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Separation and purification of curcumin using novel aqueous two-phase micellar systems composed of amphiphilic copolymer and cholinium ionic liquids

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ABSTRACT

Novel aqueous two-phase micellar systems (ATPMS) composed of Pluronic F68, a triblock amphiphilic copolymer, and cholinium-based ionic liquids (ILs) were formulated and applied for separation/purification of curcumin (CCM). CCM stability in the presence of ATPMS components was also evaluated. CCM is stable up to 24 h in copolymer (1.0 – 10.0 wt%) and ILs (0.1 – 3.0 M) aqueous solutions. Very mild phase separation conditions (close to room temperature) were achieved by adding cholinium ILs to the Pluronic F68 + McIlvaine buffer at pH 6.0 solution. The decrease of cloud-point temperature is dependent on the relative hydrophobicity of IL anion, $[Hex]^-$ > $[But]^-$ > $[Pro]^-$ > $[Ac]^-$ > Cl^-. ATPMS composed of more hydrophobic ILs ([Ch][Hex] > [Ch][But] > [Ch][Pro]) are most efficient in the partition of commercial CCM into polymeric micelles (PMs)-rich phase. The best ATPMS (0.70 M [Ch][But] and 0.60 M [Ch][Hex]-based ATPMS) were then used to purify CCM from a crude extract of *Curcuma longa* L. Both systems were very selective to separate CCM from protein-based contaminants (selectivity values ≥ 25; purification yields ≥ 12-fold). Pluronic F68-based ATPMS are promising for selective separation of hydrophobic biomolecules by using cholinium-based ILs as adjuvants to adjust phase separation temperatures and biomolecules' partition.

1. Introduction

Ionic liquid (IL)-based aqueous two-phase systems (ATPS) have shown an extremely fast progress, mainly because of biocompatible gains over conventional liquid − liquid extraction platforms [1,2]. The potential of IL-based ATPS for selective extraction/separation of a plethora of molecules/biomolecules was mostly a result of their flexibility and adjustability by a proper choice of the structure of phase-forming compounds, which can also boost ATPS' biocompatibility and lipophilic-hydrophilic range [1–4]. Outstanding performances for extraction and purification of several biological/pharmaceutical active substances were already reported [1,5,6].

From the plethora of ATPS available, a growing interest on systems that use amphiphilic polymers as phase forming compounds has been observed [7,8]. The use of copolymers on developing IL-based ATPS is an interesting approach to design "more tunable" and selective systems, mainly envisaging the separation of structurally similar and/or more hydrophobic molecules. The class of ABA triblock copolymers, commercially available as Pluronic, offers a pool of > 50 amphiphilic compounds as pharmaceutical excipients under the US and British Pharmacopeia criteria [9]. These copolymers are thermoresponsive, offering extra tunable characteristics, very attractive for the biopharmaceutical area [9–11]. Pluronic copolymers are water-soluble, polymorphic materials (A = hydrophilic block poly(ethylene oxide) (PEO) and B = hydrophobic block poly(propylene oxide) (PPO)), with lower critical solution temperature (LCST) in water, forming a biphasic regime composed of a polymeric micelles (PMs)-rich phase and a PMs-poor phase - commonly categorized as aqueous two-phase micellar systems (ATPMS) [12].

The use of copolymers in the biphasic aqueous formulations is

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supported for their: (*i*) capacity to obtain intermediate degrees of hydrophobicity [13]; (*ii*) higher biocompatible character [14]; (*iii*) amphiphilic nature, that enables the solubilization of hydrophobic molecules [11]; (*iv*) thermosensitivity, that facilitates recovery and reuse procedures [15]; (*v*) ability to form mixed micelles to purify target-molecules, and/or remove contaminants [16]; (*vi*) capability to encapsulate drugs in a polymeric (or micellar) matrix [17].

Because of countless health benefits (*e.g.* anti-inflammatory, free radical scavenging, antimicrobial, and anticancer activities) of curcumin (CCM)-related bioactive compounds (like curcuminoids from turmeric (*Curcuma longa* L.) spice [18]), the interest of nutraceutical and pharmaceutical industries on these has been growing [19–21]. Nowadays, the extraction of curcuminoids from biological sources is mostly based on solid-liquid and liquid-liquid platforms using volatile organic solvents (VOCs) [22,23], while the subsequent purification is performed by applying sequential chromatography units. Current extraction platforms exhibit several environmental and health concerns associated to the use of VOCs as major solvents (*e.g.*, toxicity; flammability; denaturing effect and waste disposal) [24,25]. Chromatographic processes are still very challenging, complex and expensive; for example, the structural similarity of curcuminoids forces coupling these methods with additional separation techniques to enhance the resolution power [18,22]. Although the scientific community has been concerned with these aspects for the separation and purification of curcuminoids, the number of works in that field is still rather scarce. An exception is the work of Silva et al. [8] that used Pluronic L35 (PEO_{11}-PPO_{16}-PEO_{11})-based ATPS for a selective separation of two structural similar flavonoids (naringin and rutin) by using cholinium bicarbonate as additive.

In order to overcome some of the issues of current CCM downstream processing, this work proposes a novel and simple process based on the application of Pluronic-based ATPMS. Novel ATPMS composed of Pluronic F68 (PEO_{76}-PPO_{29}-PEO_{76}) and cholinium-based ILs as adjuvants were formulated. In order to gather insights on how to design selective Pluronic F68 ATPMS-mediated downstream processes, the CCM stability (in the presence of phase-forming components) and partition using these ATPMS were evaluated. CCM, a yellow colored crystalline polyphenol, was chosen for the partition studies, because it is one of the major curcuminoids found in *C. longa* L. (typically about 75% [26]), with very relevant challenges regarding its extraction/purification. Additionally, CCM has a poor solubility in aqueous environments, representing an interesting model biomolecule for the development of alternative downstream processes for the purification of hydrophobic bioactive compounds of pharmaceutical interest. The potential of the best ILs-based ATPMS for the purification of CCM from a crude extract of *C. longa* L. was also evaluated.

2. Material and methods

2.1. Material

Pluronic® F68 (composed by PEO_{76}-PPO_{29}-PEO_{76}, $M_W \approx$ 8,400 g/mol), and pure commercial curcumin (CCM, purity > 99%, $M_W \approx$ 368.38 g/mol) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The powdered material dried from *Curcuma longa* L. rhizomes (*i.e.*, crude sample) was purchased from Kitano® (Cambará, PR, Brazil, Lot #L19BRPP052). The samples were stored sheltered from light and moisture before their use in the extraction studies. ILs used in this study – cholinium chloride ([Ch]Cl), cholinium acetate ([Ch][Ac]), cholinium propanoate ([Ch][Pro]), cholinium butanoate ([Ch][But]), and cholinium hexanoate ([Ch][Hex]) – were synthesized by us according to previous protocols [27]. The purity and chemical structures of Pluronic F68 and ILs are shown in Table 1. Aqueous solutions of all components were prepared in McIlvaine buffer (composed of $K_2HPO_4{\cdot}3H_2O$ + $C_6H_8O_7{\cdot}H_2O$) with distinct pH values (6, 7, and 8), using ultrapure water from Millipore Milli-Q system (Bedford, MA). All other reagents were of analytical grade and used as received.

