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Influence of Choline Chloride on the Phase Equilibria and Partition Performance of Polymer/Polymer Aqueous Biphasic Systems

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Abstract: A series of polymeric aqueous biphasic systems (ABS) were determined using polyethylene glycol (PEG) and sodium polyacrylate (NaPA) with choline chloride ([Ch]Cl) as an adjuvant. The effect of (i) PEG and NaPA molecular weights, (ii) PEG functionalization, (iii) [Ch]Cl addition (at different concentrations), and (iv) temperature (25, 37 and 50 °C) was evaluated through their ability to promote the two-phase separation. The results showed that the polymerization degree and functionalization of PEG polymers exhibit a large influence on the ABS formation, with high molecular weight PEG inducing an increase in the biphasic region. Furthermore, the addition of small amount (1–5 wt%) of [Ch]Cl also increased the liquid–liquid demixing. Temperature and the increase in the NaPA molecular weight did not influence the ABS formation ability. Finally, the partition performance of PEG/NaPA + [Ch]Cl ABS was evaluated using caffeine as a model compound. Unlike the ABS formation trend, NaPAs molecular weight significantly influenced the partitioning, which was strengthened when using NaPA-8000. Moreover, the incorporation of [Ch]Cl facilitated an inversion in the partitioning behavior of caffeine, thereby emphasizing the remarkable partitioning tailoring potential exhibited by these systems. Overall, all systems seem to be promising alternatives for the effective extraction, purification and/or concentration of different value-added biomolecules.

Keywords: aqueous two-phase systems; choline chloride; polyethylene glycol; sodium polyacrylate; caffeine



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1. Introduction

In recent years, there has been a growing recognition of the urgent need for more sustainable practices within various scientific and industrial domains. This consciousness is based on issues like resource depletion, environmental degradation, and climate change [1]. In this scenario, the adoption of greener solvents and environmentally conscious technologies has been receiving special attention. These initiatives offer the potential to reduce adverse environmental impacts, fostering a more sustainable and harmonious coexistence between human activities and the planet.

Aqueous biphasic systems (ABS) have emerged as an attractive alternative to current separation and purification methods owing to their mild conditions and biocompatible solvents [2]. ABS consists of two water-rich and immiscible phases that can be formed

by the combination of at least two different compounds, such as polymers, salts, ionic liquids (ILs), and carbohydrates [3,4]. Polymer–polymer ABS have been the most studied owing to their higher water content, resulting in a biocompatible character, although they have some limitations [4–6]. These systems usually present low selectivity due to the low polarity difference between the phases and high phase viscosity because of the polymers' high molecular weight required for liquid–liquid demixing [7]. To overcome this, while aiming to reduce the polymer concentrations and consequently increase the water content, the addition of small concentrations of inorganic salts (e.g., 1–5 wt%) as adjuvants has been explored [8–12]. When added to the polymeric aqueous solution, these minimal salt concentrations act as electrolytes, which can favor phase separation, depending on the salt nature [8,13]. This is particularly useful for enlarging the combinations of polymers that normally do not undergo liquid–liquid demixing in aqueous media at convenient concentrations for separation [5].

In this context, ionic liquids (ILs) have been investigated as an efficient alternative to common high melting point inorganic salts. ILs are commonly used as ABS phase-forming agents owing to their outstanding ability to solvate compounds with a wide range of polarities and to increase the extraction efficiency of these compounds while decreasing the viscosity and separation time of the phases [8,10–12]. The addition of small amounts of ILs as adjuvants in polymer–polymer ABS not only allows the phase separation at much lower polymer concentrations and with lower molecular weights but also allows the partitioning tailoring [7,14–20]. Within this framework, recent studies have highlighted cholinium chloride as a highly advantageous adjuvant in ABS, surpassing other ILs due to its exceptional biocompatibility, lower toxicity, superior ability to tailor phase behavior, and ability to enhance the extraction of biomolecules while maintaining an environmentally friendly nature [5,9,12,18,19,21].

Earlier reports have identified opposite effects induced by cholinium chloride ([Ch]Cl) on PEG/NaPA ABS formation, showcasing their remarkable tailoring ability [5,22,23]. To gain deeper insights into these systems, we herein investigate the phase behavior of PEG/NaPA aqueous solutions across varied molecular weights and temperatures, with and without the addition of cholinium chloride. Additionally, the effect of the addition of electrolytes on the partition of a model solute, i.e., caffeine, was evaluated. The combination of these data is of paramount importance, thus demonstrating the applicability of the PEG/NaPA/[Ch]Cl ABS for tailoring the partition of biomolecules.

2. Material and Methods

2.1. Materials

Polyethylene glycol 600 and 1000 g mol⁻¹ (PEG-600 and PEG-1000, respectively), PEG 550 g mol⁻¹ methyl ether (PEG-550-OMe), PEG 500 g mol⁻¹ dimethyl ether (MeO-PEG-500-OMe), sodium polyacrylate 8000 g mol⁻¹ at 45 wt%, and 15,000 g mol⁻¹ at 35 wt% (NaPA-8000 and NaPA-15000, respectively) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Cholinium chloride ([Ch]Cl) (purity ≥ 98%) was purchased from Sigma-Aldrich® and Iolitec® GmbH (Heilbronn, Germany), respectively. All other chemicals were of analytical grade and were used as received.

2.2. Determination of the Binodal Curves

The binodal curves were determined using the cloud point titration method [24] for different polymers. For this purpose, systems composed of PEG-600, PEG-1000, PEG-550-OMe, MeO-PEG-500-OMe, NaPA-8000, and NaPA-15000 were analyzed. The molecular weight and functionalization of the polymers were also evaluated in the presence and absence of small amounts of cholinium chloride ([Ch]Cl), namely, 0, 1, 3, and 5 wt%. Finally, different temperatures were also considered, i.e., 25, 37, and 50 °C. The selection of the specific temperature of 37 °C was motivated by its relevance in mimicking physiological conditions to facilitate the potential applications of ABS in emerging fields such as cellular micropatterning, bioprinting, and microencapsulation. In brief, dropwise addition of the

stock aqueous solutions of PEG and NaPA, with a known concentration of [Ch]Cl, was repeated until a cloudy solution was observed, followed by dropwise addition of water until a clear solution was detected. The procedure was performed under constant stirring (100 rpm) and atmospheric pressure. The system’s composition was established by the weight quantifications of all the phase-forming compounds, with an uncertainty of $\pm 10^{-4}$ g. The experimental binodal curves were correlated using the following equation:

$$Y = A \exp[(BX^{0.5}) - (CX^3)], \tag{1}$$

where X and Y are, respectively, the weight concentrations of NaPA and PEG, and A, B, and C are correlation constants.

