



## Acetonitrile as adjuvant to tune polyethylene glycol + K<sub>3</sub>PO<sub>4</sub> 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 20,000 g·mol<sup>-1</sup>) 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<sub>3</sub>PO<sub>4</sub> 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.

### 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 90s [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

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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,  $\pi^*$ , hydrogen-bond (acceptor) basicity,  $\beta$ , and hydrogen-bond (donor) acidity,  $\alpha$ , 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<sub>2</sub>O + ACN and 30 wt% of PEG 1500 + 8 wt% of  $K_3PO_4$  + H<sub>2</sub>O + ACN for the systems composed of PEG with a  $M_w = 1500 \text{ g}\cdot\text{mol}^{-1}$ ,  $K_3PO_4$ , and ACN as adjuvant at 0, 0.25, 2.5, 5 and 7.5 wt%. Aqueous solutions of protocatechuic acid and gallic acid at  $3.24 \times 10^{-3} \text{ mol}\cdot\text{L}^{-1}$  and  $2.94 \times 10^{-3} \text{ mol}\cdot\text{L}^{-1}$ , respectively, were prepared and used as part of the water content in each TL. Each-mixture was prepared gravimetrically within  $\pm 10^{-4} \text{ g}$ , vigorously stirred and centrifuged at 3500 rpm for 30 min and left to equilibrate in a water bath for 12 h and at  $(298 \pm 1) \text{ K}$  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, as 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):

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

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.

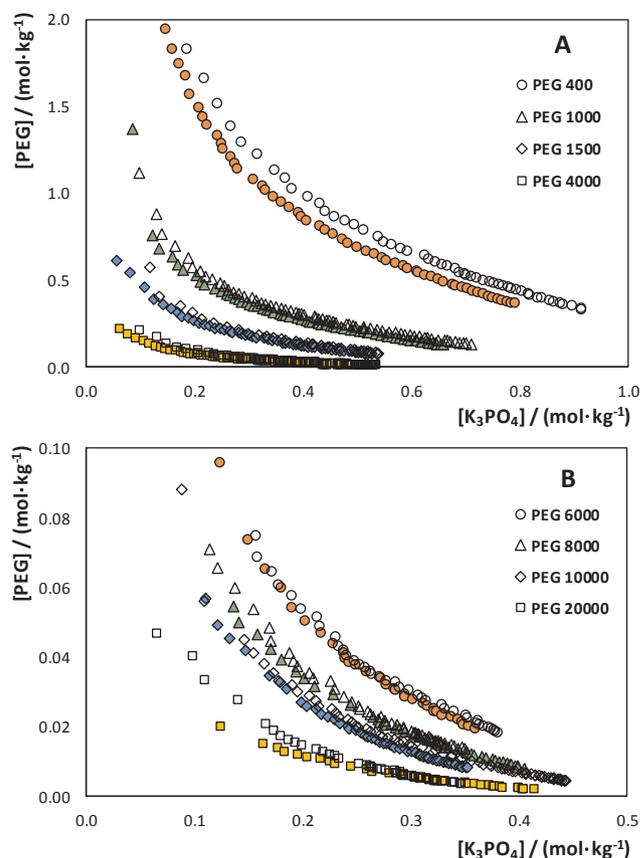


Fig. 2. The effect of ACN in ATPS composed of  $K_3PO_4$ , water and PEG with (A)  $M_w \leq 4000 \text{ g}\cdot\text{mol}^{-1}$  and (B)  $M_w > 4000 \text{ g}\cdot\text{mol}^{-1}$  at  $(298 \pm 1) \text{ K}$ : 0 wt% of ACN (open symbols); 5 wt% of ACN (full symbols).

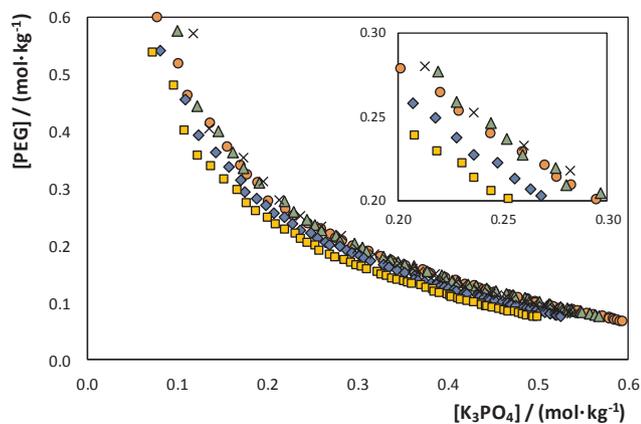
## 3. Results and discussion

### 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<sub>2</sub>O, without and with ACN at 5 wt%, were determined at  $(298 \pm 1) \text{ K}$  and atmospheric pressure and are represented in Fig. 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.