2.2. Determination of the phase diagrams

The binodal curves for the systems composed of Pluronic F68/McIlvaine buffer (pH 6.0) in the presence of ILs (concentration of IL varied from 0.1 to 3.0 M) were obtained through the cloud-point titration method [28]. Briefly, Pluronic F68 buffered solutions of known concentrations (with/without ILs) were added to graded glass tubes (15 mL) and placed in a transparent thermo-regulated device (Polyscience, model 9505). Temperature control was achieved (accuracy ± 0.015 °C) with a thermometer (Omega®, model HH40) coupled to a thermistor probe (Omega®, model ON-403-PP). Magnetic stirring was applied to ensure temperature and concentration homogeneity. The temperature was first lowered until the solution exhibited a clear monophasic phase, and then slowly raised (increase step of 0.1 °C) until the solution became cloudy. This temperature was recorded as the phase separation (cloud-point) temperature (T_{CP}). The procedure was performed in triplicate, and the corresponding binodal curve was determined by plotting the mean of T_{CP} values as a function of Pluronic F68 concentration (wt%).

2.3. CCM stability studies

Before carrying out CCM partition studies, preliminary stability studies were performed to define working experimental windows. The stability of pure commercial CCM at various pH values (6.0, 7.0, and 8.0) and temperatures (30, 50, and 70 °C) was first evaluated up to 72 h. Subsequently, the CCM stability in the presence of aqueous (McIlvaine buffer at pH 6.0 – most stable pH) solutions of Pluronic F68 (1.0 and 10.0 wt%) and each IL (0.1 and 3.0 M), during 24 h of incubation, was determined.

For all stability studies, aqueous solutions containing Pluronic F68, IL or Mcllvaine buffer, with an initial CCM concentration of 0.5 mg/mL were prepared. Aqueous solutions were then homogenized according to the procedure detailed in Section 2.4. During incubation, aliquots were withdrawn, and the respective concentration of CCM determined as described in Section 2.5.

CCM stability percentage (CCM_{stb}) was calculated according to Eq. (1):

$$CCM_{stb}\ (\%) = \frac{[CCM]_{after}}{[CCM]_{before}} \times 100 \quad (1)$$

where $[CCM]_{after}$ and $[CCM]_{before}$ correspond to CCM concentration in the sample after and before (initial concentration) incubation, respectively.

2.4. Partition/purification studies of CCM using ATPMS

For partition and separation studies, 6.0 g (± 10^{-4} g) aqueous buffered systems were prepared in graduated test tubes (15 mL) by adding known amounts of Pluronic F68, IL and McIlvaine buffer (pH 6.0). Afterwards, 3.5 mg of pure commercial CCM (partition) or CCM from crude sample (purification) were added to each system. Since CCM is light-sensitive and relatively pH unstable [29], all assays were performed in dark rooms, under pH monitoring.

ATPMS were then homogenized in an orbital shaker (Barnstead/Thermolyne, model 400110) at 15 rpm for 60 min, followed by sonication at 50 W for 45 min at 30 °C (ultrasound bath Qsonica®), and equilibrated at 5 °C (± 0.15 °C). In order to form a biphasic regime, each ATPMS was placed in a thermoregulated device (Polyscience, model 9505), at a pre-defined temperature (Table 2), during 3 h. After the equilibrium, the two coexisting (polymeric micelle (PMs)-rich and -poor) phases were carefully separated using disposable syringes and needles, and the respective CCM concentration was determined. CCM purification studies from crude extract of *C. longa* L. were only

Table 1
Chemical structure, name, abbreviation, and purity of Pluronic F68 and cholinium-based ionic liquids (ILs).

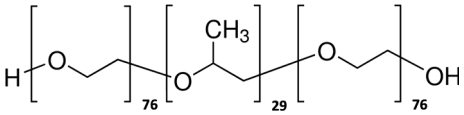
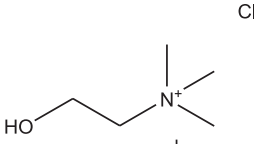
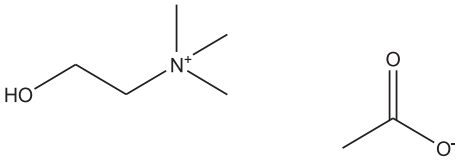
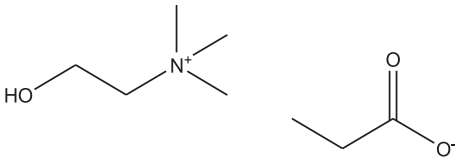
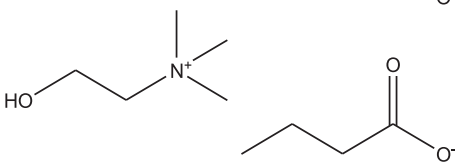
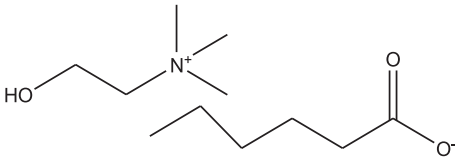
Pluronic® F68 copolymer			
Name	**Abbreviation**	**Chemical structure**	**Purity (wt%)**
Poly(ethylene glycol (PEO))-*block*-poly(propylene glycol (PPO))-*block*-poly (ethylene glycol (PEO))	F68		50.0 PEO
Ionic liquids (ILs)			
Name	**Abbreviation**	**Chemical structure**	**Purity (wt %)**
Cholinium chloride	[Ch]Cl		98.0
Cholinium acetate	[Ch][Ac]		98.7
Cholinium propanoate	[Ch][Pro]		95.2
Cholinium butanoate	[Ch][But]		98.0
Cholinium hexanoate	[Ch][Hex]		90.0

Table 2
Experimental compositions and temperatures used in the partition of curcumin (CCM) with Pluronic F68/McIlvaine buffer (pH 6.0) + ionic liquids (ILs)-based aqueous two-phase micellar systems (ATPMS).

Assays	ATPMS		T (°C)
	[F68] (wt%)	*[ILs] (M)	
1	1.0 and 5.0	2.75 M [Ch]Cl	20 and 25
2		2.60 M [Ch][Ac]	
3		2.00 M [Ch][Pro]	
4		0.70 M [Ch][But]	
5		0.60 M [Ch][Hex]	

* The binodal curves in the presence of ILs were experimentally obtained in the same region in order to guarantee the same tie-line, as well as the same polymeric micelles (PMs) concentration in the PMs-rich phase. For this reason, the ILs concentration evaluated were different between the assays.

performed for the two ATPMS with best partition performances. The experimental protocol was similar excepting that, in addition to the CCM concentration, the total protein content was also determined.