2.3. Determination of Tie-Lines

The tie-lines (TL) determination was carried out following the gravimetric method outlined by Merchuk et al. [24]. In this process, biphasic mixtures were prepared, vigorously agitated, and allowed to equilibrate for 12 h at 25 °C, resulting in the formation of two distinct and well-separated coexisting phases. After the equilibration period, both phases were meticulously separated, and their respective weights were recorded. Each individual TL value was determined using the lever arm rule, which relies on the relationship between the bottom phase’s mass composition and the overall system composition, and subsequently solving a set of four equations (Equations (2)–(5)). By simultaneously solving these equations, the composition of the respective phase-forming compounds (X_B , X_T , Y_B , and Y_T) in each of the coexisting phases was established.

$$Y_T = A \exp[(BX_T^{0.5}) - (CX_T^3)], \tag{2}$$

$$Y_B = A \exp[(BX_B^{0.5}) - (CX_B^3)], \tag{3}$$

$$Y_T = \left(\frac{Y_M}{\alpha}\right) - \left(\frac{1 - \alpha}{\alpha}\right)Y_B \tag{4}$$

$$X_T = \left(\frac{X_M}{\alpha}\right) - \left(\frac{1 - \alpha}{\alpha}\right)X_B, \tag{5}$$

where X and Y are, respectively, the weight concentrations of NaPA and PEG, and subscript letters T, B, and M represent, respectively, the top, bottom, and the initial mixture phase. The parameter α represents the ratio between the weight of the bottom phase and the total weight of the mixture.

2.4. Partitioning of Caffeine

For the partitioning of caffeine, five different mixing points in the biphasic region with varying proportions of PEG-600, NaPA-8000, and NaPA-15000 were selected, as presented in Table 1. These systems were formulated both with and without the addition of 5 wt% [Ch]Cl. All samples were prepared in graduated tubes using a caffeine aqueous solution at $0.0051 \text{ mol L}^{-1}$. The mixtures were homogenized and centrifuged to achieve the partitioning equilibrium of caffeine between phases. Caffeine was then quantified in both top and bottom phases by UV spectroscopy using an Ultrospec 2100 Pro spectrometer (Amersham Biosciences®, Buckinghamshire, UK) at 273 nm. A calibration curve for caffeine determination was previously established at the maximum absorbance peak. The partition coefficients (K) of caffeine were calculated as the ratio of the concentration of caffeine in the PEG-rich phase to that in the NaPA-rich phase, as described by the following equation:

$$K = \frac{[Cf]_{\text{PEG}}}{[Cf]_{\text{NaPA}}} \tag{6}$$

where $[Cf]_{\text{PEG}}$ and $[Cf]_{\text{NaPA}}$ are the concentration of caffeine present in the PEG-rich phase and NaPA-rich phase, respectively.

Table 1. ABS mixing points composed of PEG-600 + NaPA-8000 and 15,000 + aqueous solution of caffeine ($0.0051 \text{ mol L}^{-1}$) were used for the partition studies at $25 \text{ }^\circ\text{C}$, formulated with and without 5 wt% [Ch]Cl.

Assays	PEG-600/NaPA-8000 ABS		PEG-600/NaPA-15000 ABS	
	PEG-600 (wt%)	NaPA-8000 (wt%)	PEG-600 (wt%)	NaPA-15000 (wt%)
System 1	-	-	12	11.9
System 2	15.4	15.2	14.1	14
System 3	16.6	14.1	15.5	16
System 4	20.5	20.5	18.1	18
System 5	23.4	23.3	20.1	19.9

3. Results and Discussions

3.1. Effect of the Polymers' Molecular Weight and PEG Functionalization

First, binodal curves were determined for conventional polymer–polymer ABS in the absence of [Ch]Cl in order to evaluate the influence of the PEG alkyl side chain as well as PEG and NaPA molecular weight (M_W). Therefore, PEG-600, PEG-1000, PEG-550-OMe, and MeO-PEG-500-OMe were selected as the PEG-based polymers, whereas NaPA-8000 and NaPA-15000 were selected as the NaPA-based polymers. Figure 1 presents the effect of PEG functionalization and M_W , where it is possible to observe an increasing ability to form a biphasic system, as follows: PEG-600 < PEG-550-OMe < MeO-PEG-500-OMe \approx PEG-1000. These are quite interesting results, as PEG functionalization seems to play a more important role in the ability to increase the biphasic region than PEG M_W . When examining the methylated PEGs, they follow the well-established M_W trend commonly observed within PEG-salt ABS. This trend underscores that as the M_W of the polymers increases, the need for a salt to trigger the phase separation diminishes [22,25]. However, the substitution of a single -OH end group with a -OMe group leads to a slight enhancement in the capacity to form ABS [22]. To better illustrate this, when PEG-600 was compared with PEG-550-OMe and MeO-PEG-500-OMe, the M_W did not vary significantly. This means that the methylated counterparts triggered the phase separation at equal or lower polymer concentrations despite their lower M_W . This is clearly relevant when MeO-PEG-500-OMe and PEG-1000 are compared since the latter doubled its M_W but behaved similarly to the most functionalized PEG-500. As previously demonstrated by Pereira et al. [22], the substitution of the PEG hydroxyl group(s) with -OMe completely changed the polymer hydrogen-bonding donor/acceptor balance. The alcohol end groups of the PEG polymers were the only source of the strong hydrogen-bond-donating abilities. Therefore, when these strong hydrogen-bond donors are replaced by -OMe groups, there are considerably fewer hydrogen bond interactions between the polymer, PEG polymer, and PEG-water. Hence, phase demixing is facilitated, resulting in a wider biphasic region.

As seen in Figure 1, the experimental solubility data can be presented in a ternary diagram. Yet, to define a ternary system, only the amount of two components is needed since the third one is dependent. Consequently, an orthogonal representation of the data has been the prevailing approach in the field of ABS due to the simplicity of interpreting the data. Successively, subsequent assays were performed to evaluate different NaPA-based ABS, as presented in Figure 2. Results showed that NaPA M_W (8000 vs. 15,000 g mol^{-1}) did not influence the phase-forming capacity of the systems since the binodal curves of the different PEG/NaPA combinations overlapped. Contrary to the results observed for PEG systems, increasing the polymerization degree of NaPA does not allow the reduction of the minimum concentration required to promote phase separation. These results are in accordance with the phase separation mechanisms described by Piculell and Lindman [13] for nonionic/ionic polymer ABS. In the context of electroneutral conditions, the counterions in the polyelectrolyte must necessarily accompany the polyion. In the absence of an

electrolyte as an adjuvant, the occurrence of phase separation would inevitably result in the formation of a phase distinguished by an elevated concentration of counterions. This dissimilar concentration results in a large entropic penalty, which significantly hinders the phase separation. In instances where phase separation does take place, the separated phases differ widely in the overall polymer concentration, and the phase enriched with the uncharged polymer exhibits notably higher concentrations. In this scenario, due to the dominating influence of the entropy of counterions mixing, the degree of polymerization of the polyelectrolyte does not significantly influence the binodal curve [5,13]. Thus, in the PEG-NaPA systems without adjuvants, the molecular weight of NaPA does not significantly influence the ABS formation ability.

