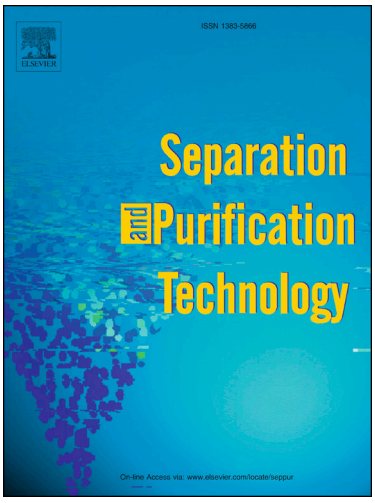

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Acetonitrile as adjuvant to tune polyethylene glycol + K_3PO_4 aqueous two-phase systems and its effect on phenolic compounds partition

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Abstract

Aqueous two-phase systems (ATPS) have been proposed as platforms for the purification of biomolecules, and the application of adjuvants to tune the properties of ATPS phases and their ability to extract and separate biological products investigated. This work addresses, for the first time, the use of acetonitrile (ACN) as adjuvant in ATPS composed of polyethylene glycol (PEG) of different molecular weights (from 400 to 20000 $g \cdot mol^{-1}$) and potassium phosphate. The effect of ACN concentration (at 0.25, 2.5, 5 and 7.5 wt %) in the liquid-liquid equilibrium is here studied by measuring the phase diagrams, the composition of the phases in equilibrium and their Kamlet-Taft parameters. The results obtained demonstrate that the ACN presence increases the biphasic region of PEG-K_3PO_4 ATPS and its distribution between the coexisting phases is dependent on its concentration and ATPS composition. The difference in the dipolarity/polarizability of the coexisting phases is significantly affected by the presence of ACN in the ternary mixture. The partition of two phenolic compounds – protocatechuic acid and gallic acid – was studied, showing that ACN improves the partition of these molecules to the salt-rich phase, and that the system selectivity can be significantly improved by changing the concentration of the phase forming compounds.

Keywords: Liquid-liquid systems, organic solvents, Kamlet-Taft parameters, extraction, phenolic compounds

1. Introduction

The separation and purification stages of biotechnological processes requires high energy and chemical consumption, which produce an important impact in the final product cost. Liquid-liquid extraction appears as a cost-effective separation technique due to its technological simplicity, easy scale-up, low cost, and high efficiency in the separation of a large range of compounds or materials. However, the liquid-liquid extraction of biomolecules is typically carried out by using volatile organic solvents due to their immiscibility with aqueous media where biomolecules are present.[1] These are often poorly biocompatible media and may lead to the denaturation of proteins and other compounds.

Aqueous two-phase systems (ATPSs) have been widely studied as benign liquid-liquid systems to separate and purify biological products. These systems consist of two immiscible aqueous phases that can be formed by the mixture of two polymers or a polymer with a salt.[2,3] Polyethylene glycol (PEG) is one of the most studied phase-forming compounds in literature. This polymer presents high biodegradability, low toxicity, large water miscibility, and low cost, and is widely used in industrial processes.[4,5] However, the hydrophilic, and poor tailoring nature of PEG is a limitation on the application of these systems to the extraction of several biomolecules. The use of additives to minimize some of the limitations associated to the application of PEG-based ATPS, was proposed for the first time in the 90's.[6,7] In these studies,[6,7] authors used small amounts of sodium chloride to increase the hydrophobicity difference between the coexisting phases of polymer-polymer and polymer-salt ATPS, influencing the partition coefficient of different proteins. Since then, several works were reported suggesting the use of salts, ionic liquids, and polymers, as additives in different types of ATPS.[4,8–10] Nowadays these additives are known as adjuvants: compounds able to fine-tune the intrinsic properties of the aqueous phases of an ATPS, without the need of changing the phase forming compounds of the system, and preventing the loss of the main characteristics of the starting mixture. Furthermore, the number of compounds that may be used as adjuvants in a specific system is very large, which means that the choice of the correct adjuvant is in itself a tool to tune the ATPS properties.

Acetonitrile (ACN) is an aprotic and strongly polar organic solvent, widely used in chemical and pharmaceutical industries[11] and in separation and purification processes as a mobile phase in high performance liquid chromatography.[12,13] Similarly to other

organic solvents, such as acetone and dimethyl sulfoxide, ACN is miscible with water in all proportions. The use of ACN as phase forming compound of ATPS was previously demonstrated, with the phases demixing induced by the addition of polymers or carbohydrates to the aqueous solution.[14–17] This type of ATPS demonstrated good performance on the extraction of biomolecules to the ACN-rich phase, and can be easily integrated into traditional solid-liquid extraction processes.[17]

In order to evaluate the potential application of organic solvents as adjuvants in ATPS, in this work is studied, for the first time, the effect of ACN as adjuvant in polymer-salt ATPS. This type of systems is more viable to be applied in downstream processing than polymer-polymer systems, due to advantages such as low interfacial tension, fast and high phase separation rates, and low cost.[4] In this context, novel ternary and quaternary systems composed of PEG at different molecular weights (M_w = 400, 1000, 1500, 4000, 6000, 8000, 10000 and 20000), potassium phosphate (K_3PO_4) and water, without and with the addition of ACN ranging from 0.25 to 7.5 wt % were determined at 298 K and atmospheric pressure. The composition and Kamlet-Taft parameters of ATPS coexisting phases were determined to infer on the effect of ACN in the phases equilibrium and properties of PEG 1500-based ATPS. The impact of ACN as adjuvant in the partition of two phenolic compounds – the gallic and the protocatechuic acids – was also evaluated. The chemical structures of the biomolecules investigated are presented in **Figure 1**.

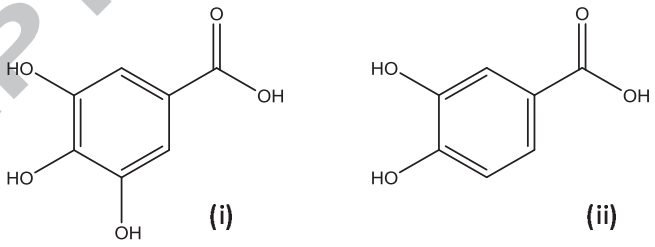

Figure 1. Chemical structure of the phenolic acids studied: (i) gallic acid; (ii) protocatechuic acid.

2. Material and Methods

2.1. Materials

The phase diagrams of ternary and quaternary systems were prepared by using aqueous solutions of PEG (95 to 99 wt % pure) of different molecular weights – 400, 1000, 1500, 4000, 6000, 8000, 10000 and 20000 $g \cdot mol^{-1}$ – ACN (HPLC grade, 99 wt % pure) from Sigma-Aldrich (Germany), and K_3PO_4 (98 wt % pure) from Vetec (Brazil). The biomolecules studied were two phenolic compounds: the protocatechuic acid (99.6 wt % pure) and the gallic acid (99.5 wt % pure), both obtained from Merck (Germany). The probes *N*,*N*-diethyl-4-nitroaniline (99 % pure) from Fluorochem (UK), 4-nitroaniline (99 % pure) from Aldrich (Germany), and pyridine-*N*-oxide (95 % pure) from Aldrich (Germany) were used on Kamlet-Taft parameters determination. All chemicals were used without further purification. Double distilled water was used in the preparation of the ATPS.

