

Effect of different organic salts on amino acids partition behaviour in PEG-salt ATPS



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ABSTRACT

The phase diagrams of six different polyethylene glycol (PEG)–salt aqueous two-phase systems (ATPS) were measured at $T = 298.15$ K and $p = 1$ bar. PEG of different molecular weight (4000, 6000 and 8000) and organic salts (potassium citrate, potassium sodium tartrate) were used to form the biphasic systems. The results were compared with data for PEG–sodium citrate ATPS, reported in the literature (PEG 4000, PEG 6000) and measured in this work (PEG 8000). The partition of four dinitrophenyl-amino acids was measured in these ATPS for different tie-line lengths. Based on these data, cation and anion effects were evaluated in terms of the relative hydrophobicity of the phases using $G^*(CH_2)$ calculations. The distribution coefficients have been obtained spectrophotometrically. Studies on the partitioning indicate the advantage of citrate salts over tartrate salts as well as sodium-based salts over potassium-based salts. This consistently results from the (liquid-liquid) phase behaviour of these systems.

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1 Introduction

The purification of biomolecules in aqueous two-phase systems (ATPS) [1] can assist in the mitigation of the two major problems that often occur when conventional separation techniques and extraction solvents are used—loss of biomolecular activity of the biocomponents and production of harmful waste. The environmental risk of classical ATPS formed by polymer and inorganic salt [2–4] can only overcome by the development, evaluation and application of new systems based on biodegradable components.

Polyethylene glycol (PEG) is a safe hydrophilic polymer, which is commonly used as a phase former in polymer-salt ATPS [5]. It can be combined with few particular non-toxic organic salts, such as citrates [6–9] or tartrates [10–12]. These biodegradable salts are commonly used as food additives and present wide range of applications [13–16]. Studies on phase separation in PEG–potassium citrate [6,8,12] and PEG–potassium sodium tartrate [11,12] show very similar, and large, liquid immiscibility regions.

It has already been reported that aqueous PEG–organic salt systems are very adequate for the partitioning of some components, e.g. amino acids [17], proteins [18–20], enzymes [21], pro-enzymes [22], antibiotics [23], among others. However, the limited comprehension of the molecular mechanisms behind the solute partitioning still narrows the application of ATPS at a large industrial scale. Through a better knowledge of these mechanisms that control the partitioning process, it is possible to further predict the partitioning behaviour in ATPS; however, this requires a significant contribution of experimental work.

Some authors have already studied the impact of PEG molecular weight on the (liquid-liquid) equilibrium (LLE) [8,12,24] and on the partitioning of different solutes [17,25,26] in PEG–salt ATPS. However, in terms of partitioning of amino acids, it has been reported that PEG molecular weight must be significantly lower than 4 kDa to observe beneficial influence on the partition coefficient [17]. Therefore, in this work more attention was given to the influence of salt constituents on the partitioning, namely cations and anions.

In this study, the relative hydrophobicities of the equilibrium phases of ATPS composed by PEG (with molecular weights of 4000, 6000, and 8000) and organic salt (potassium citrate, potassium

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sodium tartrate) were characterized using partition coefficients measured at three different tie-line compositions [8,12], for four dinitrophenyl (DNP) amino acids - n-(2,4-dinitrophenyl)-glycine, n-(2,4-dinitrophenyl)-L-alanine, n-(2,4-dinitrophenyl)-DL-norvaline, and n-(2,4-dinitrophenyl)-DL-norleucine. The results were collated with partitioning data published for systems composed by PEG (4000, 6000) and sodium citrate [17]. For the complete comparison, partition coefficients of the same DNP-amino acids for system formed by PEG 8000 and sodium citrate were also measured. The partitioning was evaluated using $G^*(CH_2)$ calculations. To the best of our knowledge, this is the first study, where hydrophobicity of the phases, in terms of Gibbs energy, for the ATPS composed by tartrate salt is evaluated, and the first one comparing the cation and anion effect of the salts (citrates and tartrates), on the partitioning of DNP-amino acids.

Table 1

Suppliers and purity of the compounds used in the experiments.