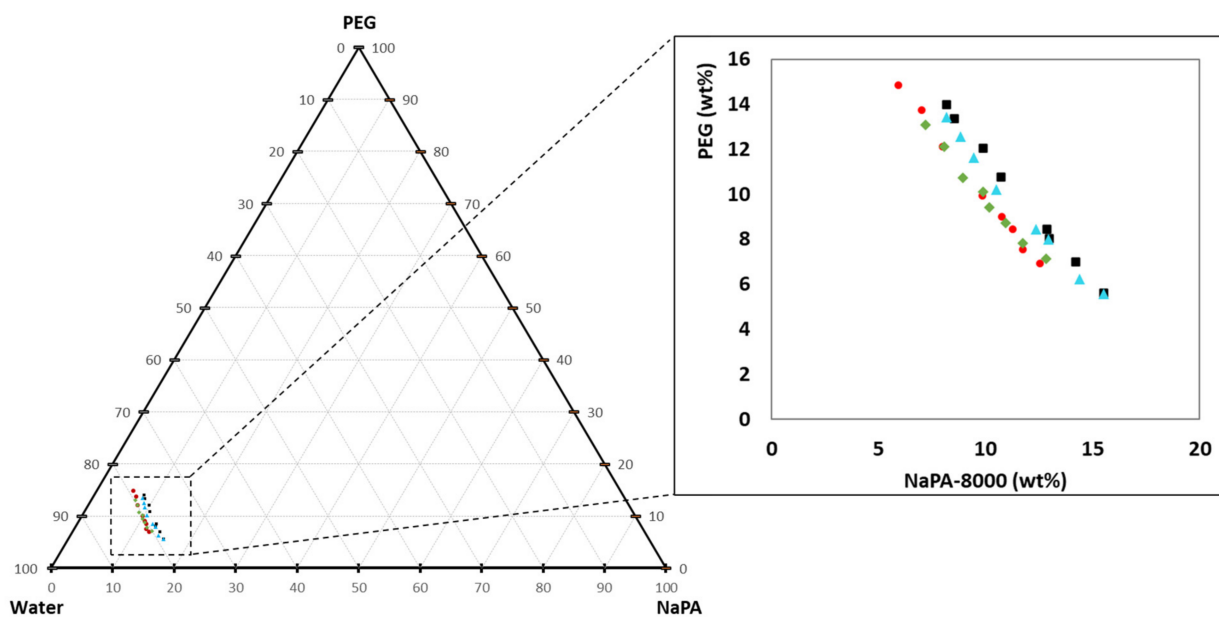


Figure 1. Experimental solubility data of the ABS composed of different PEG polymers (PEG-600 (■), PEG-550-OMe (▲), MeO-PEG-500-OMe (◆), and PEG-1000 (●)) and NaPA-8000, presented in a ternary (left side) and orthogonal diagram (right side). All diagrams were determined at 25 °C.

Figure 2 also demonstrates the effect of the polymer concentration on the phase separation behavior. By increasing the concentration of one polymer, the concentration of the second polymer required to form a biphasic system decreased. Gupta et al. [26] observed a similar trend, confirming the influence of water structures on the phase separation behavior of polyelectrolyte-PEG ABS. The inclusion of polymers in an aqueous environment causes water molecules to cluster around polymer chains [26,27]. Because of the incompatibility of distinct water structures around different polymers, phase separation occurs, with each phase being rich in a polymer, namely, a PEG-rich phase and a NaPA-rich phase. Therefore, by increasing the concentration of either polymer, the concentration of polymer-modified water structures increases, resulting in an increase in interactions between the two polymers and a greater probability of phase separation [26,28].

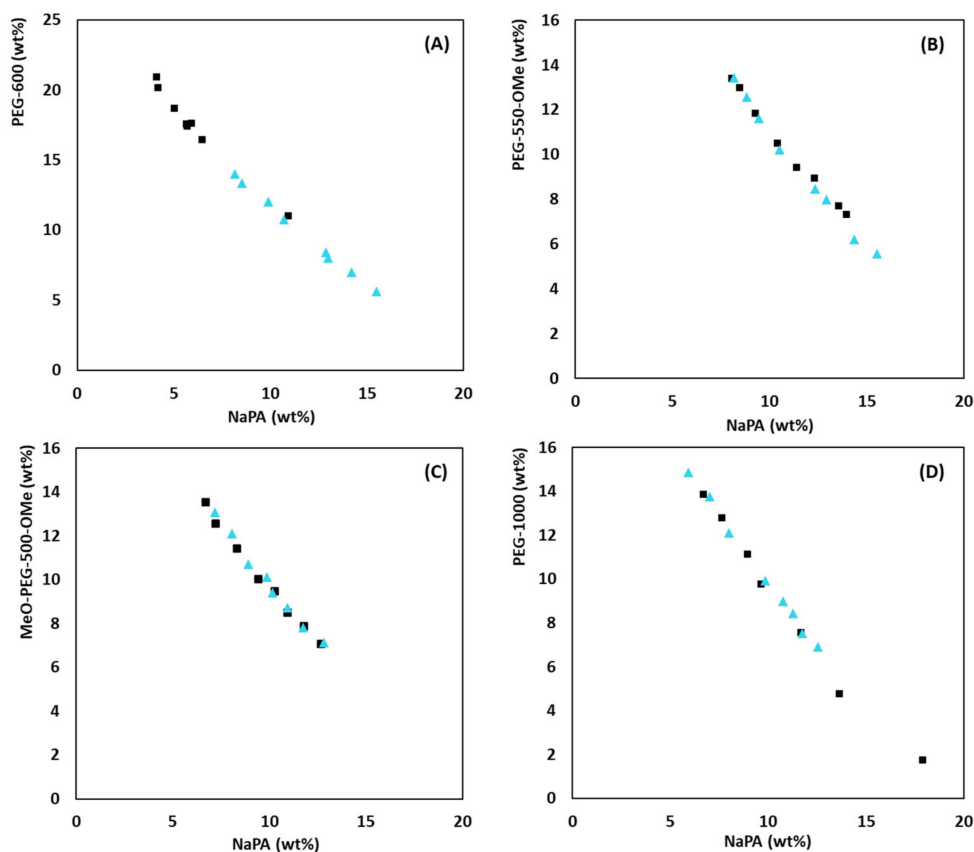


Figure 2. Experimental solubility data of ABS composed of different PEGs and NaPAs: (■) represent NaPA-15000 and (▲) represent NaPA-8000 for (A) PEG-600, (B) PEG-550-OMe, (C) MeO-PEG-500-OMe, and (D) PEG-1000. All diagrams were determined at 25 °C.