2.2. Methods

2.2.1 Determination of phase diagrams and tie-lines

The solubility curves, *i.e.* the limit between the monophasic and biphasic regions, were determined at (298 ± 1) K and atmospheric pressure through the cloud-point titration method.[18] Aqueous solutions of PEG with concentrations ranging between 30 and 60 wt % and K_3PO_4 at 40 wt %, without and with ACN at different concentrations (0.25, 2.5, 5, and 7.5 wt %) as adjuvant, were used. PEG aqueous solution was prepared in a vial, and K_3PO_4 aqueous solution was added dropwise until the mixture became cloudy, which corresponds to the transition to the biphasic region. Then, water was added dropwise until the mixture turns to a clear solution – monophasic region. All the procedure was carried out under constant stirring. The system composition was determined by weight quantification of all pure components added, with an uncertainty of $\pm 10^{-4}$ g. The ternary system composed of ACN + K_3PO_4 + H_2O was also determined.

The experimental binodal curves were fitted by the following equation [19]:

$$[PEG] = A\, exp[(B \times [K_3PO_4]^{0.5}) - (C \times [K_3PO_4]^3)] \quad \textbf{(1)}$$

where $[PEG]$ and $[K_3PO_4]$ are the PEG and the K_3PO_4 weight fraction percentages, respectively, and A, B, and C are constants obtained by the regression of the experimental binodal data.

Two different tie-lines (TLs) were determined in the mixture points 20 wt % of PEG

1500 + 8 wt % of K_3PO_4 + H_2O and 30 wt % of PEG 1500 + 8 wt % of K_3PO_4 + H_2O for the systems composed of PEG with a M_w = 1500 g·mol^{-1}, K_3PO_4, and ACN as adjuvant at 0, 0.25, 2.5, 5 and 7.5 wt %. The ternary and quaternary systems were gravimetrically prepared within ±10^{-4} g, vigorously stirred by using a vortex mixer, centrifuged at 3500 rpm for 30 minutes, and left to equilibrate in a water bath for 12 h at (298 ± 1) K, aiming at a complete separation of the coexisting phases. The top and bottom phases were then carefully separated and individually weighed.

The composition of the phases was determined analytically, quantifying the concentration of PEG and K_3PO_4 by thermogravimetric analysis (TGA), the water content by Karl Fischer titration, and the ACN by weight difference. TGA was carried out by using a Perkin Elmer TGA 400 (USA) and following the procedure previously reported by Farias et al. [20] Aqueous mixtures of known concentrations of both salt and polymer were prepared and analyzed by TGA in triplicate to infer on the capacity of this technique to quantify PEG and K_3PO_4 concentrations. The standard deviation obtained was ≤ 1 %. Karl Fischer titration (Metrohm 870 KF Titrino plus (Germany)) to water quantification was made using Hydranal™ - Composite 5 reagent from Honeywell-Fluka (Germany). In the end, a mass balance was performed between the mass of each component added to the initial mixture and the amounts quantified in the top and bottom phases to confirm the accuracy of the obtained results.

The tie-lines length (TLL) was calculated according to **Eq. (2)**:

$$TLL = \sqrt{\left([PEG]_{K_3PO_4} - [PEG]_{PEG}\right)^2 + \left([K_3PO_4]_{K_3PO_4} - [K_3PO_4]_{PEG}\right)^2} \quad \textbf{(2)}$$

where subscripts "PEG" and "K_3PO_4" represent, respectively, the PEG- and the K_3PO_4-rich phases, and $[PEG]$ and $[K_3PO_4]$ are the weight fraction percentages of polymer and inorganic salt, as described before.

The pH values (±0.02) of the PEG- and K_3PO_4-rich aqueous phases were measured at (298 ± 1) K using a SevenExcellence™ pH/Conductivity meter (Mettler Toledo (USA)).

2.2.2. Partition of ACN

For a better understanding of the behavior of ACN as adjuvant and its influence on the binodal curves and biomolecules partition, the partition coefficient of ACN was determined in the quaternary systems. As previously referred, ACN content in each

phase of the ATPS was calculated by weight difference, considering the TGA and Karl-Fisher titration results for the remaining phase forming components. The partition coefficient of ACN (K_{ACN}) was determined as the ratio between the concentration of ACN in the PEG- and in the salt-rich phase, according to **Eq. (3)**:

$$K_{mol.} = \frac{[mol.]_{PEG}}{[mol.]_{K_3PO_4}} \quad (3)$$

where $[mol.]_{PEG}$ and $[mol.]_{K_3PO_4}$ are the concentrations (wt %) of the target molecule – in this case ACN – in the PEG- and K_3PO_4-rich phases, respectively.

2.2.3. Kamlet-Taft parameters

Kamlet and Taft multiparametric approach is based on a set of probes, which allows the assessment of different interactions for the same solvent.[21–23] The probes *N*,*N*-diethyl-4-nitroaniline, 4-nitroaniline, and pyridine-*N*-oxide were used to determine the dipolarity/polarizability, π^*, hydrogen-bond (acceptor) basicity, β, and hydrogen-bond (donor) acidity, α, of both PEG- and salt-rich phases of the studied ATPS. The experimental procedure followed in this work to determine the Kamlet-Taft parameters using these probes was previously reported by Coutinho and co-workers.[24]

2.2.4. Partition of phenolic compounds

The partition of the phenolic compounds was evaluated in the mixture points 20 wt % of PEG 1500 + 8 wt % of K_3PO_4 + H_2O + ACN and 30 wt % of PEG 1500 + 8 wt % of K_3PO_4 + H_2O + ACN for the systems composed of PEG with a M_w = 1500 g·mol^{-1}, K_3PO_4, and ACN as adjuvant at 0, 0.25, 2.5, 5 and 7.5 wt %. Aqueous solutions of protocatechuic acid and gallic acid at 3.24x10^{-3} mol·L^{-1} and 2.94x10^{-3} mol·L^{-1}, respectively, were prepared and used as part of the water content in each TL. Each-mixture was prepared gravimetrically within ±10^{-4} g, vigorously stirred and centrifuged at 3500 rpm for 30 min and left to equilibrate in a water bath for 12 h and at (298 ± 1) K to achieve the complete partition of phenolic compounds between the phases. After careful separation of the phases, the concentration of each molecule in each phase was determined through UV-VIS spectroscopy (BioTeck Synergy HT microplate reader (USA)), at a wavelength of 268 nm for protocatechuic acid and 253 nm for gallic acid. Calibration curves previously established were used, and analyses were performed in triplicate. Possible interferences from the phase forming components of the ATPS with

the analytical method were considered, and control samples were prepared at the same weight fraction composition, using pure water instead of biomolecule aqueous solutions.

The partition coefficients (K) of phenolic compounds were determined as the ratio between the concentration of each biomolecule in the PEG- and in the salt-rich phase, has described in **Eq. (3)**. The selectivity ($S_{PA/GA}$) of the systems for the separation of the phenolic compounds was determined as the ratio between the partition coefficients of protocatechuic acid and gallic acid.

The extractions efficiencies ($EE\%$) were determined as the percentage ratio between the amount of the phenolic compound partitioned to the polymer-rich phase and that present in the total mixture (both phases):

$$EE\% = \frac{w_{mol.}^{PEG}}{w_{mol.}^{PEG} + w_{mol.}^{K_3PO_4}} \times 100 \quad \textbf{(4)}$$

where $w_{mol.}^{PEG}$ and $w_{mol.}^{K_3PO_4}$ are the weight (g) of the target molecule in the PEG- and K_3PO_4-rich phase, respectively. The phenolic compounds weight in each phase was determined as the product of the molecule concentration by the phase volume.

3. Results and Discussion

3.1. The effect of ACN as adjuvant in PEG-K_3PO_4 ATPS

Novel ternary and quaternary phase diagrams of ATPS composed of PEG + K_3PO_4 + H_2O, without and with ACN at 5 wt %, were determined at (298 ± 1) K and atmospheric pressure and are represented in **Figure 2**. The detailed experimental weight fraction data are given in the **Supplementary material**. Some of the ternary systems here studied were previously reported, and the results obtained in this work are in good agreement with the literature [25,26] – *cf.* **Supplementary material**.