The partition performance was evaluated considering the following parameters: CCM partition coefficient (K_{CCM}), CCM mass balance (*MB*), and percentage of CCM recovered in the PMs-rich and PMs-poor phases (REC_{Rich}, and REC_{Poor}, respectively), as described in Equations (2) to (5), respectively:

$$K_{CCM} = \frac{[CCM]_{PMs\text{-}rich}}{[CCM]_{PMs\text{-}poor}} \quad (2)$$

$$MB\ (\%) = \left(\frac{[CCM]_{PMs\text{-}rich} V_{PMs\text{-}rich} + [CCM]_{PMs\text{-}poor} V_{PMs\text{-}poor}}{[CCM]_{initial} V_{initial}} \right) \times 100 \quad (3)$$

$$REC_{Rich}\ (\%) = \left(\frac{[CCM]_{PMs\text{-}rich} V_{PMs\text{-}rich}}{[CCM]_{initial} V_{initial}} \right) \times 100 \quad (4)$$

$$REC_{Poor}\ (\%) = \left(\frac{[CCM]_{PMs\text{-}poor} V_{PMs\text{-}poor}}{[CCM]_{initial} V_{initial}} \right) \times 100 \quad (5)$$

The performance of each ATPMS to purify CCM from crude extract of *C. longa* L. was determined in terms of selectivity (*S*) and purification factor (P_F) of CCM relatively to total protein contents (as contaminants), as described by Eqs. (6) and (7), respectively.

$$S = \frac{K_{CCM}}{K_{TP}} \quad (6)$$

where K_{TP} refer to total protein (TP) partition coefficient (like in Equation (2)).

$$P_F = \frac{\frac{[CCM]_{PMs\text{-}rich}}{[TP]_{PMs\text{-}rich}}}{\frac{[CCM]_{initial}}{[TP]_{initial}}} \quad (7)$$

where $[TP]_{PMs\text{-}rich}$ and $[TP]_{initial}$ correspond to the concentration of total proteins in the PMs-rich phase and the initial total protein concentration in the system, respectively.

2.5. Quantification of CCM concentration

CCM concentration was determined by measuring the optical absorbance at a wavelength of 420 nm (Elisa Biotech Synergy HT UV–Vis spectrophotometer, Biotek, USA) using acetone and ultrapure water as blank samples. Absorbance values were correlated with CCM concentration based on a pre-established calibration curve (0 to 20 mg/mL of pure CCM).

2.6. Quantification of total protein concentration

Total protein concentration was determined through the bicinchoninic acid method (Pierce™ BCA Protein Assay Kit), according to the manufacturer's instructions. The protein was determined as visible optical absorbance at a wavelength of 562 nm by spectrophotometry (Elisa Biotech Synergy HT, Biotek, USA), using ultrapure water as blank. Absorbance values were correlated to protein concentration by using a pre-established calibration curve with bovine serum albumin (BSA) (0 to 1 mg/mL).

2.7. pH and conductivity determination

pH (± 0.03) values and conductivities were determined using a dual meter pH/conductivity device (Mettler Toledo, Columbus, OH, USA; model MPC 227).

2.8. Statistical analysis

Data were collected from three independent experimental assays and presented as mean ± standard error of the mean. p values < 0.05 were considered as statistically significant.

3. Results and discussion

3.1. CCM stability studies

In order to define the best processual conditions for partition studies, the CCM stability (CCM_{stb}) was determined at different pH values (6.0, 7.0, and 8.0) and temperatures (30, 50, and 70 °C) over 72 h (Fig. 1a and 1b), as well as in the presence of aqueous solutions of Pluronic F68 (1.0 and 10.0 wt%) and each IL (0.1 and 3.0 M) over 24 h (Fig. 2).

A general analysis of Fig. 1a and 1b allows identifying two major trends, a pH-dependent ($CCM_{stb,\ pH\ 6.0} > CCM_{stb,\ pH\ 7.0} > CCM_{stb,\ pH\ 8.0}$) and a temperature-dependent ($CCM_{stb,\ 30\ °C} > CCM_{stb,\ 50\ °C} > CCM_{stb,\ 70\ °C}$), respectively. Fig. 1a shows that CCM remains stable at acidic conditions (pH 6.0) up to 72 h of incubation (CCM_{stb} values > 90%). The increase of the pH to 7.0 and 8.0 caused a considerable decrease of CCM_{stb} (after 72 h - CCM_{stb} of 60 and 35%), respectively. Wang et al. [30] also observed higher CCM degradation (≈ 90%) at physiological conditions (*i.e.*, 0.1 M phosphate buffer at pH 7.2) than under acidic pH conditions (3.0 to 6.0). Despite the lower CCM degradation rates (a maximum of 40%), in comparison with Wang's study [30], the negative impact of pH increase is evident.

Regarding temperature influence (Fig. 1b), the incubation at a mild temperature, 30 °C, maintained CCM_{stb} (≈ 85%) up to 72 h. However, an increase to 50 and 70 °C destabilized significantly the CCM, as demonstrated by CCM_{stb} values close to 55 and 20%, after 72 h, respectively. A similar effect was observed by Wang et al. [31], when exposing CCM to 70 °C for 10 min, obtaining a sharp decrease in its concentration. Suresh et al. [32] also observed losses of *circa* 53% of CCM_{stb} as result of heating and pressure joint-effect (incubation at 100 °C, 15 psi for 10 min).

Fig. 2 depicts the CCM_{stb} in the presence of ATPMS components. With the exception of small losses in the presence of 10.0 wt% Pluronic F68 at 30 °C and 24 h of incubation (CCM_{stb} ≈ 85%) (Fig. 2a), CCM is highly stable in the presence of amphiphilic copolymer F68 buffer solutions, confirming the biocompatible and stabilization character of amphiphilic copolymers [9]. Similarly, as shown in Fig. 2b, all diluted aqueous solutions of cholinium ILs (0.1 M) were very compatible with CCM. The increase of IL concentration decreased slightly the CCM_{stb}, in particular, when the more hydrophobic ILs were added (Fig. 2c). For example, after 24 h of incubation at 30 °C with 3.0 M of [Ch][But] and [Ch][Hex] solutions, CCM_{stb} decreased to values of 82 and 80%, respectively. Anyway, these CCM losses are far less intense than those due to pH and temperature increases, confirming the biocompatible character of cholinium ILs. Recently, Magri et al. [33] demonstrated that cholinium ILs aqueous solutions maintain the enzymatic activity of L-asparaginase (ASNase). Similarly, [Ch]Cl and [Ch][Ac] have the most structural stabilizing aptitudes, while the increase of IL's anion alkyl chain length and temperature decreased the favorable stabilizing effects [33]. Like other polymers and ionic compounds [16,33,34], Pluronic F68 and cholinium-based ILs can be thus used as ATPMS-forming components, not only because of ATPMS formation but also for their biocompatible/stabilization characteristics.

3.2. Pluronic F68 + ILs ATPMS phase diagrams

The use of ionic adjuvants on the formation of ATPMS promotes electrostatic screening and, by modifying the inter- and intra-micellar interactions, it induces a subsequent change on phase separation temperature [16,35,36]. Therefore, if the ionic adjuvants decrease the T_{CP}, the separation processes could be performed at lower temperatures, facilitating operational conditions and preventing the damage of thermosensitive molecules/biomolecules. The change on phase separation behavior can also enhance the partition of solutes, which makes the addition of small amounts of ILs a simple and effective strategy to improve the extraction and purification yields. First, in order to find biphasic regions for CCM partition studies at mild conditions, the phase diagrams of all Pluronic F68/buffer + ILs-based ATPMS were determined (Fig. 3).

As shown in Fig. 3, the use of cholinium ILs as adjuvants favored the formation of ATPMS, leading to a decrease of T_{CP} and an increase of the biphasic region. The influence of cholinium-based ILs is very dependent of the anion nature, decreasing the T_{CP} as the IL anion relative hydrophobicity increases, *i.e.*: $[Hex]^- > [But]^- > [Pro]^- > [Ac]^- > Cl^-$. The formation of IL-based ATPMS results from a complex competition between the ILs and amphiphilic copolymers for water molecules, as well by the existence of specific interactions between the coexisting phase-forming components [37]. Salts and ILs can also influence the water structure according to their hydration (salting-in and salting-out) aptitudes [38–40].