3.2. Effect of [Ch]Cl in the Formation of PEG/NaPA ABS

Several combinations of different M_w of PEG and NaPA with distinct M_w were prepared to evaluate the effect of [Ch]Cl on its ability to facilitate the ABS formation. Figure 3 shows the influence of [Ch]Cl on the binodal curves. In general, it is clear that when the concentration of [Ch]Cl increases, the biphasic region of each system becomes wider. However, no significant influence was observed when 3 and 5 wt% [Ch]Cl were compared. Therefore, the ability to facilitate the ABS formation follows the trend of $0 < 1 < 3 \approx 5$ wt% [Ch]Cl. Moreover, this figure also corroborates the results discussed in the previous section in terms of PEG and NaPA M_w , as well as PEG functionalization. When Figure 3A,G were compared, or Figure 3B,H, it can be observed that independently of the [Ch]Cl influence, the biphasic region expands with the increase in PEG M_w . This has also been observed by other researchers for PEG-polymer ABS [3]. In the case of PEG-600/NaPA-15000 (Figure 3A), the PEG M_w seems to play a more important role than the concentration of [Ch]Cl. A plausible explanation is that the terminal –OH groups of the PEG molecules present a much stronger hydrogen-bond-donating ability [22] than [Ch]Cl, thus hindering the influence of the electrolyte. Regarding the influence of the NaPA M_w , which is visible by comparing all the right and left figures, there is a negligible effect in all systems. Finally, when PEG functionalization was considered, the results are shown in Figure 3A,C,E, or Figure 3B,D,F, the biphasic region increased with the number of –OMe groups introduced in the polymer, which was also independent of the presence of [Ch]Cl.

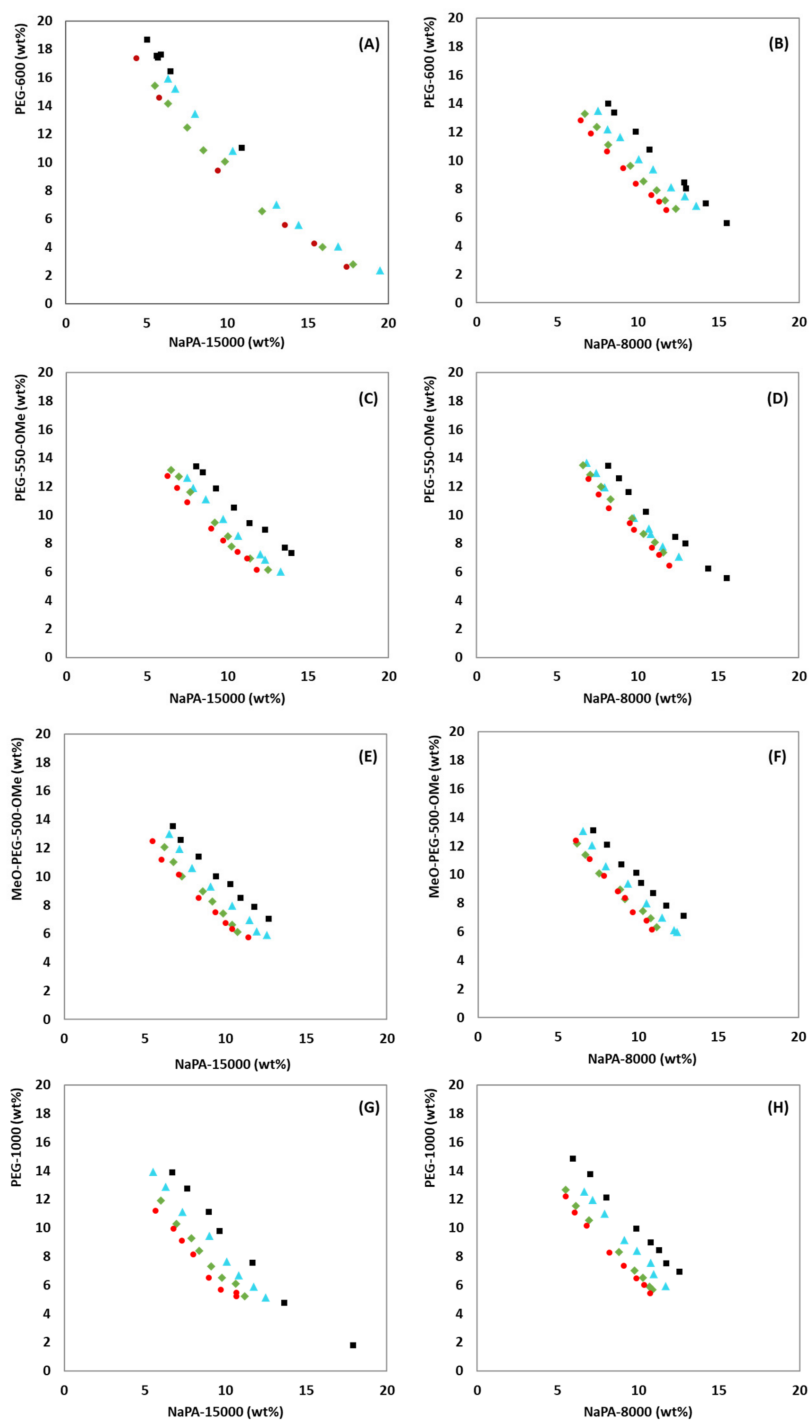


Figure 3. Experimental solubility data of ABSs composed of different PEGs and NaPAs with different concentrations (wt%) of [Ch]Cl: (A) PEG-600/NaPA-15000, (B) PEG-600/NaPA-8000, (C) PEG-550-OMe/NaPA-15000, (D) PEG-550-OMe/NaPA-8000, (E) MeO-PEG-500-OMe/NaPA-15000, (F) MeO-PEG-500-OMe/NaPA-8000, (G) PEG-1000/NaPA-15000, and (H) PEG-1000/NaPA-8000 with 0 (■), 1 (▲), 3 (◆), and 5 wt% (●) of [Ch]Cl. All diagrams were determined at 25 °C.

Overall, the molecular mechanisms governing phase separation in the presence of an electrolyte follow the phenomenon proposed by Johansson et al. [8] with inorganic salts as the electrolytes. The authors showed that the presence of the electrolyte decreased the compartmentalization entropy of the counterions in the co-existing phases, as previously discussed by Piculell and Lindman [13]. Thus, lower polymer concentrations were required

to promote the phase demixing. Other authors have correlated the electrolyte influence on ABS formation with its ability to interact with water, namely, whether the electrolyte is a water structure breaking ion or water structure making ions. The water structure-causing salts depressed the binodal curve of the system to a greater extent than the water structure-breaking salts [26]. It has also been reported that when the electrolyte is an inorganic salt, its influence follows the Hofmeister series [29,30]. Furthermore, the use of ionic surfactants and surface-active ionic liquids as electrolytes has been studied [31]. The authors observed that some cationic surfactants (such as hexadecylpyridinium chloride) could not promote the formation of two phases and that the studied anionic surfactants presented a higher ability to induce phase separation at very small concentrations, i.e., 0.1 wt%. It was also reported that the phenomenon behind the formation of ABS in PEG/NaPA systems with these electrolytes followed the mechanism described for conventional inorganic salts and short alkyl side chain imidazolium-based ionic liquids. Bernardo et al. [5] recently studied the influence of four different cholinium-based ILs as electrolytes in polymer/polymer ABS and observed a trend similar to the one observed here. [Ch]Cl and choline acetate partitioned preferentially towards the PEG-rich phase, which is the more hydrophilic phase, as these are also the more polar ILs under study, thus favoring the liquid demixing. In contrast, the less polar [Ch]-based ILs partitioned less to the PEG-rich phase, thus acting as ABS depressants.