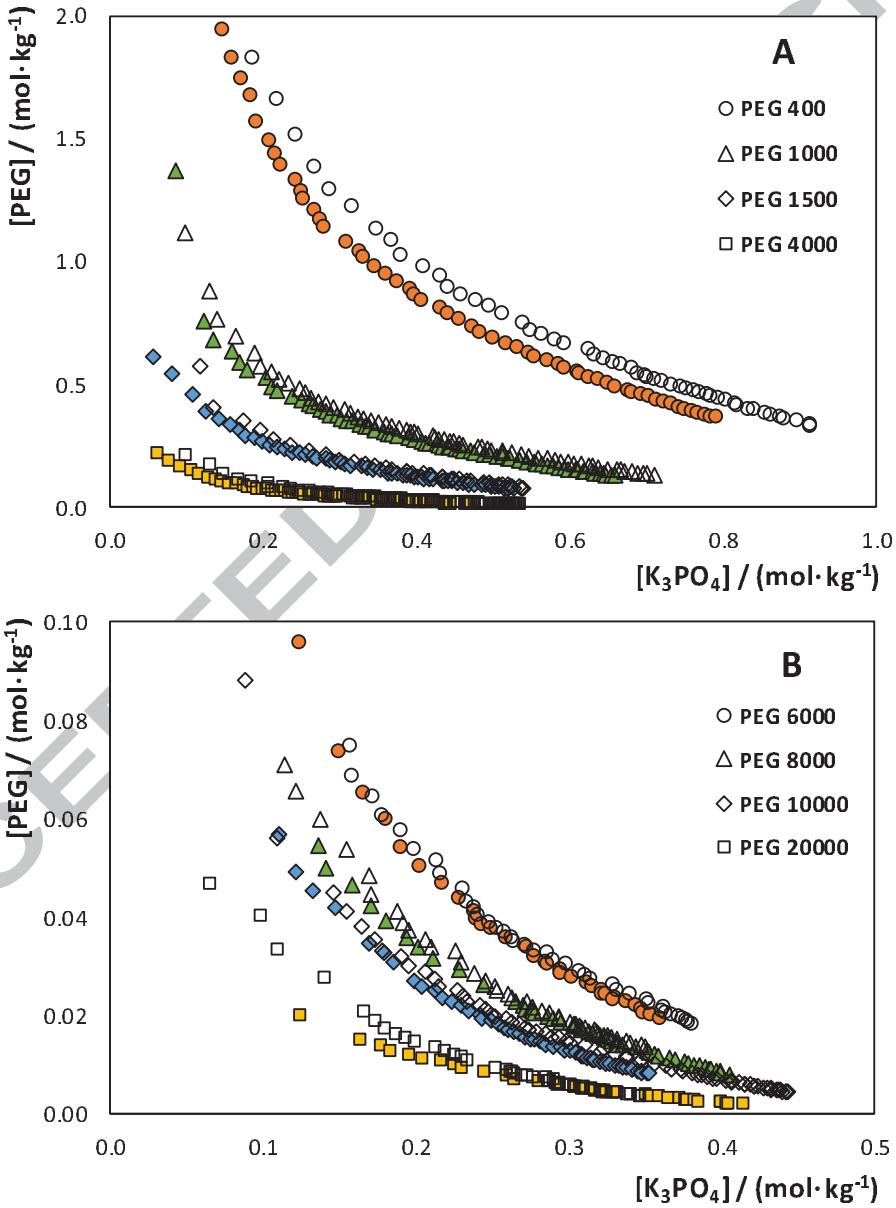

Figure 2. The effect of ACN in ATPS composed of K_3PO_4, water and PEG with **(A)** $M_w \leq 4000$ g·mol^{-1} and **(B)** $M_w > 4000$ g·mol^{-1} at (298 ± 1) K: 0 wt % of ACN (open symbols); 5 wt % of ACN (full symbols).

In **Figure 2**, the binodal curves are presented in molality units to avoid discrepancies that could result from the polymer different molecular weights, allowing a better interpretation of both polymer and salt impact on the phase diagram behavior. For all the phase diagrams determined, the biphasic region is above the solubility curve and, the larger is this region, the easier the polymer is salted-out by the salt to undergo liquid-liquid demixing. It was observed that all the ATPS formed by the mixture of PEG, K_3PO_4, and water, with or without using ACN as adjuvant, are constituted by a bottom phase that corresponds to the inorganic-salt-rich phase and a top phase rich in polymer.

Through the data presented in **Figure 2** it is possible to observe the impact of the PEG molecular weight in the binodal curves. The ability of PEG to form ATPS in presence of K_3PO_4 increases in the following order: PEG 400 < PEG 1000 < PEG 1500 < PEG 4000 < PEG 6000 < PEG 8000 < PEG 10000 < PEG 20000. The formation of an ATPS is induced by the competition of the two solutes for the formation of hydration complexes. When a high charge density salt, such as K_3PO_4, is mixed with a polymer, the salt will "capture" a large amount of water inducing the exclusion of the polymer to a second aqueous phase, occurring the salting-out effect of the salt over the polymer. This effect becomes more important as the polymer molecular weight increases, due to its higher hydrophobicity and consequently lower affinity for water.

The effect of PEG molecular weight is not changed by the addition of 5 wt % of ACN as adjuvant to the ternary system but, the ability to induce the phase separation increases in presence of ACN – *cf.* **Figure 2**. Furthermore, the influence of ACN as adjuvant seems to be higher in systems composed of PEG of lower molecular weight and has almost no effect on PEG of $M_w \geq 6000$ g·mol^{-1}. The huge impact of the high hydrophobicity of PEG with $M_w \geq 6000$ g·mol^{-1} on the binodal curves is probably masking the effect of ACN.

The effect of ACN concentration on the binodal curve of the ATPS composed of PEG 1500 + K_3PO_4 + H_2O was also evaluated, and the obtained results are presented in **Figure 3**. These data show that with the increase of ACN concentration a lower amount

of polymer and salt is necessary to induce the phase separation than for the system without adjuvant.

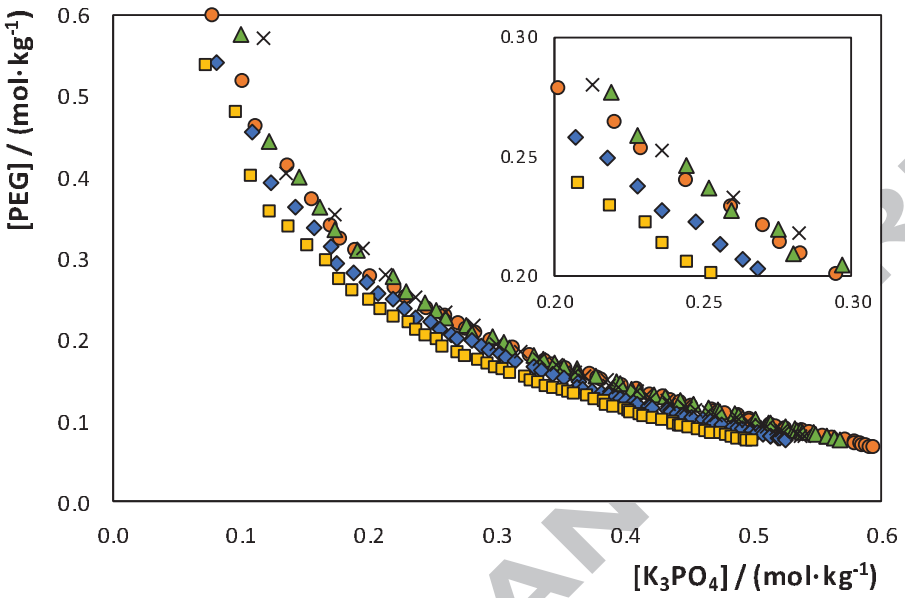

Figure 3. Phase diagrams for systems composed of PEG 1500 + K_3PO_4 + H_2O + ACN at 298 K: no ACN (x); 0.25 wt % of ACN (●); 2.5 wt % of ACN (▲); 5 wt % of ACN (◆); 7.5 wt % of ACN (■).

