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Rubisco separation using biocompatible aqueous two-phase systems

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ABSTRACT

Mild and efficient separation processes have to be developed to convert microalgal biomass into high valuable products. Aqueous two-phase system (ATPS) was adopted as a new approach in microalgae to separate hydrophilic from hydrophobic components. In this work, three biocompatible ATPSs polyethylene glycol (PEG) 400-Potassium citrate, Iolilyte 221PG-potassium citrate and PEG 400-Cholinium dihydrogen phosphate ATPS were selected based on their interaction with Ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco), a protein predominantly present in microalgae and used as ingredient in human and animal food. Binodal curves were constructed for each system and the parameters influencing phase formation were investigated. Iolilyte 221PG-potassium citrate has a stronger ability to form ATPS compared with the PEG-based systems. This stronger ability was attributed to hydrophobic and electrostatic interactions between the phase-forming components. After characterization, we investigated the performance of the ATPSs in the partitioning of Rubisco. In this study, the effect of the tie-line length (TLL), pH and type of phase-forming components on Rubisco extraction efficiency (%) was analyzed. In a single step, the appropriate parameters lead to extraction efficiencies between 80 and 100%. Additionally, stability studies were performed to see if ATPS retain the native protein structure. Iolilyte 221PG-Citrate was found to be the most efficient ATPS in Rubisco separation. However, stability studies indicated that PEG-based ATPSs have a better performance in retaining the Rubisco integrity.
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1. Introduction

Microalgae can accumulate up to 50–70% oil; 60% protein and 60% carbohydrates under different conditions [1], making it excellent candidates to supply sufficient energy and raw materials without damaging the environment. In the interest of sustainability and economic competition with other sources, a microalgae biorefinery approach should be used to valorize all the compounds inside the cell instead of one specific compound [2]. Hence mild techniques, able to break the cells and separate valuable biomolecules from cell debris need to be developed. Furthermore, such techniques need to be economical, scalable and low in energy consumption [3].

Aqueous two phase systems (ATPS) as partition technique may be used to separate biological materials from proteins to cells [4]. The technique consists of a mixture of two polymers, one polymer and a salt, or two salts that in an aqueous medium at a certain concentration separate into two phases. These systems provide a mild environment for biomolecules such as proteins due to the high quantity of water they contain and their low interfacial tension [5]. The use of traditional phase-forming components (salts/polymers) has been successfully used to separate proteins from different sources [6–8]. Traditional systems (salts/polymers) have further evolved with the use of new green solvents named "Ionic liquids (IL)". These solvents are entirely composed of ions and are fluid around or below 100 °C. They offer many advantages in green processes, but the interest in the ILs for ATPS lies mainly in their design flexibility. Furthermore, ILs have shown to enhance the extraction process and optimize selectivity and substrate solubility, overcoming the limited polarity range of traditional polymer based systems [9].

Yu et al. [10] were the first to demonstrate the extraction of proteins from biological fluids by using 1-butyl-3-methylimidazolium chloride (BmimCl) and K_2HPO_4 without altering the protein's natural properties. Subsequently, many studies focusing on protein extraction have used imidazolium based ILs [11–13]. These ILs present certain constraints due to their strong alkaline or acidic character [14,15]. Therefore, many recent studies on ionic liquid-based ATPS have explored more biodegradable and environmentally friendly ionic liquids. Their buffering capacities have also been explored to ensure a mild medium to proteins and enzymes [16].

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Different phosphonium and ammonium based ILs for protein extraction were explored [16–19] with promising results. Ammonium based ILs in combination with inorganic salts were used by [20], demonstrating their potential for the biocompatible extraction of catalytically active enzymes. On the other hand, more recently the stability of Rubisco, BSA and IgG1 in aqueous solutions of two ionic liquids: Iolilyte 221PG and Cyphos 108 was investigated [21]. This stability study indicated that high IL concentrations affect protein stability. Cholinium-based ionic liquids, a novel class of ILs with buffering characteristics, were proposed for extraction purposes. Some studies demonstrated that this class of ionic liquids can retain the protein structure and enzyme function [22–27].

To design an environmental and cost effective separation process for microalgae components, we investigated a novel approach using ATPS. An exhaustive literature review and screening of different chemicals was done to select the phase forming components (information reported in the supplementary material). We selected two ionic liquids based on their mild interaction with proteins: Iolilyte 221PG and Cholinium dihydrogen phosphate (Ch DHp). Biocompatible components: Potassium citrate and PEG 400 were selected to replace commonly used inorganic salts. Finally, the systems are: (PEG 400-Potassium citrate), (Iolilyte 221PG-Potassium citrate) and (PEG 400-Ch DHp). These systems were first characterized to delineate their potential work area and the influence of different parameters was evaluated. Ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) was used as target molecule to evaluate the performance of the three systems. The influence of TLL, pH and type of ATPS on the partitioning of the protein was investigated.

2. Experimental

2.1. Materials

Potassium citrate tribasic monohydrate, polyethylene glycol (PEG) 400 Tris (hydroxymethyl) amino methane and hydrochloric acid were purchased from Sigma-Aldrich. Citric acid was obtained from Merck. The ionic liquids Iolilyte 221PG, >95% and Choline dihydrogen phosphate, >98% were obtained from Iolitec. Table 1 presents the most relevant chemicals used to form the ATPS and their structure. The protein used for the research D-Ribulose 1,5-diphosphate oxygenase/carboxylase from spinach (Rubisco) was obtained from Sigma-Aldrich.

Table 1
Chemical structure and purity of components used to form the ATPSs.

Material	Purity	Chemical structure
Potassium citrate tribasic monohydrate	≥99%	
Citric acid	≥99.5%	
Polyethylene glycol 400 (PEG 400)	N.A[a]	
Cholinium dihydrogen phosphate (Ch DHp)	≥98%	
Iolylite 221PG	≥95%	

[a] N.A: data not available.