3.3. Effect of Temperature

In order to determine the temperature effect on the ability of liquid–liquid demixing, the ABS composed of PEG-600 and PEG-1000, both combined with NaPA-8000 and 5 wt% of [Ch]Cl, were studied at three different temperatures: 25, 37, and 50 °C. The results are shown in Figure 4. Here, the temperature clearly did not have any influence on the phase separation, as the binodal curves overlapped.

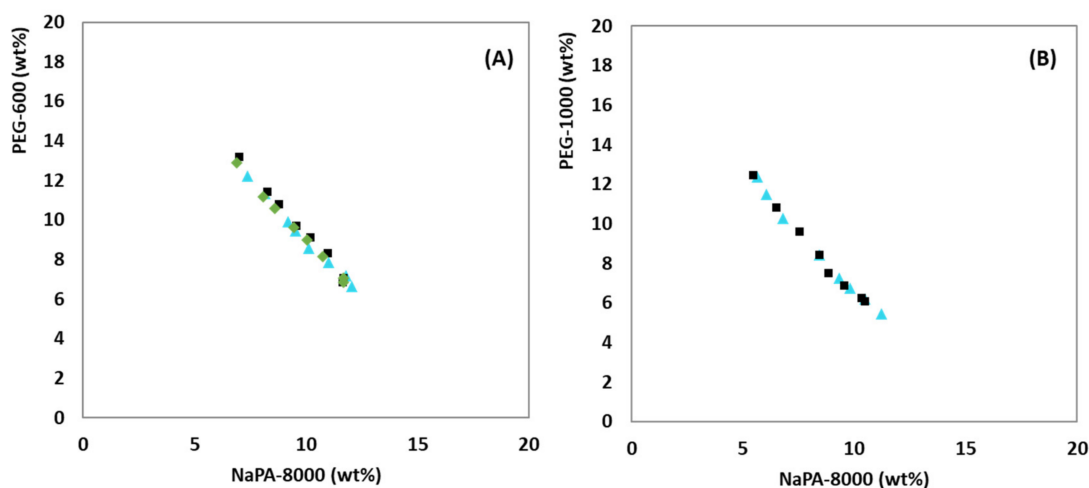


Figure 4. Experimental solubility data of ABSs composed of PEG and NaPA with 5 wt% of [Ch]Cl, in different temperatures: (A) PEG-600/NaPA-8000 at 25 (■), 37 (◆), and 50 °C (▲), and (B) PEG-1000/NaPA-8000 at 25 (■) and 50 °C (▲).

It is important to emphasize that the effect of temperature on ABS is complex and varies differently for distinct systems. Chakraborty et al. [32] studied the effect of temperature on ABSs composed of different polymer–polymer systems. An increase in temperature may promote a decrease in the phase-forming capacity of PEG/dextran ABS, whereas in PEG/maltodextrin ABS, it seems to increase the ability to form two phases. On the other hand, for PEG/maltodextrin-dextrose ABSs, the temperature has practically no influence on the liquid–liquid equilibrium [32]. Other authors have reported that the temperature increase led to the expansion of the biphasic region for all investigated polymer–polymer systems, including PEG–PEGs, polyethylene glycol dimethyl ether (PEGDME),

PEGs-polyvinyl pyrrolidone (PVP), PEGDMEs-PVP, and polypropylene glycol (PPG)-PEGDME) [33].

For the PEG-NaPA systems in the present study, temperature had no influence on the phase separation behavior, following the trend observed for the PEG/maltodextrin-dextrose system [32]. However, to the best of the authors' knowledge, there are no studies in literature that investigate the impact of temperature on the solubility curves of similar polymer-polymer systems containing adjuvants. Therefore, it is not yet possible to compare these results with literature; hence, further conclusions cannot be made.

3.4. Partitioning of Caffeine

To provide a better overview of the potential application of PEG/NaPA/[Ch]Cl ABS as novel extractive systems, the partitioning of caffeine was evaluated. Caffeine was chosen as a model solute since it is only mildly hydrophilic ($\log K_{\text{octanol/water}} = -0.07$) and does not speciate over a wide pH range [25]. The partitioning of caffeine has also been addressed as a hydrophobic probe for the resulting phases [34,35]. Two different screenings were performed: one to investigate how the concentration of the phase-forming agents affects the caffeine partitioning and another to analyze how the partition changes with the addition of adjuvants. Hence, five different mixture points within the biphasic region of PEG-600/NaPA-15000 and PEG-600/NaPA-8000 were selected in different proportions. Additionally, an aqueous solution of caffeine ($0.0051 \text{ mol L}^{-1}$) was added. The respective tie-lines of all systems were determined in order to evaluate the phase compositions. These systems were first tested without adjuvants, as shown in Figure 5.