The ternary system composed of ACN, K_3PO_4, and water was also determined. The detailed experimental weight fraction data and the phase diagram representation are given in the **Supplementary material**. Remarkably, both ACN + K_3PO_4 + H_2O and PEG + K_3PO_4 + H_2O phase diagrams present higher phases' miscibility than the quaternary system composed of PEG + ACN + K_3PO_4 + H_2O, independently of the polymer molecular weight. This behavior was previously reported for ATPS composed of PEG and different salts, where ILs were used as adjuvants.[27] It seems that, similarly to what was observed in the literature,[27] mixtures of PEG-ACN are more hydrophobic than their pure compounds being easily salted-out by the inorganic salt. Nevertheless, and contrarily to what was observed when ILs are used as adjuvants, ACN preferential partition is not always to the PEG-rich phase – as will be discussed below.

All the binodal curves determined were further fitted by the empirical relationship described by **Eq. (1)**. The regression parameters were estimated by the least-squares regression. The values of A, B and C parameters and corresponding standard deviations (σ) are given in **Table 1**. In general, the model proposed by Merchuk [19] fits well the experimental data with correlation coefficients (R^2) higher than 0.96, allowing to predict data in phase diagram regions where there is no experimental results available.

Table 1. Correlation parameters used to describe the experimental binodal data by **Eq. (1)** and respective standard deviation (σ).

PEG	ACN (wt %)	$A \pm \sigma$	$B \pm \sigma$	$10^5 \cdot (C \pm \sigma)$	R^2
400	0	111 ± 3	-0.48 ± 0.02	6 ± 1	0.996
1000		113 ± 3	-0.55 ± 0.01	10 ± 2	0.998
4000		146 ± 7	-0.79 ± 0.02	66 ± 5	0.998
6000		152 ± 22	-0.89 ± 0.08	64 ± 21	0.997
8000		96 ± 3	-0.63 ± 0.02	208 ± 17	0.998
10000		153 ± 11	-0.90 ± 0.04	133 ± 13	0.996
20000		106 ± 9	-0.63 ± 0.06	354 ± 31	0.995
400	5	114 ± 18	-0.52 ± 0.06	10 ± 6	0.963
1000	5	135 ± 10	-0.65 ± 0.04	10 ± 6	0.982
4000	5	107 ± 2	-0.71 ± 0.01	84 ± 4	0.999
6000	5	183 ± 10	-1.00 ± 0.03	48 ± 14	0.999
8000	5	108 ± 7	-0.75 ± 0.03	152 ± 9	0.999
10000	5	202 ± 47	-1.1 ± 0.1	121 ± 59	0.981
20000	5	178 ± 19	-1.07 ± 0.07	118 ± 28	0.995
1500	0	124 ± 12	-0.67 ± 0.05	22 ± 9	0.993
1500	0.25	97 ± 3	-0.56 ± 0.01	33 ± 2	0.999
1500	2.5	110 ± 4	-0.62 ± 0.02	23 ± 4	0.998
1500	5	89 ± 2	-0.55 ± 0.01	41 ± 3	0.998
1500	7.5	88 ± 3	-0.57 ± 0.02	45 ± 5	0.998

The TLs in the mixture points 20 wt % of PEG 1500 + 8 wt % of K_3PO_4 and 30 wt % of PEG 1500 + 8 wt % of K_3PO_4, with the addition of ACN at 0, 0.25, 2.5, 5 and 7.5 wt %, and their respective length (TLL) are presented in **Table 2**. In **Figure 4** is represented an example of the TLs obtained for the system composed of PEG 1500 + K_3PO_4 + H_2O + 2.5 wt % of ACN. In the **Supplementary material** it is possible to find the representation of the remaining systems. The pH of the coexisting phases was also determined and is given in **Table 2**. Due to the presence of K_3PO_4 in their composition,

the phases of these systems present alkaline pH, ranging between 11.8 and 13, with almost no differences between the bottom and top phases.

Table 2. Experimental data for TLs and TLLs of PEG 1500 + K_3PO_4 + ACN + H_2O ATPS at 298 K, and pH values of each phase.

ACN (wt %)	Top Phase (wt %)				Bottom Phase (wt %)				TLL
	[PEG]	[K_3PO_4]	[ACN]	pH	[PEG]	[K_3PO_4]	[ACN]	pH	
20 wt % of PEG 1500 + 8 wt % of K_3PO_4									
0	23.72	5.82	0	12.78	2.99	17.91	0	12.65	24.00
0.25	23.55	5.71	0.24	12.84	3.22	18.04	0.99	12.70	23.78
2.5	25.02	6.17	2.90	12.89	3.03	16.87	1.93	12.81	24.46
5	26.5	5.01	5.69	12.94	4.78	16.16	4.60	12.86	24.41
7.5	27.53	5.14	5.35	13.00	3.26	16.6	14.95	12.89	26.84
30 wt % of PEG 1500 + 8 wt % of K_3PO_4									
0	47.46	2.01	0	11.96	2.25	22.51	0	11.76	49.77
0.25	46.47	2.29	0.23	11.98	0.75	23.26	0.44	11.86	48.90
2.5	46.78	1.66	2.19	12.19	2.32	22.56	3.47	12.08	49.13
5	47.45	1.36	5.55	12.33	1.67	22.89	2.84	12.29	50.59
7.5	47.27	1.77	9.62	12.48	0.81	23.51	3.85	12.38	51.29

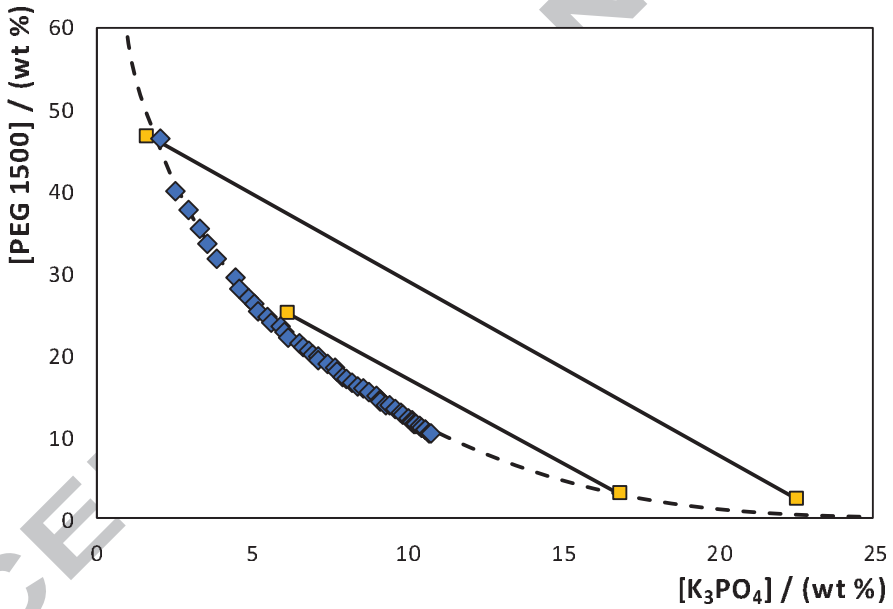

Figure 4. Phase diagram for the quaternary system composed of PEG 1500 + K_3PO_4 + 2.5 wt % ACN + H_2O, at 298 K and atmospheric pressure: binodal curve data (◆), TL (—) and TL phase composition (■), adjusted binodal data through **Eq. (1)** (- - -).