Compound	Supplier	Purity
Potassium citrate tribasic monohydrate	Sigma-Aldrich	≥99%
Potassium sodium tartrate tetrahydrate	Sigma-Aldrich	≥99%
N-(2,4-dinitrophenyl)glycine	Sigma	≥99%
N-(2,4-dinitrophenyl)-L-alanine	Research Organics	≥97%
N-(2,4-dinitrophenyl)-DL-n-valine	Sigma	≥97%
N-(2,4-dinitrophenyl)-DL-n-leucine	Research Organics	≥97%

2 Experimental section

2.1. Materials

Polyethylene glycol (PEG), with an average molecular weight (M_w) of 4000 (LOT BCBP8299V), M_w of 6000 (LOT BCBQ5326V) and M_w of 8000 (LOT SLBJ5906V), potassium citrate tribasic monohydrate ($K_3C_6H_5O_7 \cdot H_2O$, ACS reagent, ≥ 99%, $M_w = 324.41$ g/mol) and potassium sodium tartrate tetrahydrate ($C_4H_4KNaO_6 \cdot H_2O$, ACS reagent, ≥ 99%, $M_w = 282.23$ g/mol) were purchased from Sigma-Aldrich. All stock solutions were prepared in double-distilled deionized water. The concentrations of PEG and salt were measured gravimetrically and calculated from PEG/salt dry mass, obtained using a freeze-dryer (Scan Vac, CoolSafe 55–4) for PEG and potassium sodium tartrate, or after evaporating on a heating plate (Stuart hot plate SB300), for potassium citrate. DNP-amino acids: n-(2,4-dinitrophenyl)-glycine (DNP-Gly), n-(2,4-dinitrophenyl)-L-alanine (DNP-Ala), n-(2,4-dinitrophenyl)-DL-norvaline (DNP-Val), n-(2,4-dinitrophenyl)-DL-norleucine (DNP-Leu) were obtained from Sigma and Research Organics. For the solute solutions preparations (0.2 wt%) and for the dilution purposes, double-distilled deionized water was used.

All products were used as received without further purification. The purities of the compounds, together with the names of suppliers, are presented in Table 1. All weighting was carried out on an

Table 2Weight-fraction based feed compositions [9,12,17], tie-line lengths (TLL) and partition coefficients, K_i , determined for four DNP-amino acids, at $T = 298.15$ K and $p = 0.1$ MPa.^{a,b}