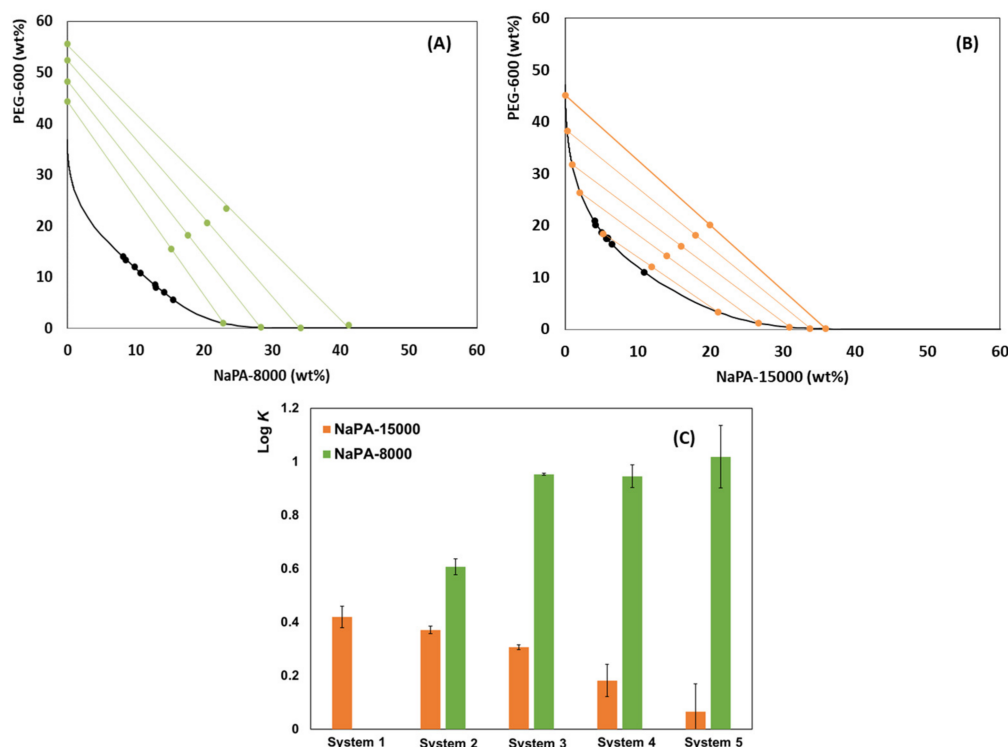


Figure 5. Experimental solubility data (●) in (A) PEG/NaPA-8000 and (B) PEG-600/NaPA-15000, with the respective tie-lines and the composition of the phases (●) for (A) and (●) for (B)); and (C) Effect of different proportions of phase-forming agents on the partitioning coefficient of caffeine (K) in PEG-600/NaPA-15000 (■) and PEG-600/NaPA-8000 systems (■). All systems were tested at 25 °C. The black line (—) represents the binodal curve correlation.

Figure 5C shows that, in both cases, caffeine was concentrated preferably in the PEG-600-rich phase (top phase). It is also verified that the ABS composed of NaPA-8000 allowed a greater partition of caffeine in relation to the system composed of NaPA-15000.

By analyzing Figure 5A,B, it becomes evident that in the NaPA-8000 systems, PEG and NaPA partition entirely to the top and bottom phases, respectively. This is indicated by the intersection of the tie-lines with the respective axis. In contrast, in the NaPA-15000 ABS, this complete partitioning does not occur for all systems. These results are interesting since the increase in NaPA M_w did not have a significant effect on the ABS formation, yet it showed a considerable influence on the caffeine partitioning, which was favored with the use of NaPA-8000. Moreover, it is evident that an increase in the concentrations of phase-forming agents resulted in a higher partition coefficient for PEG-600/NaPA-8000 systems, while for systems consisting of NaPA-15000, the opposite trend was observed. Although further research is needed to better understand the fundamental partition mechanisms in both ABS, such as determining the water content of the phases and their viscosities, the caffeine partition clearly shows that the equilibrium phase properties are completely different in each system. This evidence is extremely important since, using the same polymer base, we can obtain two distinct platforms for the partitioning of different bioproducts.

Finally, to evaluate how the addition of [Ch]Cl can change the solute partition mechanism in these systems, 5 wt% [Ch]Cl was added as an adjuvant to the PEG-600/NaPA-15000 ABS, which presented a decreased partition coefficient. The caffeine partition was evaluated at 25 °C, as shown in Figure 6.

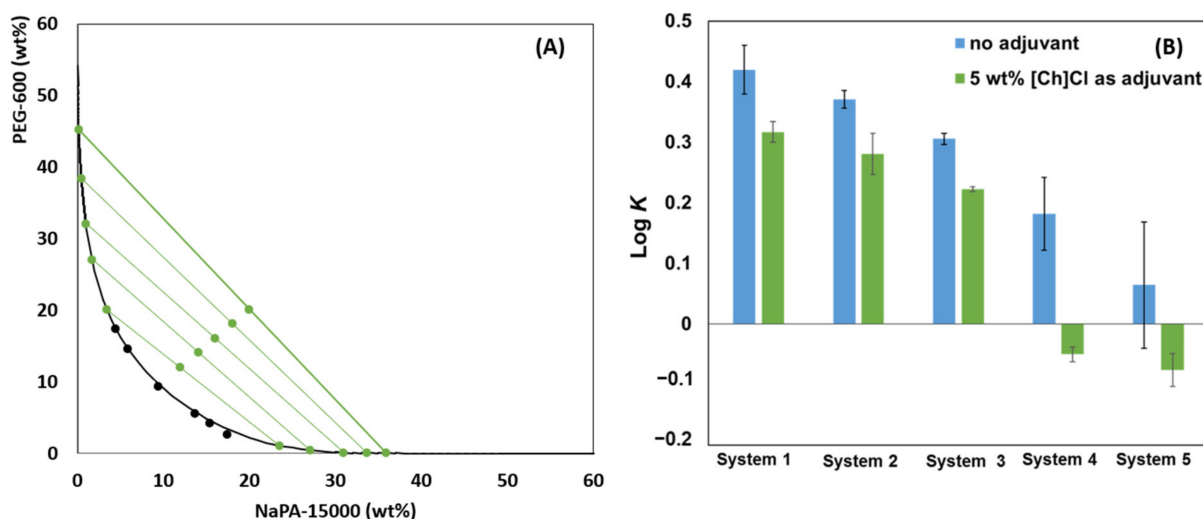


Figure 6. (A) Experimental solubility data (●) with the respective tie-lines and the composition of the phases (●) for PEG-600/NaPA-15000 with 5 wt% of [Ch]Cl; and (B) Partition of caffeine in ABS composed of PEG-600/NaPA-15000 without adjuvants (■) and with 5 wt% of [Ch]Cl (■) at 25 °C. The black line (—) represents the binodal curve correlation.

The results showed that for all systems without [Ch]Cl, caffeine was preferentially concentrated in the top phase (PEG-600-rich phase); however, for two of the systems, the addition of 5 wt% of the electrolyte led to the inversion of the migration capacity of caffeine from the PEG-600-rich phase to the NaPA-15000-rich phase. As previously discussed, it is clear that the strong hydrophobic nature of the PEG-rich phase promotes caffeine migration. Pereira et al. [23] demonstrated that the partitioning of solutes in PEG/cholinium salts ABS is related to the amount of water present in the co-existing phases. The tendency observed in Figure 6 suggests that the addition of small amounts of electrolyte changes the water distribution between the PEG and NAPA-rich phases, influencing, as a consequence, the caffeine partitioning. As caffeine typically concentrates in phases with lower water content, increasing the water content within the PEG-rich phase will start to favor the migration of caffeine toward the NaPA-rich phase. This change is in line with our previous work using PEG/cholinium salts ABS [23], in which a reduction in caffeine's partition coefficient and an inversion in the partitioning behavior of the solute as a function of the water ratio was

also observed. These results are very interesting and promising as they are a great indicator of the ability of these systems to tailor the solute partitioning.