2.2. Methods

2.2.1. Characterization of the aqueous two phase systems

The ionic liquid-based ATPSs (Iolilyte 221PG-Potassium citrate) and (PEG 400-Ch DHp) were selected after a strict screening based on protein stability. The ATPS (PEG 400-Potassium citrate) was selected to compare the IL-based ATPS with a more conventional system. Each ATPS was characterized creating its phase diagram which presents a binodal curve and four tie-lines. Two methods were used to obtain the binodal curves experimental data: the cloud point method and the titration method as described [28,29]. The concentrations of the phase-forming components was calculated based on weight quantification with an uncertainty of **±10^{-5} g**. Binodal curves were constructed for each system at 4 °C, 22 °C and 40 °C. The conductivity in each phase was measured to identify the main component.

The tie-lines (TLs) of each phase diagram were determined according to the gravimetric method proposed by Merchuk et al. [30]. A mixture was selected, which was at the tie-line. This mixture was prepared gravimetrically ±10^{-4} g, vigorously stirred and left to equilibrate until the phases completely separated. The phases were carefully separated with a glass Pasteur pipette and their weight was recorded. Eventually, the lever-arm rule was used to calculate each tie-line [30]. To calculate the tie-line length, Eq. (1) was used:

$$TLL = \sqrt{(X_T - X_B)^2 + (Y_T - Y_B)^2} \tag{1}$$

X_T, Y_T, X_B, Y_B are the phase composition, where the subscript T is top phase and B is bottom phase.

2.2.2. Rubisco partitioning: Partition coefficient and extraction efficiencies, effect of tie-lines and effect of pH

After the named systems were characterized, the partition studies were completed with a model protein (Rubisco) that was selected for being the most common biomarker protein present in microalgae and an interesting food ingredient. The total concentration of Rubisco in the mixture was 0.3 mg/mL. The mixtures were gravimetrically (10^{-4} g) prepared by using the four tie-lines reported with a volume ratio (V_r) between top and bottom phase of one. Blanks without protein were made to quantify accurately the protein. The mixtures were stirred for 10 min in a rotatory shaker and incubated at room temperature overnight to ensure phase separation. Each phase volume was then recorded and carefully separated for further analysis.

Rubisco distribution between the two phases was described by the partition coefficient *Kp* which in this context is the ratio of protein concentrations between top and bottom phase (Eq. (2)). Rubisco percentage extraction efficiency $EE_{RUBISCO}\%$ expresses the ratio of protein amount between the top phase and the total mixture (Eq. (3)).

$$Kp = \frac{C_{TOP}}{C_{BOTTOM}} \tag{2}$$

$$EE_{RUBISCO}\% = \frac{C_{TOP} * V}{C_{BOTTOM} * V + C_{TOP} * V} \tag{3}$$

Protein was quantified at 280 nm by using a Tecan infinite M200 plate reader. A calibration curve was determined for Rubisco showing a linear correlation. To accurately quantify protein, ATPS without protein was used as blank. Every sample was made in duplicate.

ATPS were prepared at different pH to study its effect in protein partitioning. The system pH was adjusted changing the salt ratio (Potassium citrate/Citric acid). In the case of PEG 400-Ch DHp the pH was adjusted using 10 M NAOH.

2.2.3. Stability studies

Size exclusion chromatography (SE-HPLC) and PAGE electrophoresis were used to analyze the effect of the two ionic liquids on the protein conformation. Top phase samples were analyzed after separation. Other experiments were performed using 2 mg/mL of Rubisco stock solution and was then mixed with ILs at concentrations from 10 to 40%w/w.

Size exclusion chromatography: SE-HPLC was performed on Shimadzu UPLC using an Agilent Bio SEC-3, 3μ particle size, 300 A, 7.8 × 300 mm. 0.1 M sodium phosphate buffer pH 7 and 0.3 M sodium chloride (mobile phase) was run isocratically with a flow rate of 1 mL/min during 25 min. The protein was detected at 280 nm keeping a constant temperature of 25 °C.

Native-PAGE: The samples were diluted with native sample buffer (1:2). After mixing the samples were applied on a 4–20% Criterion TGX (Tris-Glycine eXtended) precast gel and run in 10 X Tris glycine native buffer at 125 V for 75 min. Native gels were stained with Bio-safe coomassie and with the Pierce silver stain kit. The precast gels, buffers and Bio-safe coomassie blue were procured from Bio-Rad. The Pierce silver stain kit was obtained from Thermofisher.

3. Results and discussion

3.1. Characterization of ATPS

Phase equilibrium data is required to understand the thermodynamic behavior of the ATPSs and can be provided by the phase diagrams. These diagrams delineate the potential working area for a particular aqueous two-phase system (ATPS). The phase diagram contains a binodal curve and tie-lines. The binodal curve divides the phase diagram into two zones: below the curve is the one-phase zone and above the curve is the two-phase zone. The larger the two-phase zone is, the more easily the components can form ATPS. In other words, the closer the curve to the origin, the more easily the components can form ATPS. Fig. 1 shows the three binodal curves obtained where component X is the compound that is enriched in the lower phase and component Y is the compound that is enriched in the upper phase. When compared, these curves indicated that the order in ability to form ATPS is as follows: Iolilyte 221PG-Citrate > PEG 400-Citrate > PEG 400-Ch DHp. The binodal curves fitted parameters, standard deviations (σ) and regression coefficients (R^2) are reported in the supplementary material.

In the present study three different aqueous two phase systems (ATPS) were characterized to delineate their potential working area and to understand interactions behind phase separation. Phase separation depends on the type of salt involved and concentration, polymer molecular weight and concentration, phase volume ratio and equilibrium characteristics. Conventional polymer-based ATPS consist of two incompatible polymers or a polymer and a salting-out inducing salt. It is known that increasing the molecular weight of PEG also increases the working area of the ATPS [28]. In other words, if the working area of the ATPS is increased, less PEG and salt are required for phase separation. PEG molecular weight is directly related to PEG hydrophobicity [31,32]. Thus, higher molecular weight PEGs are more hydrophobic and have as a consequence higher phase-forming ability. Comparing our two ATPS consisting of citrate salt (Iolilyte 221PG-citrate and PEG 400-citrate), Iolilyte 221PG (n = 5–15) has a higher phase-forming ability than PEG 400 (n = 9) due to its higher molecular weight.