In Fig. 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 Fig. 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



**Fig. 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 (■).

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. Fig. 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  $M_w \geq 6000 \text{ g}\cdot\text{mol}^{-1}$ . The huge impact of the high hydrophobicity of PEG with  $M_w \geq 6000 \text{ g}\cdot\text{mol}^{-1}$  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 Fig. 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.

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 ( $\sigma$ ) 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.

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 Fig. 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

**Table 1**

Correlation parameters used to describe the experimental binodal data by Eq. (1) and respective standard deviation ( $\sigma$ ).

PEG	ACN (wt %)	$A \pm \sigma$	$B \pm \sigma$	$10^5 \cdot (C \pm \sigma)$	$R^2$
400	0	$111 \pm 3$	$-0.48 \pm 0.02$	$6 \pm 1$	0.996
1000	0	$113 \pm 3$	$-0.55 \pm 0.01$	$10 \pm 2$	0.998
4000	0	$146 \pm 7$	$-0.79 \pm 0.02$	$66 \pm 5$	0.998
6000	0	$152 \pm 22$	$-0.89 \pm 0.08$	$64 \pm 21$	0.997
8000	0	$96 \pm 3$	$-0.63 \pm 0.02$	$208 \pm 17$	0.998
10,000	0	$153 \pm 11$	$-0.90 \pm 0.04$	$133 \pm 13$	0.996
20,000	0	$106 \pm 9$	$-0.63 \pm 0.06$	$354 \pm 31$	0.995
400	5	$114 \pm 18$	$-0.52 \pm 0.06$	$10 \pm 6$	0.963
1000	5	$135 \pm 10$	$-0.65 \pm 0.04$	$10 \pm 6$	0.982
4000	5	$107 \pm 2$	$-0.71 \pm 0.01$	$84 \pm 4$	0.999
6000	5	$183 \pm 10$	$-1.00 \pm 0.03$	$48 \pm 14$	0.999
8000	5	$108 \pm 7$	$-0.75 \pm 0.03$	$152 \pm 9$	0.999
10,000	5	$202 \pm 47$	$-1.1 \pm 0.1$	$121 \pm 59$	0.981
20,000	5	$178 \pm 19$	$-1.07 \pm 0.07$	$118 \pm 28$	0.995
1500	0	$124 \pm 12$	$-0.67 \pm 0.05$	$22 \pm 9$	0.993
1500	0.25	$97 \pm 3$	$-0.56 \pm 0.01$	$33 \pm 2$	0.999
1500	2.5	$110 \pm 4$	$-0.62 \pm 0.02$	$23 \pm 4$	0.998
1500	5	$89 \pm 2$	$-0.55 \pm 0.01$	$41 \pm 3$	0.998
1500	7.5	$88 \pm 3$	$-0.57 \pm 0.02$	$45 \pm 5$	0.998

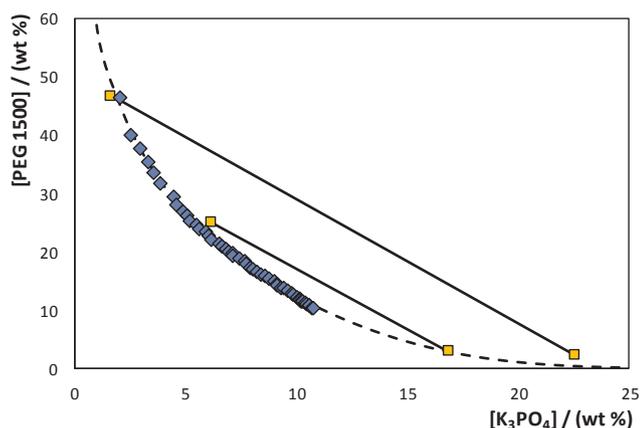
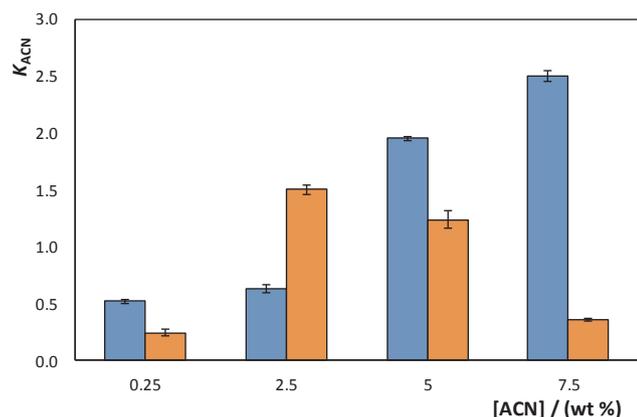
material it is possible to find the representation of the remaining systems. The pH of the coexisting phases was also determined and is given in Table 2. Due to the presence of  $K_3PO_4$  in their composition, the phases of these systems present alkaline pH, ranging between 11.8 and 13, with almost no differences between the bottom and top phases.