Feed		TLL	K _i							
W _{PEG}	W _{SALT}		DNP-Glycine		DNP-Alanine		DNP-Valine		DNP-Leucine	
PEG4000 K ₃ Citrate										
0.164	0.139	0.337	8.42	±0.01	10.53	±0.01	17.35	±0.03	24.04	±0.02
0.169	0.143	0.363	9.18	±0.01	12.80	±0.05	20.21	±0.04	30.50	±0.04
0.179	0.152	0.403	12.77	±0.03	16.60	±0.03	29.01	±0.03	47.23	±0.13
PEG6000 K ₃ Citrate										
0.175	0.141	0.381	9.52	±0.04	11.49	±0.03	18.70	±0.05	28.99	±0.08
0.181	0.145	0.399	10.58	±0.06	14.07	±0.04	22.79	±0.01	36.02	±0.02
0.186	0.150	0.417	12.72	±0.08	15.88	±0.02	27.80	±0.17	45.30	±0.12
PEG8000 K ₃ Citrate										
0.129	0.145	0.310	6.39	±0.17	8.01	±0.01	12.72	±0.01	16.52	±0.04
0.140	0.164	0.381	9.75	±0.03	13.31	±0.04	21.04	±0.03	30.57	±0.05
0.150	0.184	0.443	15.35	±0.04	19.99	±0.08	37.14	±0.12	54.02	±0.20
PEG4000 KNaTartrate										
0.162	0.123	0.246	3.76	±0.01	4.55	±0.01	6.58	±0.01	8.21	±0.01
0.168	0.127	0.288	4.64	±0.01	5.53	±0.01	8.55	±0.01	11.04	±0.02
0.175	0.134	0.333	5.68	±0.01	7.18	±0.01	11.68	±0.01	15.33	±0.08
PEG6000 KNaTartrate										
0.164	0.116	0.248	3.90	±0.02	4.48	±0.01	6.49	±0.02	8.39	±0.01
0.172	0.123	0.301	4.81	±0.01	5.80	±0.02	8.87	±0.03	11.75	±0.01
0.178	0.128	0.332	5.41	±0.01	6.77	±0.01	10.52	±0.02	14.48	±0.01
PEG8000 KNaTartrate										
0.150	0.114	0.240	3.49	±0.01	4.23	±0.01	5.84	±0.01	7.16	±0.01
0.159	0.120	0.290	4.28	±0.01	5.07	±0.03	7.73	±0.01	9.76	±0.01
0.170	0.129	0.335	5.53	±0.01	7.09	±0.03	10.54	±0.01	14.61	±0.02
PEG4000 Na ₃ Citrate ^c										
0.143	0.099	0.260	6.64	±0.04	7.86	±0.04	11.84	±0.14	15.90	±0.14
0.150	0.104	0.298	7.98	±0.16	9.48	±0.11	14.86	±0.16	21.66	±0.15
0.158	0.110	0.334	9.84	±0.15	11.57	±0.15	18.34	±0.13	29.46	±0.20
PEG6000 Na ₃ Citrate ^c										
0.135	0.095	0.253	5.62	±0.08	7.11	±0.10	9.97	±0.11	13.47	±0.08
0.143	0.100	0.290	6.77	±0.04	8.67	±0.08	12.53	±0.10	17.80	±0.10
0.156	0.110	0.341	9.61	±0.05	12.49	±0.12	18.56	±0.13	30.00	±0.21
PEG8000 Na ₃ Citrate										
0.120	0.110	0.266	7.25	±0.01	8.45	±0.04	13.46	±0.03	17.36	±0.03
0.130	0.121	0.320	9.87	±0.03	12.12	±0.08	20.02	±0.03	26.86	±0.01
0.140	0.130	0.361	12.76	±0.04	15.95	±0.08	27.73	±0.08	38.39	±0.06

^a Standard uncertainties u are $u(T) = 0.2$ K, $u(p) = 0.005$ MPa, $u(w_i) = 0.002$ (mass fractions), $u(K_i) = 0.20$.

^b Mass ratio of DNP-amino acid to total mass ATPS from 4×10^{-5} to 2×10^{-4} .

^c Partition coefficients reported in the literature [17].

Adam Equipment balance model AAA250L, with an uncertainty of ± 0.2 mg.

2.2. Partitioning of DNP-Amino acids in ATPS

Partitioning of DNP-amino acids in aqueous two-phase systems was performed according to the procedure published previously [27]. The appropriate amounts of polymer (PEG 4000/PEG 6000/PEG 8000) stock solution and salt (potassium citrate/potassium sodium tartrate) stock solution were weighed. An adequate amount

of water was added to achieve a total mass of 1 g. Feed compositions of the tie-lines used for the partitioning studies are presented in Table 2. For each tie-line, six replicates with the same feed composition were prepared and different amounts of the solute (DNP-amino acid) stock solutions (20–100 mg) and water (100–20 mg) were added. An automatic pipette (Multipipette® XStream, Eppendorf) was used to add all the components. The tubes were vigorously shaken on a vortex mixer for 2 min, and then centrifuged (Minispin, Eppendorf) at 13.4×10^3 rpm for 15 min to achieve complete phase separation. ATPS were incubated for approximately