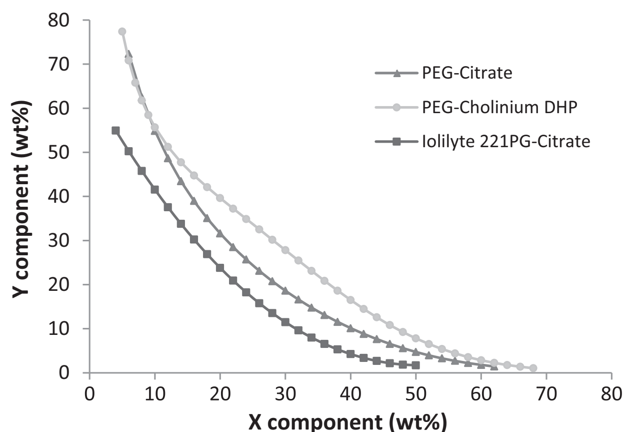

Fig. 1. Binodal curves of ATPS at 25 °C: ■, Iolilyte 221PG-Citrate; ▲, PEG400-Citrate; ●, PEG400-Ch DHp.

The effect of a salt on the miscibility of a solute in an aqueous solution (salting-out effect) has received more attention in literature as an explanation for phase-forming ability. The type of salt affects phase separation depending on its salting out ability [33,34]. This salting out effect seems to be the basis of phase formation in both ATPS: Ionic liquid-salt and Polymer-salt, this effect is correlated to the hydration strength of the salt [12]. In our study, the top phase corresponds to PEG-rich phase or ionic liquid-rich phase while the bottom phase is mainly composed of citrate salt. The addition of high charge density salts to aqueous solutions composed of certain ionic liquids leads to phase separation [35]. This is due to a preferential hydration of the high charge density salt over the ionic liquid leading therefore to the exclusion of the ionic liquid to the ionic liquid-rich phase. In agreement with this, citrate salt has a higher effect (salting-out) over Iolilyte 221PG (more hydrophobic) than over PEG 400.

In contrast, PEG 400-Ch DHp ATPS is formed by a polymer and an ionic liquid, where the top phase is PEG-rich and the bottom phase is Ch DHp-rich. The phase forming separation of this system is more complex to explain because both components significantly contribute to phase separation [36]. Phase-forming ability in our system (PEG 400-Ch DHp) is governed mainly by the specific affinity of the ionic liquid for water. Higher affinity leads to a higher phase-forming ability. This is in agreement with PEG-salt ATPS, in which the ions with higher charge density are more able to create ion-water complexes and more repulsive interactions with PEG [37]. Comparing our two ATPS using PEG 400 (Fig. 1), both curves start and end in similar points, but the middle part of the PEG400-Ch DHp ATPS becomes linear, while PEG400-Citrate system remains curved. This shows that the phase-forming ability of PEG 400-Ch DHp ATPS depends on the amount of phase-forming components added. This was also observed with other polymer/cholinium-based ionic liquids ATPS [37,38].

3.2. Effect of temperature on the ATPS formation

We studied the effect of temperature on the three systems by constructing the binodal curves at three different temperatures: 4, 25 and 40 °C (Fig. 2). Temperature seems to have more influence on the polymer-salt (PEG 400-Citrate) ATPS than on the ionic liquid based-ATPS (Iolilyte 221PG-Citrate, PEG400-Ch DHp). However, similarities were found in the two polymer (PEG 400) systems, where the binodal curves are closer to the origin at higher temperature (40 °C). Hence, the increase of temperature enhances the phase separation of the system. On the other hand, ionic liquid-salt ATPS shows the opposite effect: lower temperature (4 °C) leads to better phase separation.

In PEG 400-citrate and PEG 400-Ch DHp ATPSs increasing temperature enhance phase-separation ability. This is in agreement with other conventional polymer-salt ATPSs and polymer-ionic liquid ATPSs previously studied [37–39]. Both hydrogen-bonding and hydrophobic interactions between PEG and water are responsible