4. Conclusions

The experimental solubility data for various polymer-polymer ABS was assessed, both in the presence and absence of cholinium chloride as an adjuvant. The molecular weight and functionalization of distinct polymers were analyzed, as well as the effect of different temperatures on the binodal curves. The critical parameters influencing the phase separation ability of the PEG/NaPA/[Ch]Cl ABS are the molecular weight of PEG, its functionalization, and the addition of the electrolyte used. An increase in PEG molecular weight or its functionalization favors liquid–liquid demixing, expanding the biphasic region of the system. Furthermore, the addition of [Ch]Cl also favors phase separation, with this effect becoming more pronounced as the salt concentration increases. In contrast, temperature variations do not significantly influence liquid–liquid demixing. Experimental results regarding caffeine partitioning indicate a preference for the more hydrophobic PEG-rich phase. Nevertheless, the addition of small quantities of [Ch]Cl causes an inversion in this partitioning tendency, highlighting the remarkable ability of these systems to tailor the solute partitioning. These ABS, composed of biocompatible and biodegradable salts and polymers, exhibit great industrial potential as promising alternatives to conventional liquid–liquid extraction processes. Therefore, their potential application in separation and purification processes for a wide range of value-added compounds from biological or synthetic media is the step to follow.

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References

1. Khajuria, A.; Atienza, V.A.; Chavanich, S.; Henning, W.; Islam, I.; Kral, U.; Liu, M.; Liu, X.; Murthy, I.K.; Oyedotun, T.D.T.; et al. Accelerating Circular Economy Solutions to Achieve the 2030 Agenda for Sustainable Development Goals. *Circ. Econ.* **2022**, *1*, 100001. [[CrossRef](#)]
2. Saha, N.; Sarkar, B.; Sen, K. Aqueous Biphasic Systems: A Robust Platform for Green Extraction of Biomolecules. *J. Mol. Liq.* **2022**, *363*, 119882. [[CrossRef](#)]
3. Freire, M.G.; Cláudio, A.F.M.; Araújo, J.M.M.; Coutinho, J.A.P.; Marrucho, I.M.; Lopes, J.N.C.; Rebelo, L.P.N. Aqueous Biphasic Systems: A Boost Brought about by Using Ionic Liquids. *Chem. Soc. Rev.* **2012**, *41*, 4966–4995. [[CrossRef](#)] [[PubMed](#)]
4. Singla, M.; Sit, N. Theoretical Aspects and Applications of Aqueous Two-Phase Systems. *ChemBioEng Rev.* **2022**, *10*, 65–80. [[CrossRef](#)]

5. Bernardo, S.C.; Capela, E.V.; Pereira, J.F.B.; Ventura, S.P.M.; Freire, M.G.; Coutinho, J.A.P. Opposite Effects Induced by Cholinium-Based Ionic Liquid Electrolytes in the Formation of Aqueous Biphasic Systems Comprising Polyethylene Glycol and Sodium Polyacrylate. *Molecules* **2021**, *26*, 6612. [[CrossRef](#)] [[PubMed](#)]
6. Torres-Bautista, A.; Torres-Acosta, M.A.; González-Valdez, J. Characterization and Optimization of Polymer-Polymer Aqueous Two-Phase Systems for the Isolation and Purification of CaCo₂ Cell-Derived Exosomes. *PLoS ONE* **2022**, *17*, e0273243. [[CrossRef](#)]
7. Marchel, M.; João, K.G.; Marrucho, I.M. On the Use of Ionic Liquids as Adjuvants in PEG-(NH₄)₂SO₄ Aqueous Biphasic Systems: Phase Diagrams Behavior and the Effect of IL Concentration on Myoglobin Partition. *Sep. Purif. Technol.* **2019**, *210*, 710–718. [[CrossRef](#)]
8. Johansson, H.-O.; Feitosa, E.; Junior, A.P. Phase Diagrams of the Aqueous Two-Phase Systems of Poly(Ethylene Glycol)/Sodium Polyacrylate/Salts. *Polymers* **2011**, *3*, 587–601. [[CrossRef](#)]
9. Farias, F.O.; Oliveira, G.; Leal, F.G.; Wojeicchowski, J.P.; Yamamoto, C.I.; Igarashi-Mafra, L.; Mafra, M.R. Cholinium Chloride Effect on Ethanol-Based Aqueous Biphasic Systems: Liquid-Liquid Equilibrium and Biomolecules Partition Behavior. *Fluid Phase Equilibria* **2020**, *505*, 112363. [[CrossRef](#)]
10. Santos, J.H.P.M.; Martins, M.; Silva, A.R.P.; Cunha, J.R.; Rangel-Yagui, C.O.; Ventura, S.P.M. Imidazolium-Based Ionic Liquids as Adjuvants to Form Polyethylene Glycol with Salt Buffer Aqueous Biphasic Systems. *J. Chem. Eng. Data* **2020**, *65*, 3794–3801. [[CrossRef](#)]
11. da Cruz Silva, K.; Abreu, C.S.; Vieira, A.W.; Mageste, A.B.; Rodrigues, G.D.; de Lemos, L.R. Aqueous Two-Phase Systems Formed by Different Phase-Forming Components: Equilibrium Diagrams and Dye Partitioning Study. *Fluid Phase Equilibria* **2020**, *520*, 112664. [[CrossRef](#)]
12. Neves, C.M.S.S.; de Cássia, S.; Sousa, R.; Pereira, M.M.; Freire, M.G.; Coutinho, J.A.P. Understanding the Effect of Ionic Liquids as Adjuvants in the Partition of Biomolecules in Aqueous Two-Phase Systems Formed by Polymers and Weak Salting-out Agents. *Biochem. Eng. J.* **2019**, *141*, 239–246. [[CrossRef](#)] [[PubMed](#)]
13. Piculell, L.; Lindman, B. Association and Segregation in Aqueous Polymer/Polymer, Polymer/Surfactant, and Surfactant/Surfactant Mixtures: Similarities and Differences. *Adv. Colloid Interface Sci.* **1992**, *41*, 149–178. [[CrossRef](#)]
14. Rocha, I.L.D.; Lopes, A.M.C.; Ventura, S.P.M.; Coutinho, J.A.P. Selective Separation of Vanillic Acid from Other Lignin-Derived Monomers Using Centrifugal Partition Chromatography: The Effect of PH. *ACS Sustain. Chem. Eng.* **2022**, *10*, 4913–4921. [[CrossRef](#)]
15. Magalhães, F.F.; Pereira, M.M.; de Cássia Superbi de Sousa, R.; Tavares, A.P.M.; Coutinho, J.A.P.; Freire, M.G. Tailoring the partitioning of proteins using ionic liquids as adjuvants in polymer-polymer aqueous biphasic systems. *Green Chem. Eng.* **2022**, *3*, 328–337. [[CrossRef](#)]
16. Ng, H.S.; Kee, P.E.; Wu, Y.-C.; Chen, L.; Wong, S.Y.W.; Lan, J.C.-W. Enhanced recovery of astaxanthin from recombinant *Kluyveromyces marxianus* with ultrasonication-assisted alcohol/salt aqueous biphasic system. *J. Biosci. Bioeng.* **2021**, *132*, 513–518. [[CrossRef](#)] [[PubMed](#)]
17. Ng, H.S.; Kee, P.E.; Yim, H.S.; Tan, J.S.; Chow, Y.H.; Lan, J.C.-W. Characterization of alcohol/salt aqueous two-phase system for optimal separation of gallic acids. *J. Biosci. Bioeng.* **2021**, *131*, 537–542. [[CrossRef](#)]
18. Capela, E.V.; Valente, A.I.; Nunes, J.C.F.; Magalhães, F.F.; Rodríguez, O.; Soto, A.; Freire, M.G.; Tavares, A.P.M. Insights on the laccase extraction and activity in ionic-liquid-based aqueous biphasic systems. *Sep. Purif. Technol.* **2020**, *248*, 117052. [[CrossRef](#)]
19. Dimitrijević, A.; Tavares, A.P.M.; Jocić, A.; Marić, S.; Trtić-Petrović, T.; Gadžurić, S.; Freire, M.G. Aqueous Biphasic Systems Comprising Copolymers and Cholinium-Based Salts or Ionic Liquids: Insights on the Mechanisms Responsible for Their Creation. *Sep. Purif. Technol.* **2020**, *248*, 117050. [[CrossRef](#)]
20. Tang, N.; Wang, Y.; Li, M.; Liu, L.; Yin, C.Y.; Yang, X.; Wang, S. Ionic Liquid as Adjuvant in an Aqueous Biphasic System Composed of Polyethylene Glycol for Green Separation of Pd(II) from Hydrochloric Solution. *Sep. Purif. Technol.* **2020**, *246*, 116898. [[CrossRef](#)]
21. Veríssimo, N.V.; Vicente, F.A.; de Oliveira, R.C.; Likozar, B.; de Oliveira, R.C.; Pereira, J.F.B. Ionic Liquids as Protein Stabilizers for Biological and Biomedical Applications: A Review. *Biotechnol. Adv.* **2022**, *61*, 108055. [[CrossRef](#)] [[PubMed](#)]
22. Pereira, J.F.B.; Kurnia, K.A.; Freire, M.G.; Coutinho, J.A.P.; Rogers, R.D. Controlling the Formation of Ionic-Liquid-Based Aqueous Biphasic Systems by Changing the Hydrogen-Bonding Ability of Polyethylene Glycol End Groups. *ChemPhysChem* **2015**, *16*, 2219–2225. [[CrossRef](#)] [[PubMed](#)]
23. Pereira, J.F.B.; Magri, A.; Quental, M.V.; Gonzalez-Miquel, M.; Freire, M.G.; Coutinho, J.A.P. Alkaloids as Alternative Probes to Characterize the Relative Hydrophobicity of Aqueous Biphasic Systems. *ACS Sustain. Chem. Eng.* **2016**, *4*, 1512–1520. [[CrossRef](#)]
24. Merchuk, J.C.; Andrews, B.A.; Asenjo, J.A. Aqueous Two-Phase Systems for Protein Separation. *J. Chromatogr. B Biomed. Sci. Appl.* **1998**, *711*, 285–293. [[CrossRef](#)]
25. Pereira, J.F.B.; Rebelo, L.P.N.; Rogers, R.D.; Coutinho, J.A.P.; Freire, M.G. Combining Ionic Liquids and Polyethylene Glycols to Boost the Hydrophobic–Hydrophilic Range of Aqueous Biphasic Systems. *Phys. Chem. Chem. Phys.* **2013**, *15*, 19580–19583. [[CrossRef](#)]
26. Gupta, V.; Nath, S.; Chand, S. Role of Water Structure on Phase Separation in Polyelectrolyte–Polyethyleneglycol Based Aqueous Two-Phase Systems. *Polymer* **2002**, *43*, 3387–3390. [[CrossRef](#)]
27. Titus, A.R.; Madeira, P.P.; Ferreira, L.A.; Chernyak, V.; Uversky, V.N.; Zaslavsky, B.Y. Mechanism of Phase Separation in Aqueous Two-Phase Systems. *International J. Mol. Sci.* **2022**, *23*, 14366. [[CrossRef](#)] [[PubMed](#)]