3.2. Partition of ACN

The partition coefficient (K) of ACN to PEG-rich phase was determined in the mixture points reported in **Table 2**. The results obtained are presented in **Figure 5** and additional details on the experimental data are given in the **Supplementary material**.

As previously mentioned and in contrast with the observed when ILs are used as adjuvants in polymer-salt-type ATPS,[4,27] ACN preferential partition is not always to the PEG-rich phase. Through the data presented in **Figure 5** it is possible to conclude that the ACN partition depends on both the amount of ACN added to the system and on the composition of the ternary system. In the TL composed of 30 wt % of PEG 1500 + 8 wt % of K_3PO_4 (with longer TLL), the partition of ACN increases with its concentration. Considering the ACN weight percentage in the top and bottom phases presented in **Table 2**, after the addition of 2.5 wt % of ACN to the ternary system is possible to observe the saturation of the bottom phase, while the concentration on the top phase keeps increasing with the amount of ACN added to the system. However, for the TL composed of 20 wt % of PEG 1500 + 8 wt % of K_3PO_4 (with shorter TLL), there is a maximum on the partition coefficient of ACN for the PEG-rich phase, with K = 1.50 ± 0.04 at 2.5 wt % of ACN. Contrasting with the observed for the other TL, in this system occurs the saturation of the top phase after 5 wt % of ACN is added to the mixture, with a significant increase of ACN concentration in the bottom phase, which could justify the decrease of the ACN partition coefficient at these conditions. This type of behavior suggests that the mechanisms that rule the ACN partition are highly dependent on the ternary system composition. It is well known that the partition of molecules in ATPS could be highly dependent of the TLL. In fact, the longer is a TL, the greater the differences in the composition of the coexisting phases, and consequently their properties, and the interactions that induce the partition of a molecule to a specific phase will be stronger improving its extraction. Furthermore, above a certain concentration of salt and ACN, K_3PO_4 may be inducing the salting-out of ACN to the polymer-rich phase.

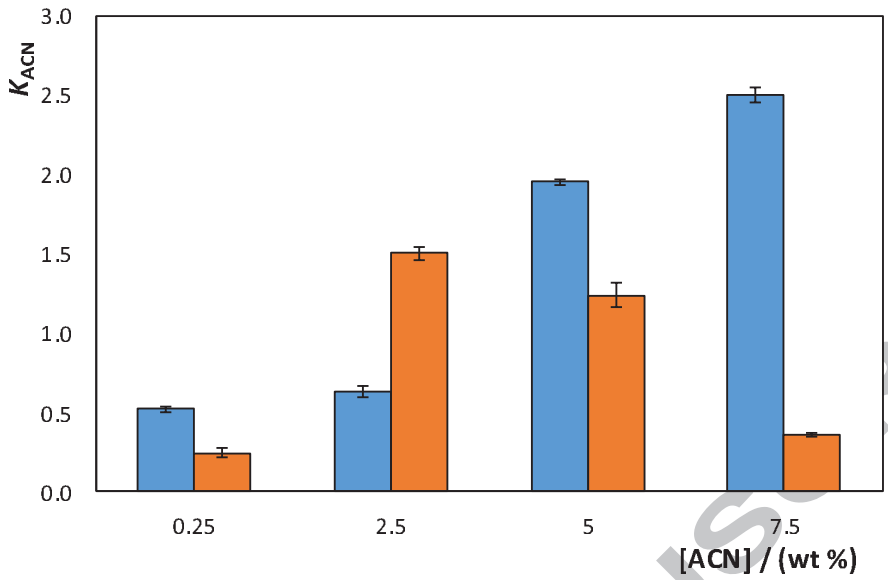

Figure 5. Partition coefficient (*K*) of ACN to PEG 1500-rich phase in the quaternary systems composed of 30 wt % of PEG 1500 + 8 wt % of K_3PO_4 + H_2O (blue) and 20 wt % of PEG 1500 + 8 wt % of K_3PO_4 + H_2O (orange).

3.3. Kamlet-Taft parameters

Since the description of the multiple possible solute-solvent interactions by a single parameter is not possible, Kamlet and Taft proposed a multiparametric approach based on the use of a set of probes to determine different interactions, namely dipolarity/polarizability (π^*), solvent hydrogen-bond acidity (α), and solvent hydrogen-bond basicity (β).[21–23] This approach has been successfully used for the description of solutes partition in ATPS composed of polymers and salts.[28,29]

Kamlet-Taft parameters of the coexisting phases of PEG 1500 + K_3PO_4 + H_2O ATPS, without and with the addition of ACN as adjuvant at different concentrations, were determined at the two different mixture points. For a correct interpretation of the results obtained, hereafter "solvent" refers to the global phase mixture, and not to an individual component, such as water, polymer, salt or ACN. The results obtained for π^*, α, and β, as well as their respective differences (Δ, calculated as the difference between the parameter values in the PEG- and the salt-rich phases) are presented in **Table 3**.

Table 3. Kamlet–Taft parameters and their differences (Δ) between the coexisting phases of PEG 1500 + K_3PO_4 ATPS at 278 K.

ACN (wt %)	β_{PEG}	β_{salt}	$\Delta\beta$	π_{PEG}^*	π_{salt}^*	$\Delta\pi^*$	α_{PEG}	α_{salt}	$\Delta\alpha$
	20 wt % of PEG 1500 + 8 wt % of K_3PO_4								
0	0.42	0.31	0.12	1.22	1.26	-0.04	1.28	1.26	0.02
0.25	0.44	0.30	0.13	1.22	1.27	-0.05	1.32	1.32	0.00
2.5	0.41	0.29	0.13	1.22	1.27	-0.05	1.22	1.26	-0.04
5	0.46	0.30	0.17	1.18	1.27	-0.09	1.18	1.32	-0.14
7.5	0.45	0.31	0.15	1.17	1.27	-0.10	1.28	1.30	-0.02
	30 wt % of PEG 1500 + 8 wt % of K_3PO_4								
0	0.55	n.s.[a]	---	1.14	n.s.[a]	---	1.15	1.29	-0.14
0.25	0.49	n.s.[a]	---	1.17	n.s.[a]	---	1.22	1.33	-0.11
2.5	0.53	n.s.[a]	---	1.11	n.s.[a]	---	1.15	1.31	-0.16
5	0.51	n.s.[a]	---	1.13	n.s.[a]	---	1.20	1.31	-0.11
7.5	0.49	n.s.[a]	---	1.09	n.s.[a]	---	1.14	1.31	-0.17

[a] Not soluble (n.s.).

Considering the results obtained in the mixture point 20 wt % of PEG 1500 + 8 wt % of K_3PO_4, independently of the amount of ACN added to the ternary mixture, the solvent dipolarity/polarizability of salt-rich phase is always higher than in the PEG-rich phase, which is in good agreement with the data previously reported in the literature for others PEG-salt-type ATPS.[28,29] Furthermore, the salt-rich phase presents π^* values very close to the dipolarity/polarizability of pure water ($\pi_{H_2O}^* = 1.26$) and the addition of ACN seems to have no effect on this parameter. On the other side, the π^* of PEG-rich phase decreases with the addition of ACN, resulting in a significant increase of $\Delta\pi^*$ absolute value. The trends are presented in **Figure 6**. The Kamlet-Taft parameters were previously determined for mixtures of ACN and water by Marcus et al.[30] and the results here reported are in good agreement with the literature data. The authors observed that π^* decreases significantly with the addition of ACN to the water. However, in the case of polymer-salt ATPS, this effect is only observed in the polymer-rich phase (*cf.* **Figure 6**), which could be related with the effect of the presence of a third compound, such as the polymer and/or the salt, in the phases of the system.