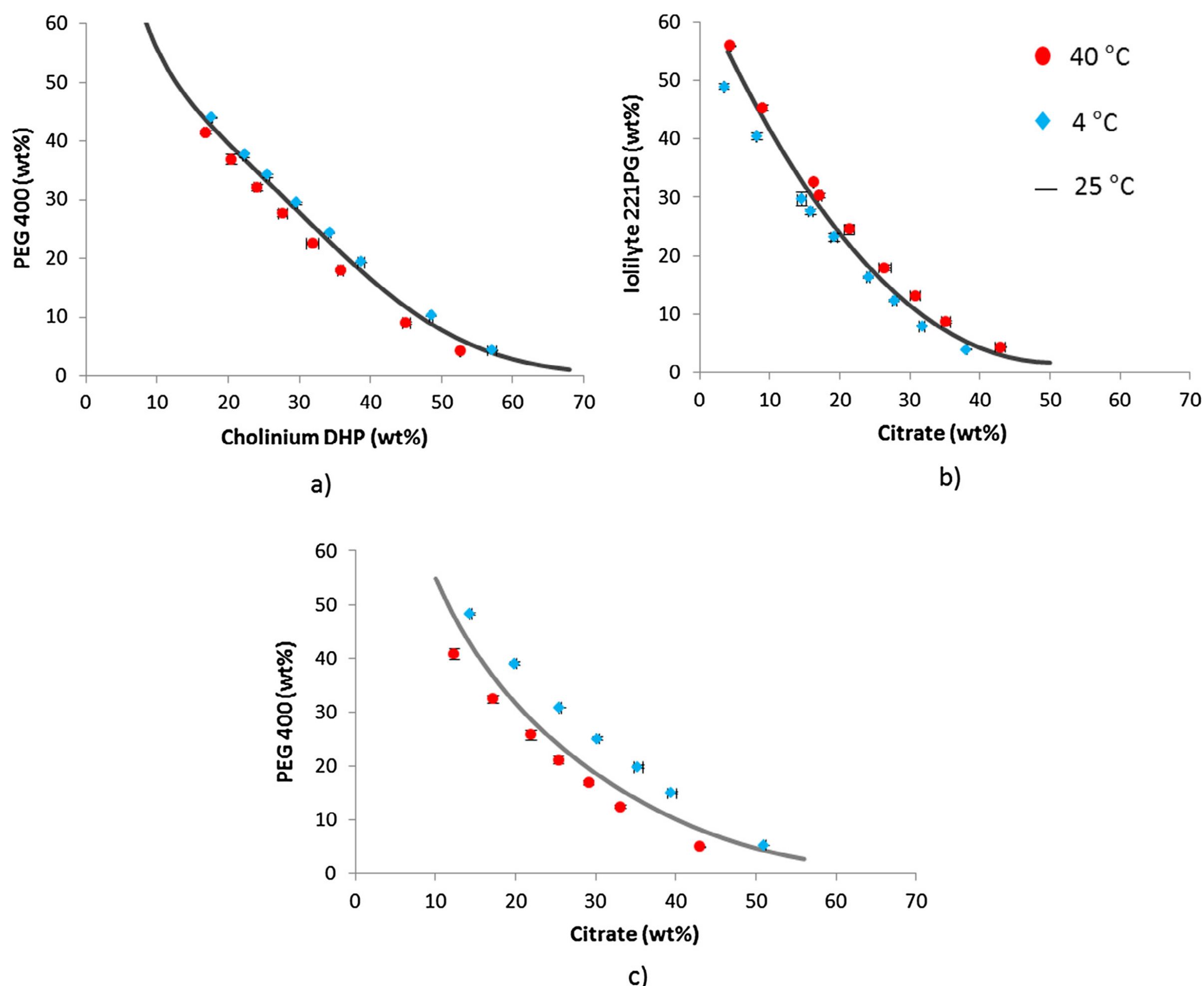

Fig. 2. Effect of temperature on the binodal curves of the ATPS: (a) PEG400-Ch DHp; (b) Iolilyte 221PG-Citrate; (c) PEG400-Citrate; ●, 40 °C; ■, 25 °C; ◆, 4 °C.

for the temperature effect on phase-formation. In PEG 400-citrate and PEG 400-Ch DHp ATPS higher temperatures lead to a breakdown of the hydrogen bonds and consequently to easier phase-separation [40].

In the Iolilyte 221PG-citrate ATPS a decrease in the temperature is favorable for phase-separation, showing an opposite behavior than the other two ATPS studied. The interactions between hydrophilic ionic liquids and water are low with the presence of a salting out salt (citrate). Based on Fig. 2, decreasing the temperature to 4 °C, these interactions are lower. This leads to enhance the phase-forming ability. This behavior was found in other studies in which the effect of the temperature on phase-separation depends on the type of salt employed [20]. Sadeghi et al. [39] discussed this effect based on salting-out coefficient of the salt, showing that by increasing the temperature, the salting out coefficient decreases, resulting in a better phase-formation.

Based on these results, temperature is an important parameter to understand the driving forces of phase-separation. However, looking towards the design of an effective separation process IL-based ATPS did not show an important shift in the binodal curves at different temperatures. Rubisco extraction studies were performed at room temperature (no need of heating or cooling).

3.3. Tie-lines

Fig. 3 illustrates the binodal curve and tie-lines at 25 °C of each system. Four tie-lines were determined for each system to evaluate the effect of the ATPS composition on the extraction studies. The tie-line determines the two phase composition in equilibrium. Once this is determined, we also obtained the tie-line length (TLL), an important parameter that represents the composition and thermodynamic difference of the two phases. The detailed weight fraction data and respective correlations, tie-lines, slope of the tie-line and tie-line lengths are provided in the supplementary material.

3.4. Extraction of Rubisco in ATPS

Rubisco was selected as a model protein to evaluate the performance of three ATPS in a microalgae biorefinery framework. A conventional ATPS was selected to compare with two IL-based ATPS at 25 °C. Table 1 shows the effect of the tie-line length on the Rubisco partition coefficient and extraction efficiency percentage in the three ATPS. The partition coefficient of Rubisco increases with the TLL and this effect seems to be consistent in the three systems. Increasing the salt concentration from ~30 wt% to ~40 wt% in the three systems will enhance the salting out effect, resulting in higher exclusion of the protein to the opposite phase.

Partition coefficient values demonstrate the preference of Rubisco for the top phase in the three cases. The best partitioning was obtained by the ionic liquid based-ATPS (Iolilyte 221PG-Citrate) with a maximal extraction efficiency of 98.8% by a single extraction step (see Table 2).

The partitioning behavior of proteins is influenced by their physicochemical properties and their interactions with the system