28. Saravanan, S.; Rao, J.R.; Nair, B.U.; Ramasami, T. Aqueous Two-Phase Poly(Ethylene Glycol)–Poly(Acrylic Acid) System for Protein Partitioning: Influence of Molecular Weight, PH and Temperature. *Process. Biochem.* **2008**, *43*, 905–911. [[CrossRef](#)]
29. Lopes, A.M.; Molino, J.V.D.; dos Santos-Ebinuma, V.C.; Pessoa, A.; Valentini, S.R.; Pereira, J.F.B. Effect of Electrolytes as Adjuvants in GFP and LPS Partitioning on Aqueous Two-Phase Systems: 1. Polymer-Polymer Systems. *Sep. Purif. Technol.* **2018**, *206*, 39–49. [[CrossRef](#)]
30. Santos-Ebinuma, V.C.; Lopes, A.M.; Pessoa, A.; Teixeira, M.F.S. Extraction of Natural Red Colorants from the Fermented Broth of *Penicillium Purpurogenum* Using Aqueous Two-Phase Polymer Systems. *Biotechnol. Prog.* **2015**, *31*, 1295–1304. [[CrossRef](#)] [[PubMed](#)]
31. Santos, J.H.P.M.; Martins, M.; Silvestre, A.J.D.; Coutinho, J.A.P.; Ventura, S.P.M. Fractionation of Phenolic Compounds from Lignin Depolymerisation Using Polymeric Aqueous Biphasic Systems with Ionic Surfactants as Electrolytes. *Green Chem.* **2016**, *18*, 5569–5579. [[CrossRef](#)]
32. Chakraborty, A.; Sen, K. Impact of PH and Temperature on Phase Diagrams of Different Aqueous Biphasic Systems. *J. Chromatogr. A* **2016**, *1433*, 41–55. [[CrossRef](#)] [[PubMed](#)]
33. Sadeghi, R.; Maali, M. Toward an Understanding of Aqueous Biphasic Formation in Polymer–Polymer Aqueous Systems. *Polymer* **2016**, *83*, 1–11. [[CrossRef](#)]
34. Almeida, M.R.; Rufino, A.F.C.S.; Belchior, D.C.V.; Carvalho, P.J.; Freire, M.G. Characterization of Cholinium-Carboxylate-Based Aqueous Biphasic Systems. *Fluid Phase Equilibria* **2022**, *558*, 113458. [[CrossRef](#)]
35. Basaiahgari, A.; Priyanka, V.P.; Ijardar, S.P.; Gardas, R.L. Aqueous Biphasic Systems of Amino Acid-Based Ionic Liquids: Evaluation of Phase Behavior and Extraction Capability for Caffeine. *Fluid Phase Equilibria* **2020**, *506*, 112373. [[CrossRef](#)]

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