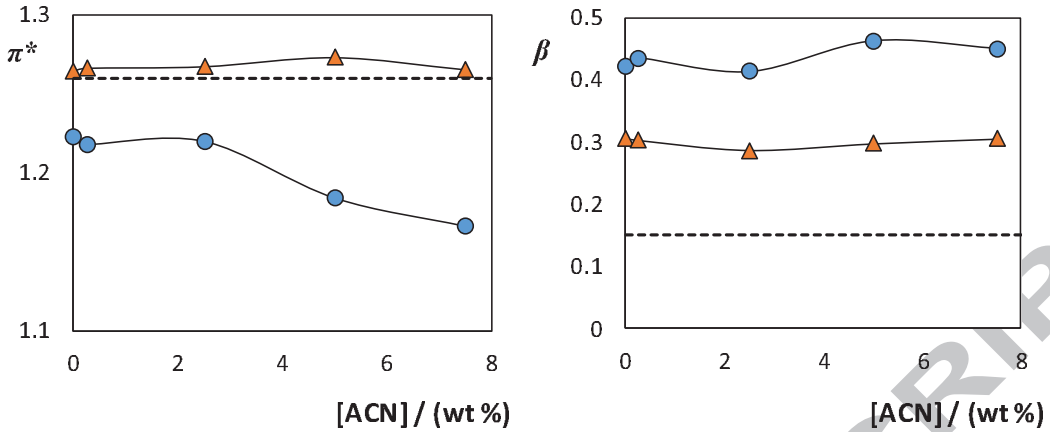

Figure 6. Kamlet-Taft parameters for ATPS composed of 20 wt % of PEG 1500 + 8 wt % of K_3PO_4 + ACN + H_2O at different ACN concentrations: salt-rich phase (orange triangles); PEG-rich phase (blue circles); water (dashed line).

The hydrogen-bond basicity of both top and bottom phases of PEG 1500 + K_3PO_4 ATPS is significantly higher than pure water, with the PEG-rich phase presenting the highest values – *cf.* **Table 3** and **Figure 6**. Similarly to the results obtained for the solvent dipolarity/polarizability, these trends are also in good agreement with literature.[28,29] However, it is more difficult to identify a trend on the $\Delta\beta$. Nevertheless, it seems to occur a slight increase of this parameter with the ACN concentration. As expected, $\Delta\alpha$ slightly decreases with the amount of ACN added to the ternary system, presenting the opposite behavior of $\Delta\beta$.

The data presented in **Table 3**shows that when the TLL increases, and consequently the composition of the coexisting phases becomes richer in PEG or K_3PO_4, there are significant changes on the Kamlet-Taft parameters. However, due to the high concentration and strong salting-out effect of the inorganic salt K_3PO_4, the probes used to determine the parameters β and π^* are not soluble on the salt-rich phase of the mixture point 30 wt % of PEG 1500 + 8 wt % of K_3PO_4, preventing the determination of $\Delta\pi^*$ and $\Delta\beta$ for these systems.

The changes observed on the Kamlet-Taft parameters are small. However, when ATPS are used to separate biomolecules with similar structures, a fine manipulation of ATPS phases properties may allow an improvement in the partition/separation of these molecules. Thus, the application of ACN as adjuvant could be of high interest for the fine tuning of the properties of polymer-salt-based ATPS.

3.4. Phenolic compounds partition

To evaluate the effect of ACN as adjuvant in the partition of biomolecules in polymer-salt-based ATPS, the partition coefficients and extraction efficiencies of two phenolic compounds – the protocatechuic acid and the gallic acid – were determined in the systems composed of PEG 1500 + K_3PO_4 + H_2O previously reported in **Table 2**. The results obtained are presented in **Figure 7**, and more details are given in the **Supplementary material**.

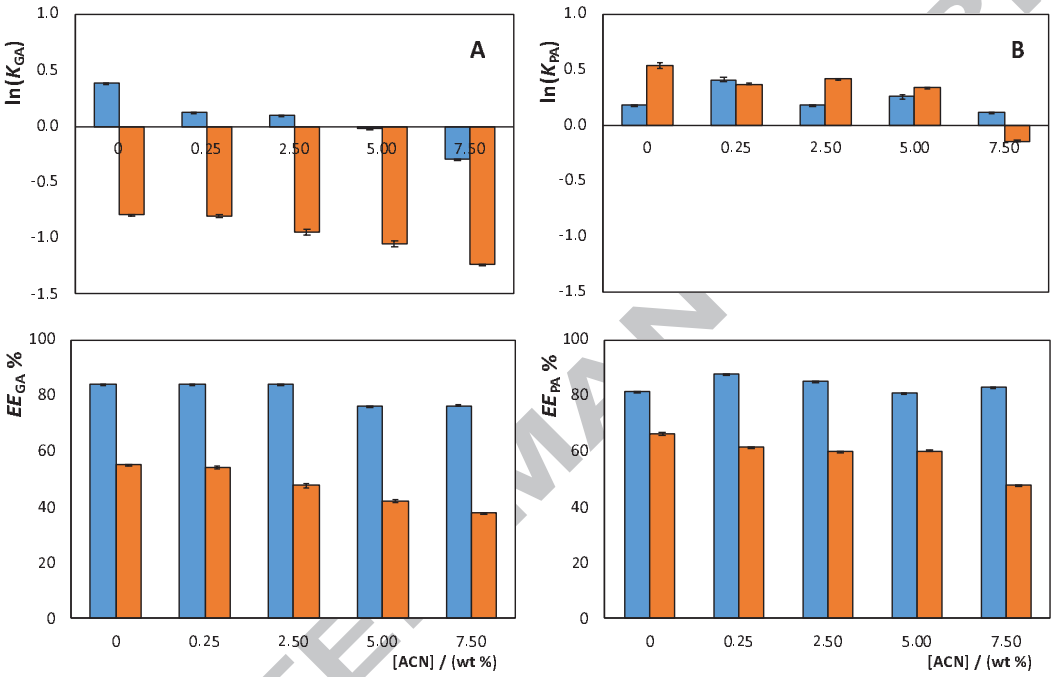

Figure 7. Partition coefficient (K) and extraction efficiency percentages (EE%) of (**A**) gallic acid (GA) and (**B**) protocatechuic acid (PA) in ATPS composed of 30 wt % of PEG 1500 + 8 wt % of K_3PO_4 + H_2O (blue bars) and 20 wt % of PEG 1500 + 8 wt % of K_3PO_4 + H_2O (orange bars) at different concentrations ACN.