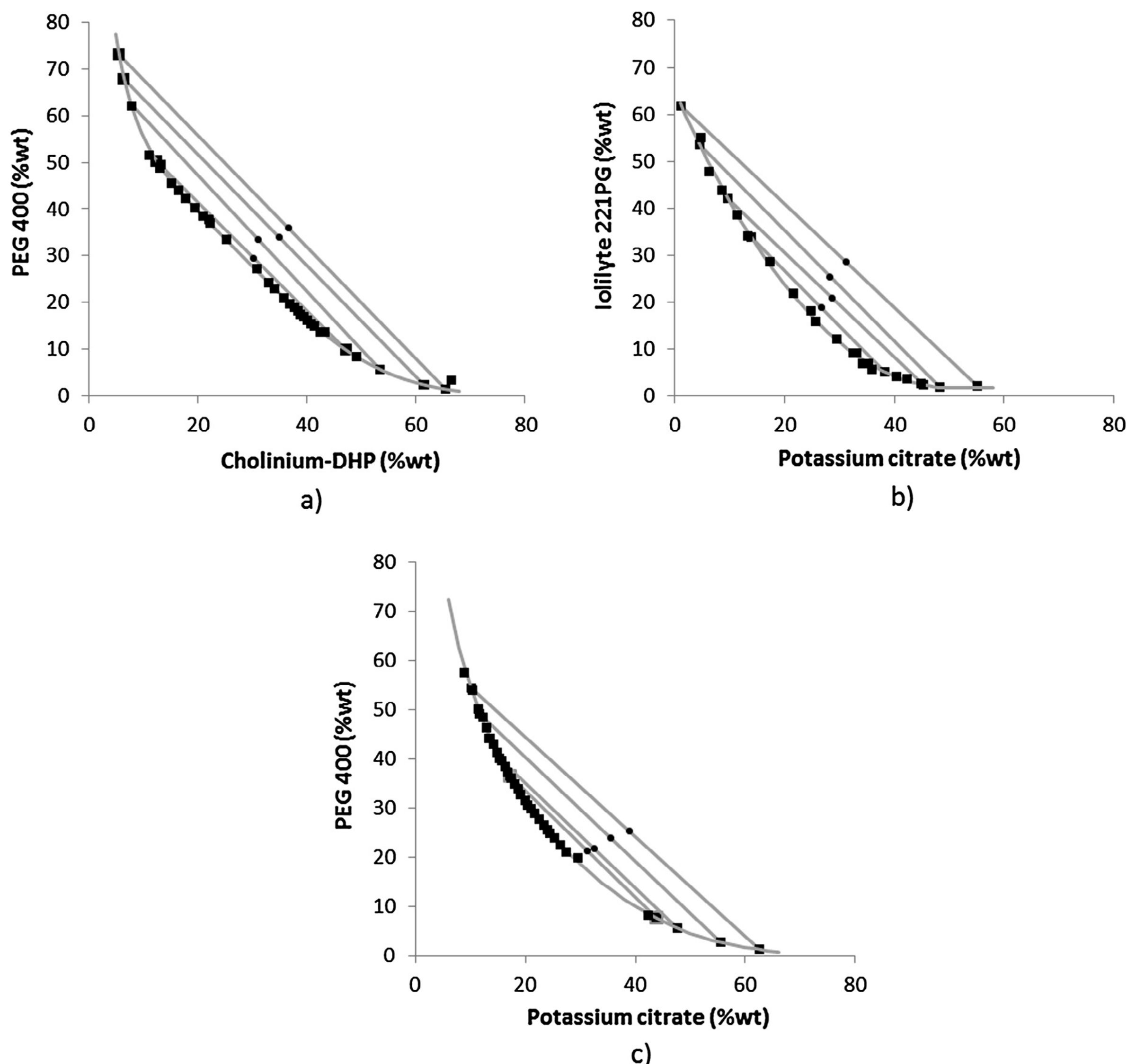

Fig. 3. Phase diagram of the ATPS: (a) PEG400-Cholinium DHp; (b) Iolilyte 221PG-Citrate; (c) PEG400-Citrate; ■, binodal curve; ●, total composition.

components (Polymer, salt, ionic liquid and water). Protein properties like hydrophobicity, net charge, electrostatic forces, size and solubility have been studied to understand the main driving forces for the partitioning behavior of proteins in ATPS [41,42]. Several authors agree that hydrophobic interactions between the protein and the phase-forming components are one of the main driving forces in the partitioning behavior [11,43]. These interactions are responsible for the protein partition preference for the top phase. In contrary to this, Dreyer et al. found that the main driving force in protein partitioning is the electrostatic interactions between the amino acids of the protein surface and the ionic liquid cation [9]. We conclude that the higher Rubisco extraction efficiency on Iolilyte 221PG-Citrate system compared to the other ATPS is a result of higher salting out in that system and higher hydrophobicity of the ionic liquid. Furthermore, electrostatic interactions between the IL cation and negatively charged amino acid residues at the surface of Rubisco influence positively the extraction efficiency.

Table 2
Effect of tie-line length (TLL) on Rubisco partition at 25 °C. TLL is described in Eq. (1). Kp is the Rubisco partition coefficient (Eq. (2)) and $EE_{RUBISCO}\%$ stands for Rubisco percentage extraction efficiency (Eq. (3)).

ATPS	TLL	Kp	$EE_{RUBISCO}\%$
PEG 400-Citrate	39.30	7.00	88.3
	47.25	11.28	90.7
	63.75	17.19	94.1
	74.63	21.50	96.6
Iolilyte 221PG-Citrate	37.53	38.42	91.8
	53.23	27.54	93.3
	67.70	32.32	96.0
	80.30	93.80	98.8
PEG 400-Cholinium DHp	53.13	2.59	72.5
	72.92	2.81	72.1
	85.33	3.21	77.6
	93.28	3.63	79.6

The maximal extraction efficiency using PEG 400-Ch DHp was 79.6%. The protein is partitioned preferentially to the PEG-rich phase and only 20.4% of protein go to the IL-rich phase. This preference is a consequence of the salting-out effect of the cholinium-based ionic liquid over Rubisco which leads to its migration to the top phase (the more hydrophobic phase). This is in agreement with the partition preference of the protein in the PEG-salt and Ionic liquid-salt ATPSs previously discussed. On the contrary, Li et al.

[22] and Quental et al. [25] found preferential partitioning of BSA protein for the IL-rich phase (less hydrophobic phase) when using PPG-cholinium-based ILs ATPS. The type of polymer influences the partition preference of the proteins. Salabat et al. [44] showed that the recovery of the proteins in the ionic liquid-rich phase was higher than in the polymer-rich phase when using PPG than when using PEG [44]. This difference seems to be related with the higher hydrophobicity of PPG compared with PEG. In addition, the protein structure and its hydrophobic interactions with the ATPS components affect notably the partitioning preference [11,43]. Protein partitioning is then the result of a more complex phenomenon, where hydrogen bonding and molecular interactions between the protein and ATPS components are the main driving forces.

3.5. Effect of the pH

pH is an important parameter that influences protein charge and conformation. Three different pH conditions were studied 6, 7 and 8 to look for the optimal conditions for Rubisco partitioning in ATPS. The pH studied are higher than Rubisco isoelectric point (pI), which is between 5.5 and 5.7. This means that the protein has a net negative charge in the ATPSs. Rubisco partitioned preferentially to the top phase. This can be explained based on the interactions between the protein that is negatively charged and the compound that is enriched in the upper phase (PEG 400 and Iolilyte 221PG). Fig. 4 shows the effect of pH on Rubisco extraction efficiency in the three systems studied. A system with pH 8 drives more protein to the top phase, showing a higher extraction efficiency. This increase is easier to observe in the PEG-based ATPSs than in the Iolilyte 221PG-citrate system, which efficiency is already close to 100% at pH 6. The effect of the system pH on Rubisco partitioning indicates that negatively charged characteristics of the protein contribute to an enrichment in the upper phase. This can be explain by stronger attraction between the charged exposed groups of the proteins and the phase forming compounds when the protein became more negatively charged (higher pH). Other investigations using PEG-salt and Iolilyte 221PG-salt indicated a similar behavior [9,45].