The results shown in Fig. 3 seem to indicate that the hydrophobic interactions between PMs-based Pluronic F68 and hydrophobic portion of IL anionic alkyl chain are predominant for the T_{CP} decrease. When increasing the IL anion alkyl chain length, the relative hydrophobicity of IL also increases, consequently strengthening hydrophobic type interactions with the hydrophobic portion of micelles [41]. This effect can be confirmed by the significant increase of the biphasic region with [Ch][Hex] and [Ch][But] as adjuvants. It is likely that these ILs interact more easily with the PMs because of their higher relative hydrophobicity. Parmar et al. [42] confirmed that the presence of

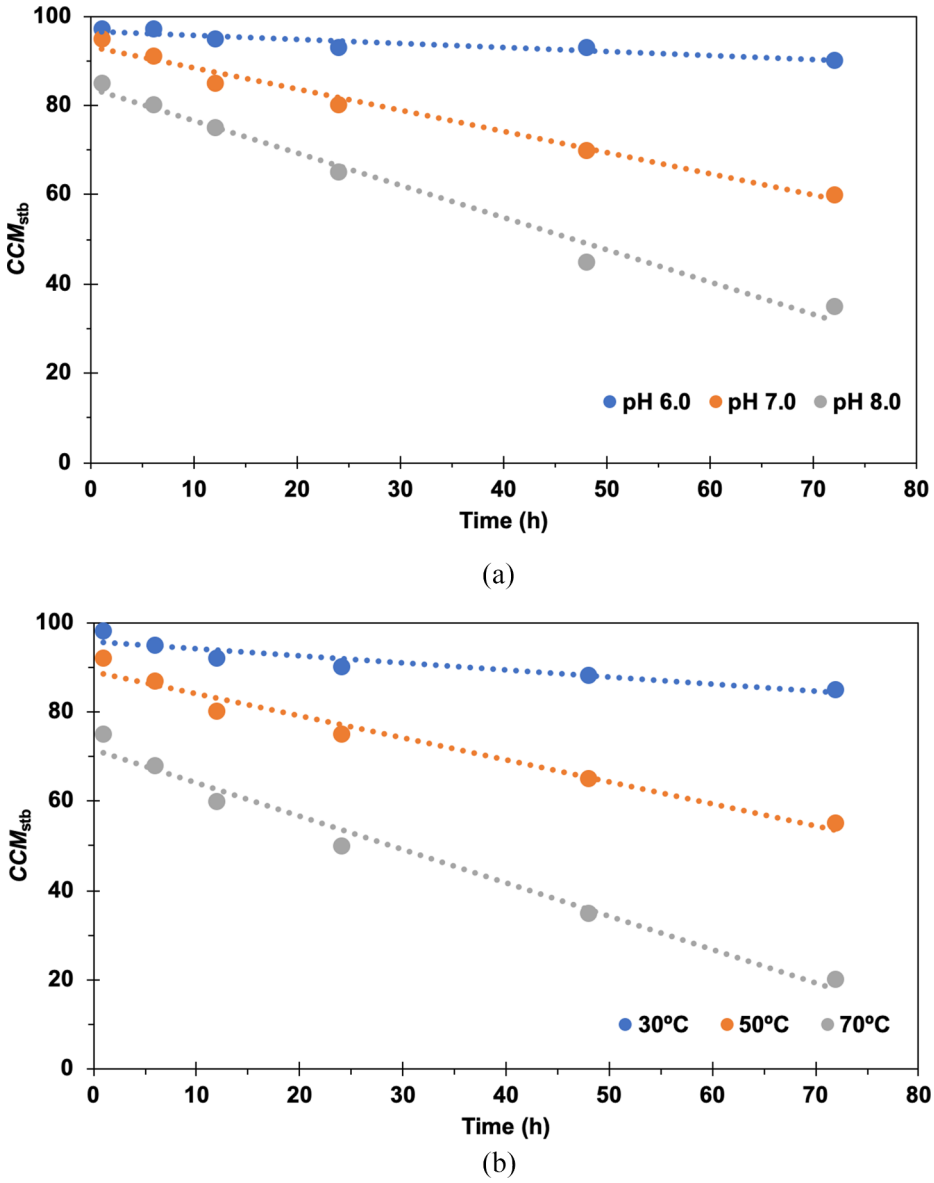


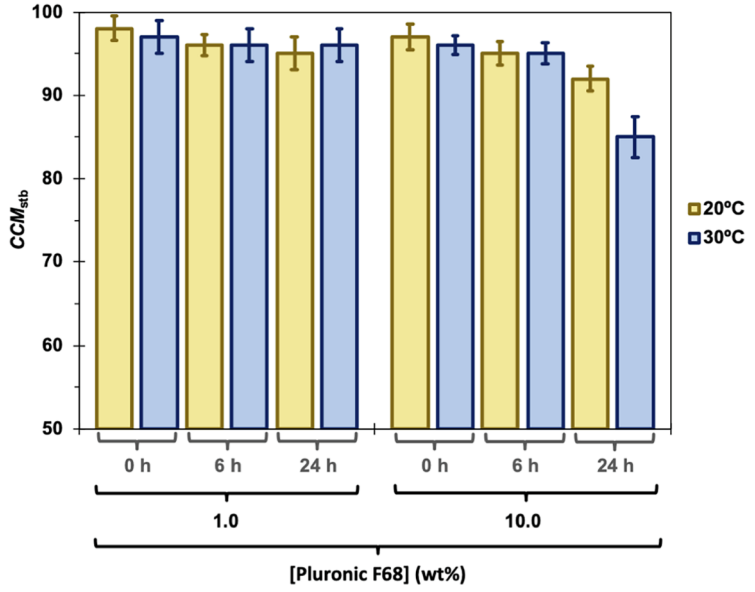
Fig. 1. CCM stability (CCM_{stb}) during 72 h: (**a**) at 25 °C in the buffered (Mcllvaine at pH 6.0, 7.0, and 8.0) solutions; (**b**) pH 6.0 and in the temperatures of 30, 50 and 70 °C. The respective errors correspond to the 95% confidence levels for three independents measurements, but error bars are smaller than the markers.

hydrophobic 1-alkyl-3-methyl imidazolium tetrafluoroborates ($[C_n mim][BF_4]$, n = 4, 6, and 8) modify the shape and size of the amphiphilic copolymer Pluronic P103 (composed of PEO_{17}-PPO_{60}-PEO_{17}), promoting the formation of mixed micelles.