### 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 Fig. 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 Fig. 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  $K = 1.50 \pm 0.04$  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.

**Table 2**Experimental data for TLs and TLLs of PEG 1500 + K<sub>3</sub>PO<sub>4</sub> + ACN + H<sub>2</sub>O ATPS at 298 K, and pH values of each phase.

ACN (wt %)	Top Phase (wt %)				Bottom Phase (wt %)				TLL
	[PEG]	[K <sub>3</sub> PO <sub>4</sub> ]	[ACN]	pH	[PEG]	[K <sub>3</sub> PO <sub>4</sub> ]	[ACN]	pH	
<b>20 wt% of PEG 1500 + 8 wt% of K<sub>3</sub>PO<sub>4</sub></b>									
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
<b>30 wt% of PEG 1500 + 8 wt% of K<sub>3</sub>PO<sub>4</sub></b>									
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

**Fig. 4.** Phase diagram for the quaternary system composed of PEG 1500 + K<sub>3</sub>PO<sub>4</sub> + 2.5 wt% ACN + H<sub>2</sub>O, at 298 K and atmospheric pressure: binodal curve data (blue ♦), TL (—) and TL phase composition (yellow ■), adjusted binodal data through Eq. (1) (---).**Fig. 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<sub>3</sub>PO<sub>4</sub> + H<sub>2</sub>O (blue) and 20 wt% of PEG 1500 + 8 wt% of K<sub>3</sub>PO<sub>4</sub> + H<sub>2</sub>O (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 ( $\pi^*$ ), solvent hydrogen-bond acidity ( $\alpha$ ), and solvent hydrogen-bond basicity ( $\beta$ ) [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<sub>3</sub>PO<sub>4</sub> + H<sub>2</sub>O 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  $\pi^*$ ,  $\alpha$ , and  $\beta$ , as well as their respective differences ( $\Delta$ , calculated as the difference between the parameter values in the PEG- and the salt-rich phases) are presented in Table 3.

Considering the results obtained in the mixture point 20 wt% of PEG 1500 + 8 wt% of K<sub>3</sub>PO<sub>4</sub>, 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  $\pi^*$  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  $\pi^*$  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 Fig. 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  $\pi^*$  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. Fig. 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

**Table 3**Kamlet-Taft parameters and their differences ( $\Delta$ ) between the coexisting phases of PEG 1500 + K<sub>3</sub>PO<sub>4</sub> ATPS at 278 K.

ACN (wt %)	$\beta_{PEG}$	$\beta_{salt}$	$\Delta\beta$	$\pi_{PEG}^*$	$\pi_{salt}^*$	$\Delta\pi^*$	$\alpha_{PEG}$	$\alpha_{salt}$	$\Delta\alpha$
<b>20 wt% of PEG 1500 + 8 wt% of K<sub>3</sub>PO<sub>4</sub></b>									
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
<b>30 wt% of PEG 1500 + 8 wt% of K<sub>3</sub>PO<sub>4</sub></b>									
0	0.55	n.s. <sup>a</sup>	−	1.14	n.s. <sup>a</sup>	−	1.15	1.29	−0.14
0.25	0.49	n.s. <sup>a</sup>	−	1.17	n.s. <sup>a</sup>	−	1.22	1.33	−0.11
2.5	0.53	n.s. <sup>a</sup>	−	1.11	n.s. <sup>a</sup>	−	1.15	1.31	−0.16
5	0.51	n.s. <sup>a</sup>	−	1.13	n.s. <sup>a</sup>	−	1.20	1.31	−0.11
7.5	0.49	n.s. <sup>a</sup>	−	1.09	n.s. <sup>a</sup>	−	1.14	1.31	−0.17

<sup>a</sup> Not soluble (n.s.).