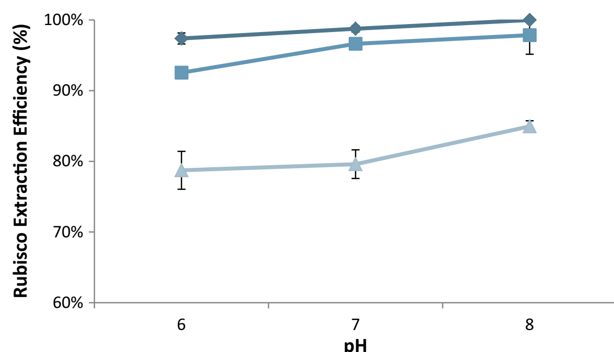

Fig. 4. Effect of pH on Rubisco extraction efficiency in: ◆, Iolilyte 221PG-Citrate; ■, PEG400-Citrate; ▲, PEG400-Ch DHp.

3.6. Stability studies

Native-PAGE was used to evaluate the conformation of Rubisco after separation in ATPS. The gel in Fig. 5(a) shows the top phase of the three systems at optimal conditions, same phase-forming components composition and pH 8. Fig. 5(b) shows Rubisco interaction with PEG400-Ch DHp at different pH conditions. Although Iolilyte 221PG-Citrate shows the best extraction efficiency compared with the other two systems, the band of Rubisco was very soft in the gel (well 2). Opposite to this, in PEG-based systems (PEG400-Citrate and PEG400-Ch DHp) Rubisco bands are more intense (well 1, 3). The native form of Rubisco is influenced by the pH of the ATPS (well 4, 5 and 6). This can be observed with the difference in the Rubisco band visibility when lowering the pH from 8 to 6. The visibility in the gel can also be affected by the low concentration of protein in the top phase when decreasing the pH of the system.

Choline dihydrogen phosphate (Ch DHp) has been identified as one of the most promising media for the stabilization of proteins and other biomolecules [25,27,46,47]. This protein stabilization can be related according the Hofmeister series, which describes the ability of anions to stabilize/destabilize the native state of proteins [48,49]. It is already known that Rubisco is more vulnerable to form aggregates in presence of ionic liquids than other proteins due to its subunits and size/complexity [21]. Samples of the protein were prepared with increasing amount of ionic liquid (10–40 w/w%) to evaluate the interaction of Rubisco with the

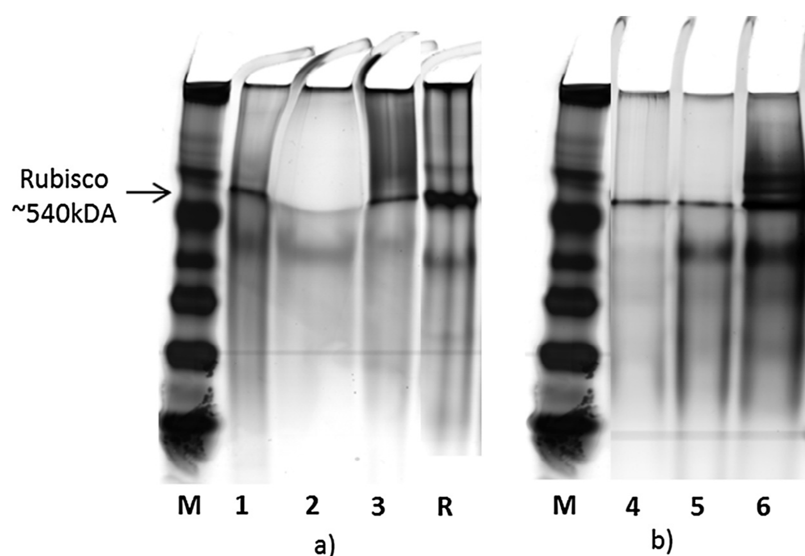

Fig. 5. Native-PAGE of top phase after Rubisco separation in ATPSs. (a) Three ATPS using the same conditions. Marker (M), PEG400-citrate pH 8 (1); Iolilyte 221PG-citrate pH 8 (2); PEG400-Ch DHph pH 8 (3); Rubisco (R). b) PEG 400-Ch DHP ATPS at three pH conditions. Marker (M); PEG400-Ch DHph pH 6 (4); PEG400-Ch DHph pH 7 (5); PEG400-Ch DHph pH 8 (6).