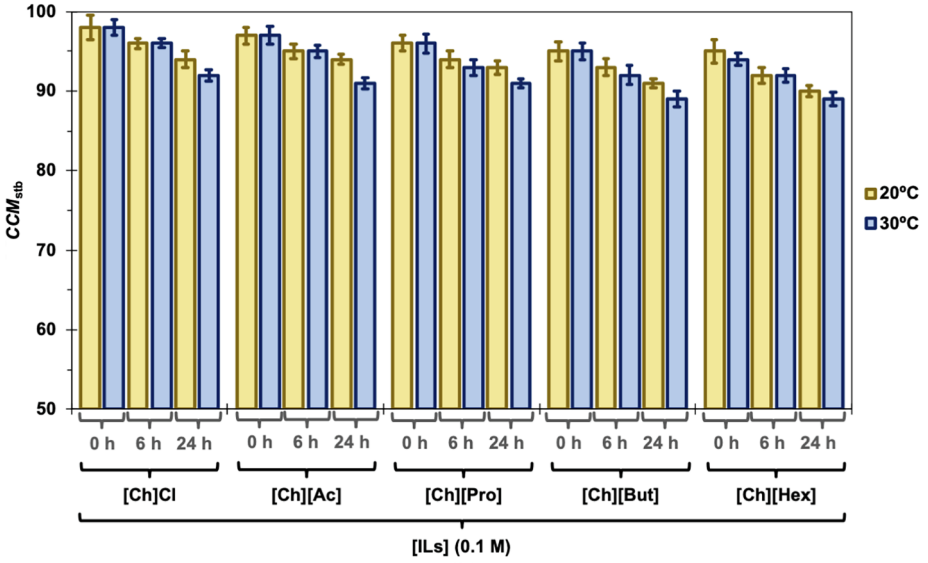
On the other hand, concerning the dehydration of PEO_x-PPO_y-PEO_x copolymer by adding ILs to aqueous solution, a recent study on the critical micellization temperature of Pluronic F108 aqueous solutions showed that the addition of cholinium bitartrate ([Ch][Bit]) and cholinium dihydrogen citrate ([Ch][DHCit]) are more prone to dehydrate the copolymer than [Ch][Ac] and [Ch]Cl (with strong tendency of their ions to be hydrated) [43]. Despite the lower abilities of [Ch][Ac] and [Ch]Cl to form ATPMS, in comparison with the other cholinium ILs (Fig. 3), those ILs enlarged the biphasic region in comparison with the system without ionic additives (Pluronic F68 + Mcllvaine buffer). [Ch]Cl and [Ch][Ac] ions compete for water molecules around PEO chains of amphiphilic copolymers, increasing the micelle-micelle interactions and decreasing the T_{CP} (enlargement of the two-phase region) [5,8]. Our results are in agreement with findings of Teixeira-Pinto et al. [16], that observed a reduction of T_{CP} in $C_{10}E_4$ nonionic surfactant aqueous solutions + salts ($SO_4^{2-} > Cl^- > NO_3^- > I^-$), and Silva et al. [8] that studied cholinium-based ILs + Pluronic L35 (composed of PEO_{11}-PPO_{16}-PEO_{11}) ATPMS, observing an increase of the biphasic region as a function of the nature of the IL anion ($[DHP]^- > [Bit]^- > [DHCit]^- > [Bic]^- > [Ac]^- > Cl^-$).

3.3. Partition of pure commercial CCM using Pluronic F68 + ILs ATPMS

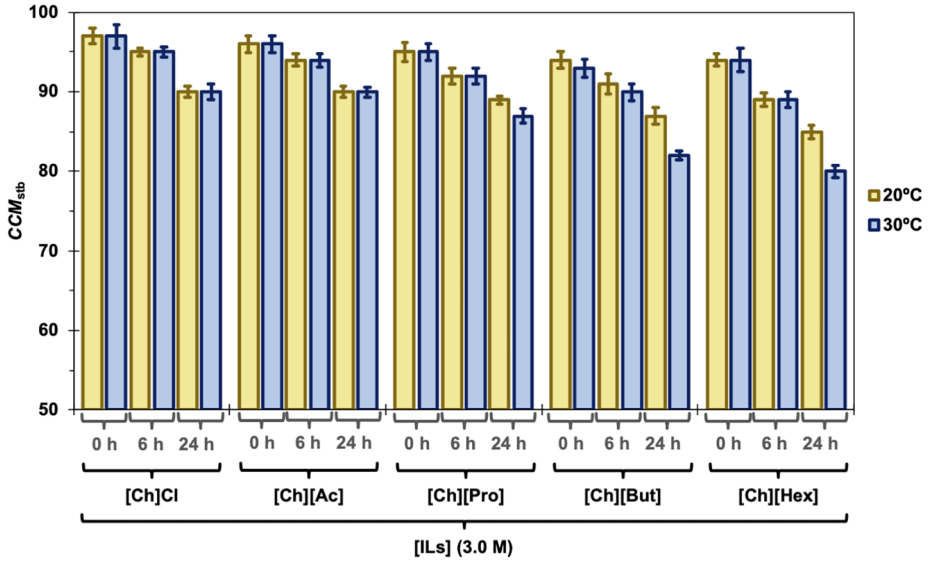
Considering stability and ATPMS phase diagrams results, the partition of commercial CCM was performed at low temperatures (20 < T (°C) < 30), short incubation time (3 h), and low concentrated Pluronic F68 + ILs aqueous solutions. In order to infer the effect of Pluronic F68 in the partition of CCM, ATPMS composed with two distinct polymer concentrations (*i.e.*, 1.0 and 5.0 wt%) were prepared, and mixed with different IL concentrations at mild temperatures (*i.e.*, from 20 to 30 °C), as detailed in Table 2. CCM recovery in the PMs-rich phase (REC_{Rich}) and CCM partition coefficients (K_{CCM}) values are depicted in Fig. 4a and 4b, respectively. In order to guarantee equivalent tie-lines and micelles concentration (ϕ_c) in the PMs-rich phase in the presence of ILs, the binodal curves were experimentally obtained in the same region (Fig. 4c). Note that mass balances of CCM (*MB*) were determined for all



(a)



(b)



(c)

(caption on next page)

Fig. 2. CCM stability (CCM_{stb}) during 24 h (at 20 and 30 °C) in the buffered (McIlvaine at pH 6.0) solutions of: (**a**) Pluronic F68 (at 1.0 and 10.0 wt%); (**b**) 1.0 M and (**c**) 3.0 M of the ionic liquids (ILs) [Ch]Cl, [Ch][Ac], [Ch][Pro], [Ch][But], and [Ch][Hex]. The respective errors correspond to the 95% confidence levels for three independents measurements.

assays and found to be approximately 100% (data not shown).

Fig. 4a and 4b show that, independently of the Pluronic F68 concentration, K_{CCM} (in PMs-rich phase) increases with the increase of the IL anion alkyl chain length. CCM was preferentially partitioned in the PMs-rich phase, with REC_{Rich} (from 45 to 88%) and K_{CCM} (from 3.5 to 22). ATPMS containing 0.70 M of [Ch][But] and 0.60 M of [Ch][Hex] exhibited the highest K_{CCM}, respectively, $15 < K_{CCM} < 22$ (at 1.0 wt % Pluronic F68) and $12 < K_{CCM} < 16$ (at 5.0 wt% Pluronic F68). Since CCM is a hydrophobic compound, its preferential partition to the more hydrophobic phase (*i.e.*, PMs-rich phase) is probably a result of favorable hydrophobic interactions. The Pluronic F68 is a triblock copolymer composed of PEO_{76}-PPO_{29}-PEO_{76} and, therefore, the hydrophobic PPO block must interact with the CCM molecules through hydrophobic interactions, favoring CCM migration to the PMs-rich phase. Letchford et al. [44] demonstrated that the amount of hydrophobic drugs (*i.e.*, indomethacin, plumbagin, paclitaxel, etoposide, including CCM) solubilized in methoxy poly(ethylene glycol)-polycaprolactone (MePEG-*b*-PCL) micelles is directly related to the length of the hydrophobic block (PCL), resulting in higher drug loading in micelles with longer hydrophobic block lengths. The presence of ions affected not only ATPMS equilibria but also the solutes' partition, *i.e.* electrolyte solutions can affect their solubility in the coexisting phases [6,8,45]. It is evident that with the addition of more hydrophobic ILs ([Ch][Hex] > [Ch][But] > [Ch][Pro] > [Ch][Ac] ≈ [Ch]Cl), a more hydrophobic environment is created in the micelle-rich phase, increasing CCM partition.