The addition of ACN to the ternary system seems to present a small effect on the partition and extraction efficiency of the phenolic compounds. Nevertheless, it is possible to observe an increase on the partition of gallic acid to the salt-rich phase, which is more evident for the shorter TL (mixture point 20 wt % of PEG 1500 + 8 wt % of K_3PO_4). For protocatechuic acid, this effect is not clear, and small oscillations can be observed at both mixture points used. Furthermore, this phenolic compound is always extracted to the polymer-rich phase with the exception of the mixture point 20 wt % of PEG 1500 + 8 wt % of K_3PO_4 + 7.5 wt % of ACN. The obtained results are in good

agreement with phenolic compounds octanol-water partition coefficients. Protocatechuic acid is slightly more hydrophobic than gallic acid – log K_{OW} is equal to 1.16 and 0.91 for protocatechuic and gallic acid, respectively [31] – which may justify its preferential partition for PEG-rich phase. Additionally, and considering the speciation curves of these compounds,[31] at the pH of the coexisting phases (pH ≅ 12, *cf.* **Table 2**), protocatechuic acid and gallic acid are negatively charged. As previously demonstrated by Claudio et al.,[32] at these conditions gallic acid preferentially partitions to the salt-rich phase – the most hydrophilic phase – what could explain the low partition of these phenolic compounds to the polymer-rich phase.

Despite the low effect of ACN in the extraction of the phenolic compounds, the change of the mixture point used in the extractions presents a huge impact on the gallic acid partition. Since the protocatechuic acid partition is not significantly changed by the mixture point, the selectivity of these systems to the separation of the two phenolic compounds here studied can be improved, as shown in **Figure 8**. By changing the mixture point from 30 wt % of PEG 1500 + 8 wt % of K_3PO_4 to 20 wt % of PEG 1500 + 8 wt % of K_3PO_4 the selectivity can be increased up to 4.6 times – cf. the **Supplementary material**. The obtained results also demonstrate that ACN addition has no influence on the selectivity of PEG + K_3PO_4 ATPS for the extraction of the studied phenolic compounds.

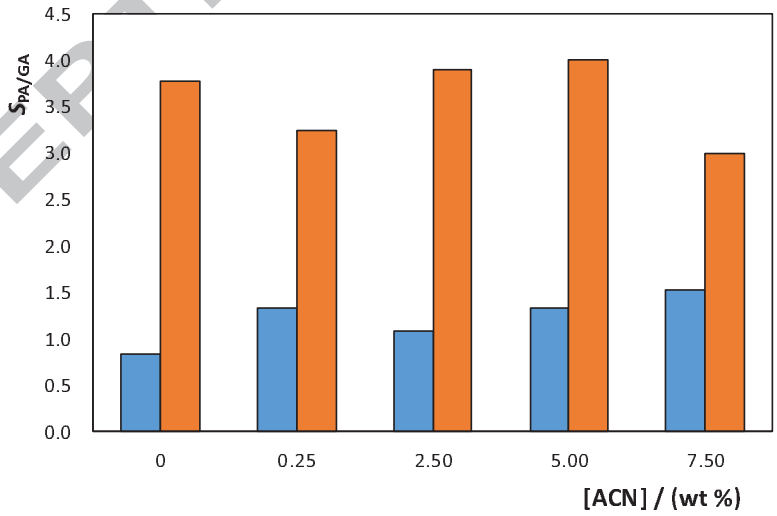

Figure 8. The effect of ACN on the selectivity ($S_{PA/GA}$) of ATPS composed of 30 wt % of PEG 1500 + 8 wt % of K_3PO_4 + H_2O (blue bars) and 20 wt % of PEG 1500 + 8 wt % of K_3PO_4 + H_2O (orange bars) for protocatechuic acid (PA) and gallic acid (GA).

4. Conclusion

The use of ACN as adjuvant in ternary systems composed of PEG, K_3PO_4 and water was studied in this work. By using PEG with different molecular weights, it was observed that the addition of 5 wt % of ACN improves the phase demixing of PEG-K_3PO_4 ATPS, presenting a higher effect in the systems composed of lower molecular weight polymer, and with almost no effect on systems composed of PEG with $M_w \geq$ 6000 g·mol^{-1}. The results reported suggest that the mixture of ACN-PEG is easier to be salted-out by K_3PO_4 than the individual compounds, since the mixture results in an ATPS with a larger biphasic region than the individual ternary systems. It was also observed that ACN partition between the coexisting phases of the systems is highly dependent of the ACN concentration and the ATPS starting composition. However, independently of its distribution, ACN addition significantly increase the difference in the dipolarity/polarizability of the system coexisting phases, and has almost no effect on the solvent hydrogen-bond basicity and acidity. Finally, the evaluation of protocatechuic acid and gallic acid partition in function of ACN concentration, shows that ACN improves the partition of these biomolecules to the salt-rich phase. Nevertheless, by changing the mixture point, it was possible to significantly improve the selectivity of the system for the two phenolic compounds. The obtained results show how the ACN can be used to do the fine-tuning of the properties and ability of polymer-salt ATPS to partition and selectively separate a mixture of biomolecules.

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6. References

[1] M. Martínez-Aragón, S. Burghoff, E.L.V. Goetheer, A.B. de Haan, Guidelines for solvent selection for carrier mediated extraction of proteins, Sep. Purif. Technol. 65 (2009) 65–72. doi:10.1016/j.seppur.2008.01.028.

[2] P.A. Albertsson, Partition of cell particles and macromolecules, 3rd ed., Wiley, New York, 1986.

[3] B. Zavlasky, Aqueous Two-Phase Partitioning: Physical Chemistry and Bioanalytical Applications, M. Dekker, New York, 1994. doi:10.1080/01932699508943691.

[4] J.F.B. Pereira, A.S. Lima, M.G. Freire, J.A.P. Coutinho, Ionic liquids as adjuvants for the tailored extraction of biomolecules in aqueous biphasic systems, Green Chem. 12 (2010) 1661–1669. doi:10.1039/c003578e.

[5] S. Raja, V.R. Murty, V. Thivaharan, V. Rajasekar, V. Ramesh, Aqueous two phase systems for the recovery of biomolecules – a review, Sci. Technol. 1 (2012) 7–16. doi:10.5923/j.scit.20110101.02.

[6] C.-K. Lee, S.I. Sandler, Vancomycin partitioning in aqueous two-phase systems: Effects of pH, salts, and an affinity ligand, Biotechnol. Bioeng. 35 (1990) 408–416. doi:10.1002/bit.260350408.

[7] O. Cascone, B.A. Andrews, J.A. Asenjo, Partitioning and purification of thaumatin in aqueous two-phase systems, Enzyme Microb. Technol. 13 (1991) 629–635. doi:10.1016/0141-0229(91)90076-M.

[8] M. Vahidnia, G. Pazuki, S. Abdolrahimi, Impact of polyethylene glycol as additive on the formation and extraction behavior of ionic-liquid based aqueous two-phase system, AIChE J. 62 (2016) 264–274. doi:10.1002/aic.15035.

[9] C.P. Song, R.N. Ramanan, R. Vijayaraghavan, D.R. MacFarlane, E.-S. Chan, P.L. Show, S.T. Yong, C.-W. Ooi, Effect of salt-based adjuvant on partition behaviour of protein in aqueous two-phase systems composed of polypropylene glycol and cholinium glycinate, Sep. Purif. Technol. 196 (2018) 281–286. doi:10.1016/J.SEPPUR.2017.09.017.

[10] I. V. Lucena, I. V. Brandão, S. Mattedi, R.L. Souza, C.M.F. Soares, A.T. Fricks, Á.S. Lima, Use of protic ionic liquids as adjuvants in PEG-based ATPS for the purification of radish peroxidase, Fluid Phase Equilib. 452 (2017) 1–8. doi:10.1016/J.FLUID.2017.08.012.

[11] D. Zhang, Y. Zhang, Y. Wen, K. Hou, J. Zhao, Intrinsic kinetics for the synthesis

of acetonitrile from ethanol and ammonia over Co–Ni/γ–Al2O3 catalyst, Chem. Eng. Res. Des. 89 (2011) 2147–2152. doi:10.1016/J.CHERD.2011.01.029.