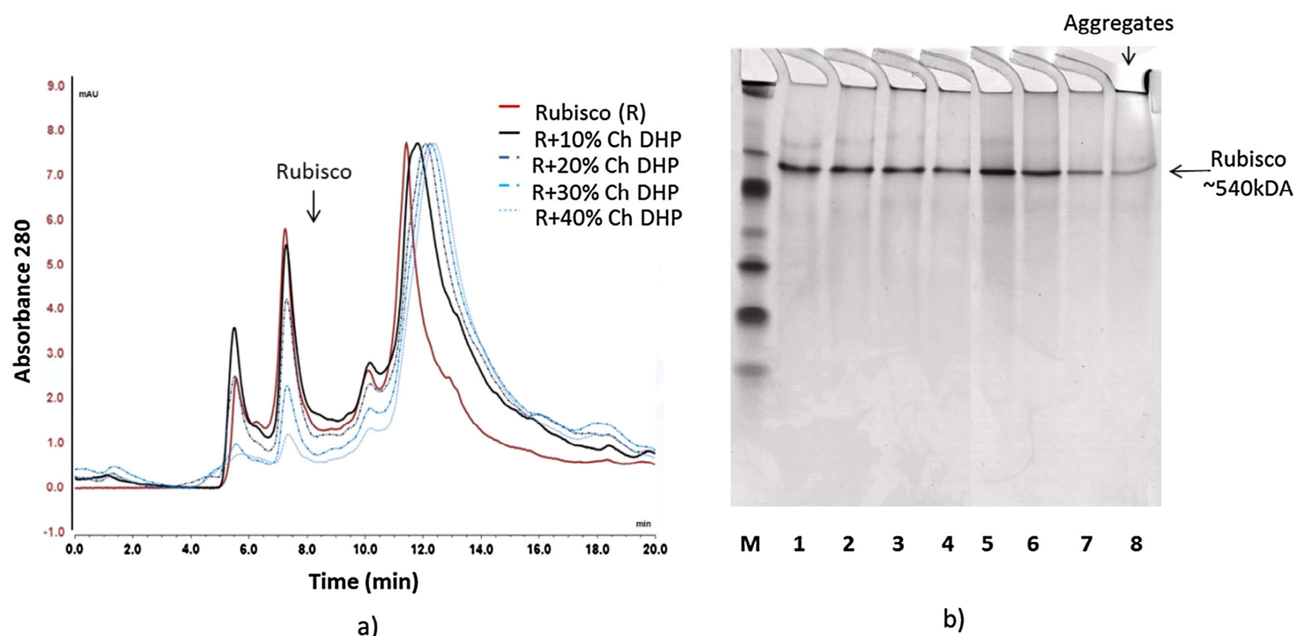

Fig. 6. (a) SEC-HPLC of Rubisco at increasing concentrations of Ch DHph. (b) Native page: effect of increasing amount of IL on Rubisco native form: Marker (M); Rubisco + 10% Ch DHp (1); Rubisco + 20% Ch DHp (2); Rubisco + 30% Ch DHp (3); Rubisco + 40% Ch DHp (4); Rubisco + 10%Iolilyte 221PG (5); 20%Iolilyte 221PG (6); 30%Iolilyte 221PG (7); 40%Iolilyte 221PG (8).

two ionic liquids used: Iolilyte 221PG and Ch DHp. Size exclusion chromatography shows a decreasing of Rubisco signal when increasing the amount of Ch DHp (Fig. 6a). However, comparing the two ionic liquids, Native-PAGE shows no big difference in the bands when increasing Ch DHp concentration. On the other hand, Iolilyte 221PG shows a clear decrease in the Rubisco band and increase in aggregates (well 8) (Fig. 6b). This suggests that Iolilyte 221PG leads to denaturation of the protein at lower concentration (30% of IL) than Ch DHp. We conclude that PEG-based ATPSs (PEG400-citrate and PEG400-Ch DHp) keep the native form of Rubisco and do not destabilize Rubisco by formation of aggregates compared to Iolilyte 22PG-Citrate, which promotes formation of aggregates [21]. The fact that Rubisco prefers to separate in the top phase in the PEG-based ATPSs is beneficial for the stability of the protein due to PEG which is the main component of that phase and stabilizes the protein.

3.7. Conclusions

In this research, two biocompatible ionic liquid-based ATPSs were investigated and compared with a traditional PEG-salt ATPS. We selected biocompatible phase-forming components and ionic liquids suitable for protein separation. The potential of the ATPSs was demonstrated based on the characterization of the systems. Phase equilibrium data of the three systems were here reported for the first time and the parameters evaluated were useful to understand the phenomena behind each ATPS. Iolilyte 221PG-Citrate had the highest phase-forming ability and was the most efficient ATPS in Rubisco separation. This performance is related with the higher salting out effect of potassium citrate over Iolilyte 221PG and the higher hydrophobicity of the ionic liquid. In addition, electrostatic interactions between the IL and Rubisco positively influence the extraction efficiency. However, Iolilyte 221PG-citrate ATPS was not able to keep the protein in its native form at high concentration due to formation of aggregates.

Keeping the functionality of the proteins is very important in the development of an effective separation method for proteins. PEG400-citrate and PEG400-Ch DHp are good alternatives to replace the commonly used inorganic salts, which can cause environmental problems. These alternatives can replace also highly toxic ionic liquids. Additionally, these systems are able to keep the native conformation of the protein after separation. Rubisco prefers to separate into the polymer-rich (PEG400) phase, which is a nice environment for Rubisco. Cholinium-based ATPS could be very promising in the separation of microalgae components as well. This work reported a good start in the development of an effective and environmentally friendly separation process for microalgae components. In future studies the behavior of other microalgae components in ATPS will be investigated. Furthermore, techniques for recovery and reuse of the ILs need to be explored to design an economically feasible process.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.seppur.2017.05.001.

References

[1] R.B. Draaisma, R.H. Wijffels, P.M. Slegers, L.B. Brentner, A. Roy, M.J. Barbosa, Curr. Opin. Biotechnol. 24 (2013) 169–177.
[2] I. Hariskos, C. Posten, Biotechnol. J. 9 (2014) 739–752.
[3] M. Vanthoor-Koopmans, R.H. Wijffels, M.J. Barbosa, M.H.M. Eppink, Biores. Technol. 135 (2013) 142–149.
[4] B.Y. Zaslavsky, Aqueous Two-Phase Partitioning: Physical Chemistry and Bioanalytical Applications, M. Dekker, New York, 1995.
[5] J.A. Asenjo, B.A. Andrews, J. Chromatogr. A 1218 (2011) 8826–8835.
[6] J. Huddleston, J.C. Abelaira, R. Wang, A. Lyddiatt, J. Chromatogr. B Biomed. Appl. 680 (1996) 31–41.