Fig. 4c shows that as the temperature increases, from 20 °C ($\phi_c \approx$ 7.0 wt%) to 25 °C ($\phi_c \approx$ 8.5 wt%), the K_{CCM} into the PMs-rich phase is increased for both Pluronic F68 concentrations. In this case, with the temperature increase, there is an increase of excluded-volume effect (*EV*) [12,34,45], as shown by high ϕ_c values in the PMs-rich phase, which can explain the difference between the K_{CCM} values obtained.

Fig. 4a and 4b showed that the decrease of Pluronic F68 concentration favor the CCM in the PMs-rich phase, *i.e.*, K_{CCM} at 1 wt% Pluronic F68 > K_{CCM} at 5 wt% Pluronic F68. In order to understand if other effects are affecting K_{CCM}, the pH/conductivity values of the PMs-rich and -poor phases were analyzed (Fig. 5). It was observed that the difference in the conductivity (σ) of both coexisting phases for the ATPMS containing 1.0 wt% ($\sigma_{PMs\text{-}poor\ phase} > > > \sigma_{PMs\text{-}rich\ phase}$, Fig. 5a) is higher than for the ATPMS containing 5.0 wt% of Pluronic F68 ($\sigma_{PMs\text{-}poor\ phase} > \sigma_{PMs\text{-}rich\ phase}$, Fig. 5b). These results seem to indicate that repulsive electrostatic interactions are enhancing the K_{CCM} towards the PMs-rich phase, in particular when used [Ch]Cl and [Ch][Ac] as adjuvants. On the other hand, the σ values of the PMs-rich phase increased with the increase of the IL anion alkyl chain length (namely, [Ch][Pro] < [Ch][But] < [Ch][Hex]), confirming higher incorporation rates of the more hydrophobic ILs in the micelle-rich phase. Recent experimental data of our group corroborate this phenomenon (unpublished data), in which an increase of the hydrodynamic diameter (D_H) of micelles (with Pluronic L35) in the presence of [Ch][But] and [Ch][Hex] is observed, *i.e.* ILs that interact more effectively with copolymer molecules tend to form mixed micelles. In these systems, as the difference in σ tends to decrease, the effect of K_{CCM} is mostly dominated by the aptitude of the CCM to promote hydrophobic interactions with the PMs (as discussed above).

3.4. Purification of CCM from crude extract of Curcuma longa L.

After the partition studies using pure commercial CCM, the two ATPMS with the highest K_{CCM} and REC_{Rich} values were selected to purify CCM from a real matrix. The recovery of CCM from crude samples (powdered material dried from *C. longa* L.) using the ATPMS composed of Pluronic F68 (1.0 wt%) + [Ch][But] (0.70 M) and Pluronic F68 (1.0 wt%) + [Ch][Hex] (0.60 M) were performed at 25 °C, and the respective partition and purification parameters were determined (Table 3).

Both ATPMS were very effective in recovering CCM from crude samples of *C. longa* L., as shown by high REC_{Rich} yields (88 ± 1.1% in the system with [Ch][But] and 92 ± 1.3% with [Ch][Hex]). Interestingly, even when extracting CCM from a more complex matrix, the ability of both ATPMS to partition the CCM into PMs-rich phase was enhanced. Slight increases of the K_{CCM} from the crude sample ($K_{CCM,\ with\ 0.60\ M\ [Ch][Hex]} = 25 > K_{CCM,\ with\ 0.70\ M\ [Ch][But]} = 20$) in comparison to those obtained with pure CCM ($K_{CCM,\ with\ 0.60\ M\ [Ch][Hex]} = 22 > K_{CCM,\ with\ 0.70\ M\ [Ch][But]} = 18$) were observed. It seems that the presence of substantial amounts of hydrophilic contaminants in the crude sample (0.55 mg/mL) is repelling the CCM molecules from the PMs-poor phase.

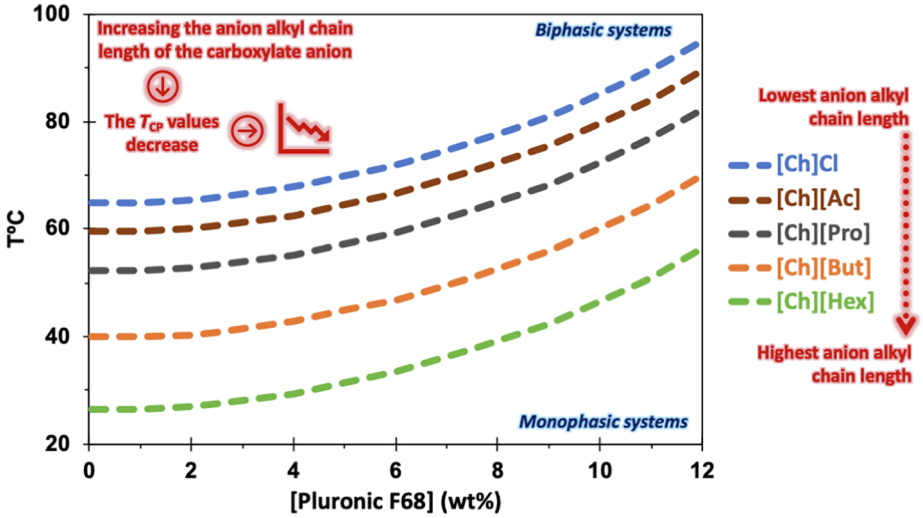


Fig. 3. Binodal curves obtained by cloud point (T_{CP}) method for aqueous two-phase micellar systems (ATPMS) composed of Pluronic F68 and McIlvaine buffer (pH 6.0) + 0.5 M of ionic liquids (ILs), namely: [Ch]Cl (blue); [Ch][Ac] (brown); [Ch][Pro] (gray); [Ch][But] (orange); and [Ch][Hex] (green).