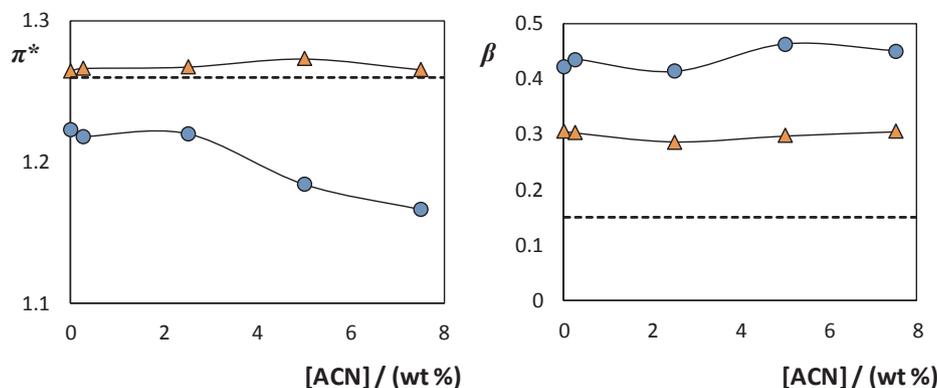


Fig. 6. Kamlet-Taft parameters for ATPS composed of 20 wt% of PEG 1500 + 8 wt% of  $K_3PO_4$  + ACN +  $H_2O$  at different ACN concentrations: salt-rich phase (orange triangles); PEG-rich phase (blue circles); water (dashed line).

system.

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 Fig. 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  $\beta$  and  $\pi^*$  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 Fig. 7, and more details are given in the Supplementary material.

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 ( $pH \cong 12$ , 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 Fig. 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.

## 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  $M_w \geq 6000 \text{ g}\cdot\text{mol}^{-1}$ . 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.

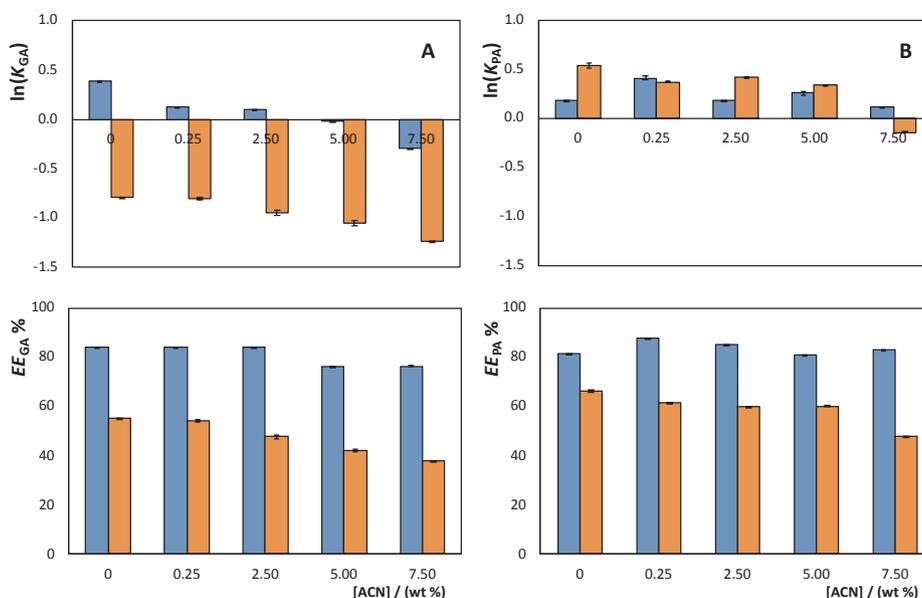


Fig. 7. Partition coefficient ( $K$ ) and extraction efficiency percentages ( $EE$ ) of (A) gallic acid (GA) and (B) protocatechuic acid (PA) in ATPS composed of 30 wt% of PEG 1500 + 8 wt% of  $K_3PO_4$  +  $H_2O$  (blue bars) and 20 wt% of PEG 1500 + 8 wt% of  $K_3PO_4$  +  $H_2O$  (orange bars) at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

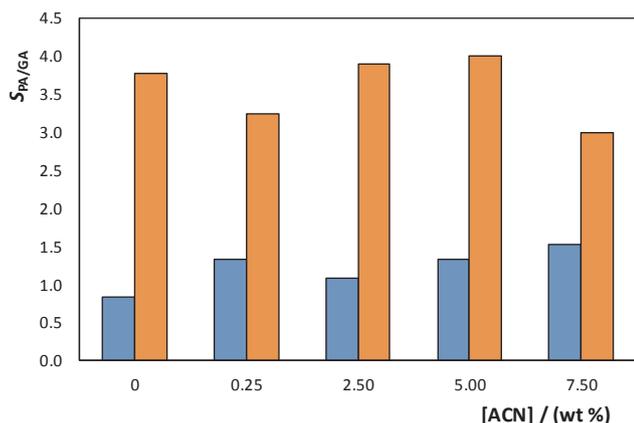


Fig. 8. The effect of ACN on the selectivity ( $S_{PA/GA}$ ) of ATPS composed of 30 wt % of PEG 1500 + 8 wt% of  $K_3PO_4$  +  $H_2O$  (blue bars) and 20 wt% of PEG 1500 + 8 wt% of  $K_3PO_4$  +  $H_2O$  (orange bars) for protocatechuic acid (PA) and gallic acid (GA).

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.seppur.2019.04.062>.

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