[7] P. Selvakumar, T.C. Ling, S. Walker, A. Lyddiatt, Sep. Purif. Technol. 90 (2012) 182–188.
[8] D. Balasubramaniam, C. Wilkinson, K. Van Cott, C. Zhang, J. Chromatogr. A 989 (2003) 119–129.
[9] S. Dreyer, P. Salim, U. Kragl, Biochem. Eng. J. 46 (2009) 176–185.
[10] Z. Du, Y.L. Yu, J.H. Wang, Chem. Eur. J. 13 (2007) 2130–2137.
[11] Y. Pei, J. Wang, K. Wu, X. Xuan, X. Lu, Sep. Purif. Technol. 64 (2009) 288–295.
[12] M.G. Freire, A.F.M. Cláudio, J.M.M. Araújo, J.A.P. Coutinho, I.M. Marrucho, J.N.C. Lopes, L.P.N. Rebelo, Chem. Soc. Rev. 41 (2012) 4966–4995.
[13] X. Lin, Y. Wang, Q. Zeng, X. Ding, J. Chen, Analyst 138 (2013) 6445–6453.
[14] W.Y. Lou, M.H. Zong, T.J. Smith, H. Wu, J.F. Wang, Green Chem. 8 (2006) 509–512.
[15] S. Cantone, U. Hanefeld, A. Basso, Green Chem. 9 (2007) 954–971.
[16] M. Taha, F.A.E. Silva, M.V. Quental, S.P.M. Ventura, M.G. Freire, J.A.P. Coutinho, Green Chem. 16 (2014) 3149–3159.
[17] J. Chen, Y. Wang, Q. Zeng, X. Ding, Y. Huang, Anal. Methods 6 (2014) 4067–4076.
[18] Y. Ito, Y. Kohno, N. Nakamura, H. Ohno, Int. J. Mol. Sci. 14 (2013) 18350–18361.
[19] M.M. Pereira, S.N. Pedro, M.V. Quental, Á.S. Lima, J.A.P. Coutinho, M.G. Freire, J. Biotechnol. 206 (2015) 17–25.
[20] S. Dreyer, U. Kragl, Biotechnol. Bioeng. 99 (2008) 1416–1424.
[21] R.K. Desai, M. Streefland, R.H. Wijffels, M.H.M. Eppink, Green Chem. 16 (2014) 2670–2679.
[22] Z. Li, X. Liu, Y. Pei, J. Wang, M. He, Green Chem. 14 (2012) 2941–2950.
[23] S. Huang, Y. Wang, Y. Zhou, L. Li, Q. Zeng, X. Ding, Anal. Methods 5 (2013) 3395–3402.
[24] S.Y. Lee, F.A. Vicente, F.A.E. Silva, T.E. Sintra, M. Taha, I. Khoiroh, J.A.P. Coutinho, P.L. Show, S.P.M. Ventura, ACS Sustain. Chem. Eng. 3 (2015) 3420–3428.
[25] M.V. Quental, M. Caban, M.M. Pereira, P. Stepnowski, J.A.P. Coutinho, M.G. Freire, Biotechnol. J. 10 (2015) 1457–1466.
[26] C.P. Song, R.N. Ramanan, R. Vijayaraghavan, D.R. Macfarlane, E.S. Chan, C.W. Ooi, ACS Sustain. Chem. Eng. 3 (2015) 3291–3298.
[27] M. Taha, M.V. Quental, I. Correia, M.G. Freire, J.A.P. Coutinho, Process Biochem. 50 (2015) 1158–1166.
[28] P.Å. Albertsson, Partition of Cell Particles and Macromolecules: Separation and Purification of Biomolecules, Cell Organelles, Membranes, and Cells in Aqueous Polymer Two-Phase Systems and Their Use in Biochemical Analysis and Biotechnology, Wiley, New York, 1986.
[29] R. Hatti-Kaul, Aqueous two-phase systems: methods and protocols, Springer Science & Business Media, 2000.
[30] J.C. Merchuk, B.A. Andrews, J.A. Asenjo, J. Chromatogr. B Biomed. Appl. 711 (1998) 285–293.
[31] T. Arakawa, S.N. Timasheff, Biochemistry 24 (1985) 6756–6762.
[32] S.A. Oelmeier, F. Dismer, J. Hubbuch, BMC Biophys. 5 (2012) 1.
[33] J.G. Huddleston, H.D. Willauer, R.D. Rogers, J. Chem. Eng. Data 48 (2003) 1230–1236.
[34] N.J. Bridges, K.E. Gutowski, R.D. Rogers, Green Chem. 9 (2007) 177–183.
[35] K.E. Gutowski, G.A. Broker, H.D. Willauer, J.G. Huddleston, R.P. Swatloski, J.D. Holbrey, R.D. Rogers, J. Am. Chem. Soc. 125 (2003) 6632–6633.
[36] T. Mourão, L.C. Tomé, C. Florindo, L.P.N. Rebelo, I.M. Marrucho, ACS Sustain. Chem. Eng. 2 (2014) 2426–2434.
[37] J.F.B. Pereira, K.A. Kurnia, O.A. Cojocaru, G. Gurau, L.P.N. Rebelo, R.D. Rogers, M. G. Freire, J.A.P. Coutinho, Phys. Chem. Chem. Phys. 16 (2014) 5723–5731.
[38] X. Liu, Z. Li, Y. Pei, H. Wang, J. Wang, J. Chem. Thermodyn. 60 (2013) 1–8.
[39] R. Sadeghi, R. Golabiazar, H. Shekaari, J. Chem. Thermodyn. 42 (2010) 441–453.
[40] V. Fischer, W. Borchard, M. Karas, J. Phys. Chem. 100 (1996) 15992–15999.
[41] B.A. Andrews, A.S. Schmidt, J.A. Asenjo, Biotechnol. Bioeng. 90 (2005) 380–390.
[42] G. Tubio, B. Nerli, G. Picó, J. Chromatogr. B 799 (2004) 293–301.
[43] F. Hachem, B.A. Andrews, J.A. Asenjo, Enzyme Microb. Technol. 19 (1996) 507–517.
[44] A. Salabat, M. Abnosi, A. Motahari, J. Iran. Chem. Soc. 7 (2010) 142–149.
[45] J.A. Asenjo, A.S. Schmidt, F. Hachem, B.A. Andrews, J. Chromatogr. A 668 (1994) 47–54.
[46] R.M. Vrikkis, K.J. Fraser, K. Fujita, D.R. MacFarlane, G.D. Elliott, J. Biomech. Eng. (2009) 131.
[47] K.D. Weaver, R.M. Vrikkis, M.P. Van Vorst, J. Trullinger, R. Vijayaraghavan, D.M. Foureau, I.H. McKillop, D.R. MacFarlane, J.K. Krueger, G.D. Elliott, Phys. Chem. Chem. Phys. 14 (2012) 790–801.
[48] D. Constantinescu, H. Weingärtner, C. Herrmann, Angew. Chem. – Int. Ed. 46 (2007) 8887–8889.
[49] D. Constatinescu, C. Herrmann, H. Weingärtner, Phys. Chem. Chem. Phys. 12 (2010) 1756–1763.