[12] Y. Gu, P.-H. Shih, Salt-induced phase separation can effectively remove the acetonitrile from the protein sample after the preparative RP-HPLC, Enzyme Microb. Technol. 35 (2004) 592–597. doi:10.1016/J.ENZMICTEC.2004.08.011.

[13] M. Taha, H.-L. Teng, M.-J. Lee, Phase diagrams of acetonitrile or (acetone + water + EPPS) buffer phase separation systems at 298.15 K and quantum chemical modeling, J. Chem. Thermodyn. 54 (2012) 134–141. doi:10.1016/J.JCT.2012.03.026.

[14] G. de B. Cardoso, I.N. Souza, M.M. Pereira, M.G. Freire, C.M.F. Soares, Á.S. Lima, Aqueous two-phase systems formed by biocompatible and biodegradable polysaccharides and acetonitrile, Sep. Purif. Technol. 136 (2014) 74–80. doi:10.1016/J.SEPPUR.2014.08.020.

[15] G. De Brito Cardoso, T. Mourão, F.M. Pereira, M.G. Freire, A.T. Fricks, C. Mara, F. Soares, Á.S. Lima, Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin, Sep. Purif. Technol. 104 (2013) 106–113. doi:10.1016/j.seppur.2012.11.001.

[16] P.B. Dhamole, P. Mahajan, H. Feng, Phase separation conditions for sugaring-out in acetonitrile-water systems, J. Chem. Eng. Data. 55 (2010) 3803–3806. doi:10.1021/je1003115.

[17] B. Wang, T. Ezejias, H. Feng, H. Blaschek, Sugaring-out: A novel phase separation and extraction system, Chem. Eng. Sci. 63 (2008) 2595–2600. doi:10.1016/j.ces.2008.02.004.

[18] S.P.M. Ventura, C.M.S.S. Neves, M.G. Freire, I.M. Marrucho, J. Oliveira, J.A.P. Coutinho, Evaluation of anion influence on the formation and extraction capacity of ionic-liquid-based aqueous biphasic systems, J. Phys. Chem. B. 113 (2009) 9304–9310. doi:10.1021/jp903286d.

[19] J.C. Merchuk, B. a Andrews, J. a Asenjo, Aqueous two-phase systems for protein separation. Studies on phase inversion., J. Chromatogr. B. Biomed. Sci. Appl. 711 (1998) 285–93.

[20] F.O. Farias, F.H.B. Sosa, L. Igarashi-Mafra, J.A.P. Coutinho, M.R. Mafra, Study of the pseudo-ternary aqueous two-phase systems of deep eutectic solvent (choline chloride:sugars) + K2HPO4 + water, Fluid Phase Equilib. 448 (2017) 143–151. doi:10.1016/J.FLUID.2017.05.018.

[21] M.J. Kamlet, R.W. Taft, The solvatochromic comparison method. 1. The .beta.-scale of solvent hydrogen-bond acceptor (HBA) basicities, J. Am. Chem. Soc. 98 (1976) 377–383. doi:10.1021/ja00418a009.

[22] R.W. Taft, M.J. Kamlet, The solvatochromic comparison method. 2. The alpha-scale of solvent hydrogen-bond donor (HBD) acidities, J. Am. Chem. Soc. 98 (1976) 2886–2894. doi:10.1021/ja00426a036.

[23] M.J. Kamlet, J.L. Abboud, R.W. Taft, The solvatochromic comparison method. 6. The .pi.* scale of solvent polarities, J. Am. Chem. Soc. 99 (1977) 6027–6038. doi:10.1021/ja00460a031.

[24] H. Passos, T.B. V. Dinis, E. V. Capela, M. V. Quental, J. Gomes, J. Resende, P.P. Madeira, M.G. Freire, J.A.P. Coutinho, Mechanisms ruling the partition of solutes in ionic-liquid-based aqueous biphasic systems – the multiple effects of ionic liquids, Phys. Chem. Chem. Phys. 20 (2018) 8411–8422. doi:10.1039/C8CP00383A.

[25] J. G. Huddleston, A. Heather D. Willauer, R.D. Rogers, Phase diagram data for several PEG + salt aqueous biphasic systems at 25 °C, J. Chem. Eng. Data. 48 (2003) 1230–1236. doi:10.1021/JE034042P.

[26] S. Hamzehzadeh, A. Majouy, B. Mokhtarani, Effect of the ionic liquid 1-butyl-3-methylimidazolium bromide as an additive on the formation of {polyethylene glycol + tri-potassium phosphate} aqueous biphasic systems: the role of polymer molecular weight, J. Mol. Liq. 213 (2016) 235–246. doi:10.1016/J.MOLLIQ.2015.10.039.

[27] M.R. Almeida, H. Passos, M.M. Pereira, Á.S. Lima, J.A.P. Coutinho, M.G. Freire, Ionic liquids as additives to enhance the extraction of antioxidants in aqueous two-phase systems, Sep. Purif. Technol. 128 (2014) 1–10. doi:10.1016/J.SEPPUR.2014.03.004.

[28] J.G. Huddleston, H.D. Willauer, R.D. Rogers, The solvatochromic properties, α, β, and π*, of PEG-salt aqueous biphasic systems, Phys. Chem. Chem. Phys. 4 (2002) 4065–4070. doi:10.1039/B203018G.

[29] O. Rodríguez, S.C. Silvério, P.P. Madeira, J.A. Teixeira, E.A. Macedo, Physicochemical characterization of the PEG 8000-Na2SO4 aqueous two-phase system, Ind. Eng. Chem. Res. 46 (2007) 8199–8204. doi:10.1021/ie070473f.

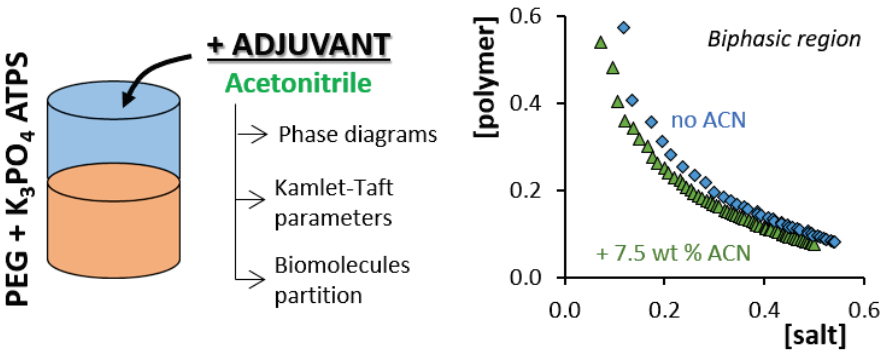
[30] Y. Marcus, Y. Migron, Polarity, hydrogen bonding, and structure of mixtures of water and cyanomethane, J. Phys. Chem. 95 (1991) 400–406.

doi:10.1021/j100154a070.

[31] ChemSpider - The free chemical database, (2015). www.chemspider.com.

[32] A.F.M. Cláudio, A.M. Ferreira, C.S.R. Freire, A.J.D. Silvestre, M.G. Freire, J.A.P. Coutinho, Optimization of the gallic acid extraction using ionic-liquid-based aqueous two-phase systems, Sep. Purif. Technol. 97 (2012) 142–149. doi:10.1016/J.SEPPUR.2012.02.036.

Graphical Abstract



Highlights

> Acetonitrile effect as an adjuvant is studied in PEG + K_3PO_4 + H_2O ATPS.

> Liquid-liquid demixing is improved with the increase of acetonitrile concentration.

> Acetonitrile influences the Kamlet-Taft solvent parameters of the coexisting phases.

> Phenolic compounds partition is changed by the addition of acetonitrile.