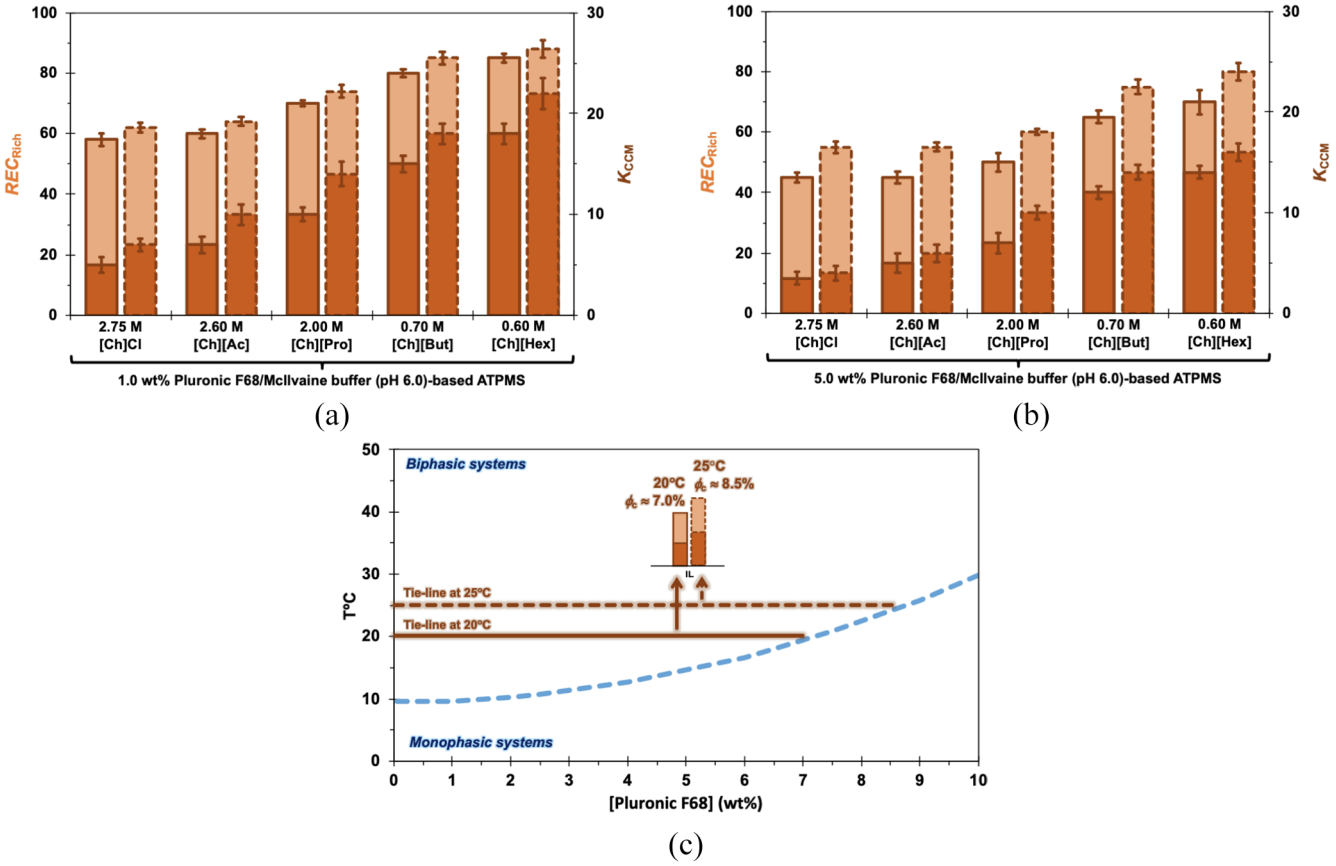


Fig. 4. Partition of curcumin (CCM) in aqueous two-phase micellar systems (ATPMS) composed of (**a**) 1.0 wt%, and (**b**) 5.0 wt% Pluronic F68 and different ionic liquids (ILs), namely: 2.75 M [Ch]Cl; 2.60 M [Ch][Ac]; 2.00 M [Ch][Pro]; 0.70 M [Ch][But]; and 0.60 M [Ch][Hex] at 20 °C (solid lines) and 25 °C (dashed lines). Partition parameters analyzed: CCM recovery (REC_{Rich}, lighter bars), and CCM partitioning coefficient (K_{CCM}, darker bars). (**c**) The binodal curves in the presence of ILs were experimentally obtained in the same region. The ϕ_c corresponds to the Pluronic F68 concentration in the polymeric micelles (PMs)-rich phase, in this case ϕ_c values at 20 °C and 25 °C were estimated in 7.0 and 8.5 wt%, respectively. The respective errors correspond to the 95% confidence levels for three independents measurements.

In addition to the excellent partition abilities, both ATPMS were also very efficient in purifying CCM from the protein-based contaminants, as confirmed by high selectivity values ($S_{\text{with 0.60 M [Ch][Hex]}} = 30 \pm 0.75 > S_{\text{with 0.70 M [Ch][But]}} = 25 \pm 0.45$) and purification yields ($P_{F,\text{ with 0.60 M [Ch][Hex]}} = 15 \pm 0.65 > P_{F,\text{ with 0.70 M [Ch][But]}} = 12 \pm 0.50$). Because of the more hydrophilic character of the contaminant proteins, these are preferentially partitioned into the PMs-poor phase, while the hydrophobic CCM is concentrated in the more hydrophobic environment.

CCM is a major active component of the Indian spice turmeric (*C. longa*). In this sense, commercially available crude CCM contains significant amounts of structurally similar compounds, known as curcuminoids. This class of bioactive compounds is mostly comprised by three fundamental components, namely, CCM (≈ 75 wt%), demethoxycurcumin (DMC, ≈ 10–25 wt%), and bisdemethoxycurcumin (BDMC, ≈ 5 wt%), included in the diferuloylmethane group of phenolic compounds [26,46]. The three major curcuminoids are structurally very similar, distinguished only by (the presence/absence of) a methoxy functional group on each of the aromatic rings [18]. Therefore, more than the removal of hydrophilic contaminants, it is the selective purification of similar curcuminoids the biggest challenge. To confirm if these ATPMS are efficient in the selective purification of CCM from the other major curcuminoids, a careful analysis (by HPLC) of curcuminoids content of the crude sample (*C. longa* L.) before and after the purification using ATPMS was carried out. Initially, the crude sample was composed of 70 wt% CCM, 12 wt% DMC, and 5.5 wt% of BDMC. After the purification step, the PMs-rich phase included 77 wt% CCM and 18 wt% DMC, with no detectable concentration of BDMC. Although selective separation of curcuminoids was not the objective of this work, these preliminary results are very promising, demonstrating that at least one of the major curcuminoids was fully removed (*i.e.*, BMDC) by using a single ATPMS extraction step, as illustrated in Fig. 6. In further studies, we will design and propose additional strategies to obtain selective separation of individual curcuminoids using single or integrated ATPMS-based platforms.

Regarding the purification of curcuminoids, several promising downstream processing platforms have been proposed in the last 5 years [18,26,46–50]. Despite of good CCM recovery yields and purification factors, most previous studies applied very energetic operations, like subcritical solvent extraction [47], cooling crystallization [46], ultrasound-assisted methods [49,50] or combining multiple, time-consuming and laborious stages [18,26]. The high recovery/purification performances together with the milder processing conditions (mild temperatures and VOCs-free) make these Pluronic F68/water + ILs-based ATPMS very interesting platforms for the purification of CCM from *C. longa* L., at least, to replace some of the more energy-consuming and environmentally heavier current downstream units.

