)	PF_{CPS}
298	60.95 ± 1.29	90.57 ± 0.48	3.26 ± 0.08
318	67.71 ± 0.96	90.93 ± 0.12	3.20 ± 0.10

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