4. Conclusions

These results demonstrated the importance of a proper IL-based ATPMS design, namely, by increasing the relative hydrophobicity of the IL anion, to increase the partition of hydrophobic molecules (*e.g.*, curcuminoids, flavonoids, among other bioactive molecules) into the PMs-rich phase. These ATPMS required low concentrations of copolymer and ILs (*i.e.*, 1.0 wt% and 0.70 M for [Ch][But] and 0.60 M for [Ch][Hex],

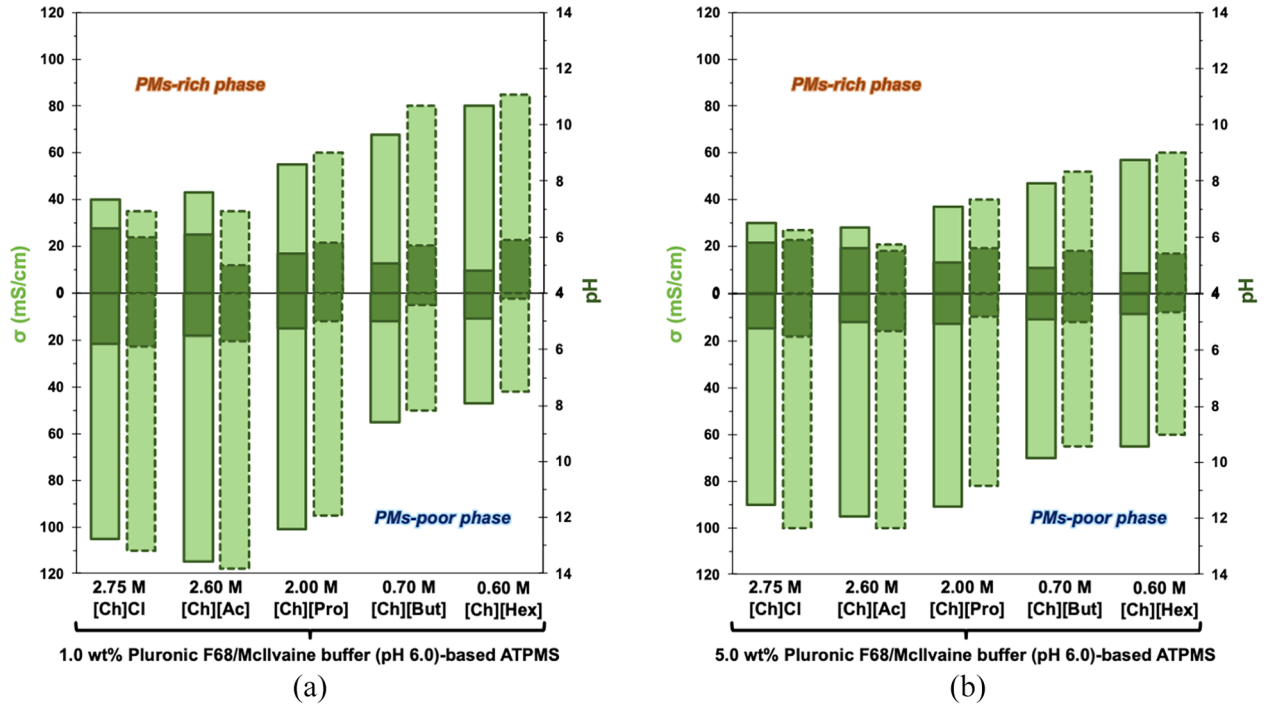


Fig. 5. pH and conductivity (σ) values of polymeric micelles (PMs)-rich and -poor phases of the aqueous two-phase micellar systems (ATPMS) composed of 1.0 wt% (**a**) and 5.0 wt% (**b**) of Pluronic F68 + ionic liquids (ILs) at different concentrations: 2.75 M [Ch]Cl; 2.60 M [Ch][Ac]; 2.00 M [Ch][Pro]; 0.70 M [Ch][But]; and 0.60 M [Ch][Hex] at 20 °C (solid lines) and 25 °C (dashed lines).

Table 3
Experimental results of curcumin partition (K_{CCM} and REC_{Rich}), purification factor (P_F), and selectivity (S) from crude sample of *C. longa* L. using aqueous two-phase micellar systems (ATPMS) composed of 1 wt% Pluronic F68 + ionic liquids (ILs) at 25 °C and pH 6.0. The respective errors correspond to the 95% confidence levels for three independents measurements.

Parameters	Pluronic F68 (1 wt%) + ILs-based ATPMS	
	[Ch][But] (0.70 M)	[Ch][Hex] (0.60 M)
K_{CCM}	20 ± 0.04	25 ± 0.05
REC_{Rich}	88 ± 1.1%	92 ± 1.3%
P_F	12 ± 0.50	15 ± 0.65
S	25 ± 0.45	30 ± 0.75

respectively) to form biphasic regimes, as well as to guarantee high CCM recovery yields. The partition/purification of CCM results confirm the potential of IL-based ATPMS as a simple, mild, safe and environmentally friendly platform for the replacement of common VOCs-based extraction methods and chromatographic-based purification processes. This work demonstrated that Pluronic F68/buffer + ILs-based ATPMS are feasible options for the purification of bioactive compounds from complex media by a selective removal of hydrophobic molecules from the more hydrophilic protein-based contaminants. In addition to the use of ATPMS as extraction platforms, these systems are also promising to encapsulate hydrophobic molecules/biomolecules within nanostructures (*i.e.*, polymeric micelles – PMs) and to develop novel drug delivery systems (DDS).

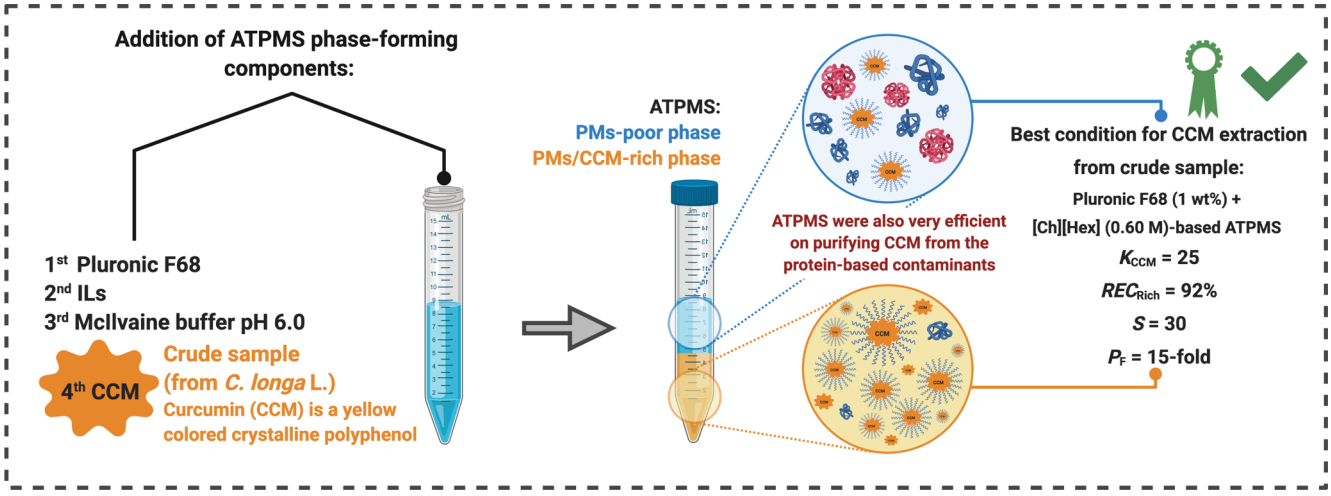


Fig. 6. A schematic representation of the separation process for the partition/purification of curcumin (CCM) using aqueous two-phase micellar systems (ATPMS). Experimental results of CCM partition (K_{CCM} and REC_{Rich}), purification factor (P_F), and selectivity (S) from crude sample of *C. longa* L. using the best ATPMS (1 wt% Pluronic F68 + 0.60 M [Ch][Hex] at 25 °C and pH 6.0). PMs correspond to polymeric micelles.

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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