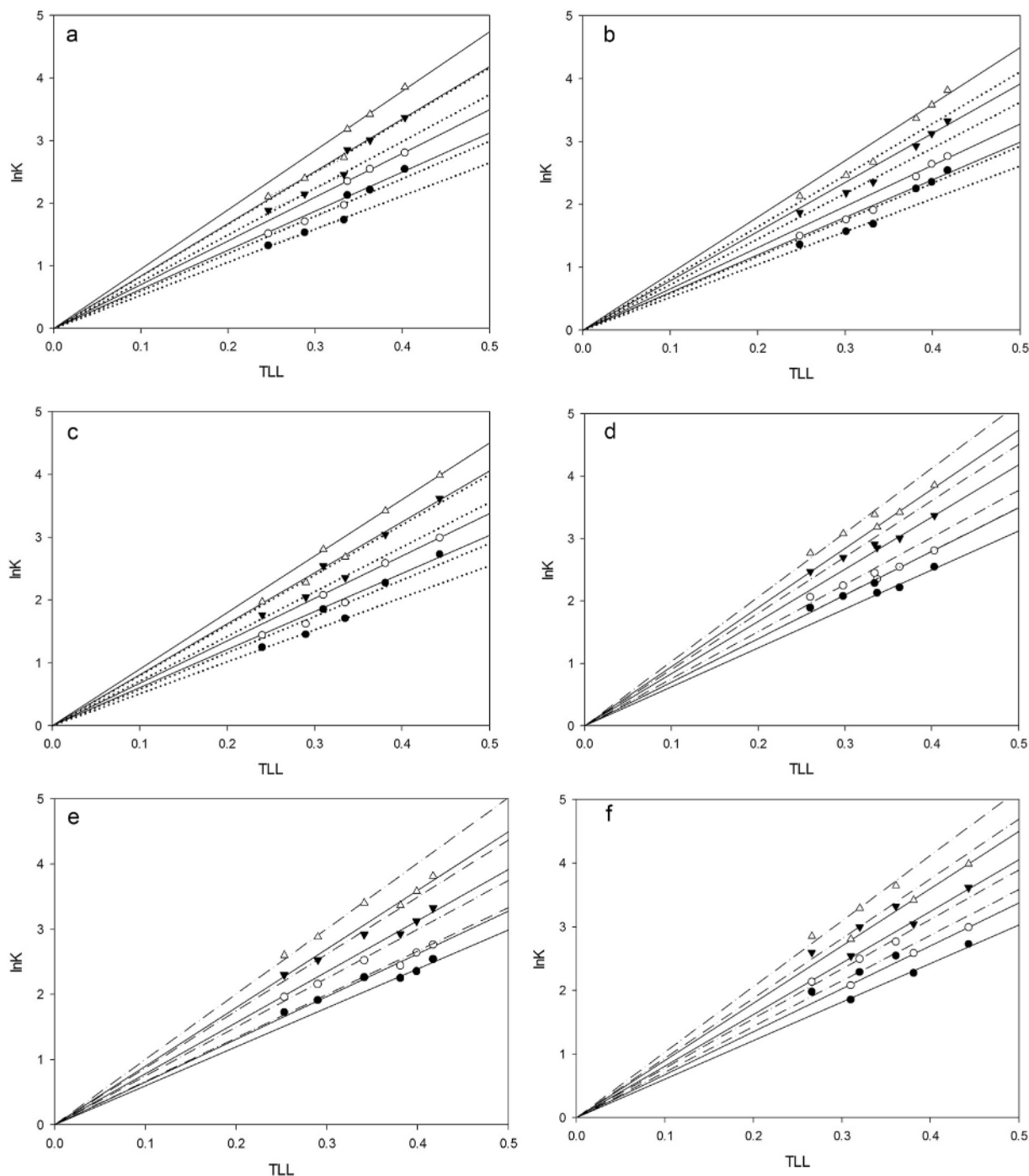


Fig 1 Logarithm of partition coefficients of the DNP-amino acids (● Gly; ○ Ala; ▼ Val; (b,e), PEG 8000 (c,f) and organic salt: potassium citrate (—), potassium sodium tartrate (— — —), compared to data obtained for sodium citrate (— — —) and polymer: PEG 8000 (this work), PEG (4000, 6000) from the literature [17].

Leu) as a function of TLL for the ATPS formed by polymer: PEG 4000 (a,d), PEG 6000 (b,e), PEG 8000 (c,f) and organic salt: potassium citrate (—), potassium sodium tartrate (— — —), compared to data obtained for sodium citrate (— — —) and polymer: PEG 8000 (this work), PEG (4000, 6000) from the literature [17].

2 h, at $T = 298.15$ K, controlled with air conditioning and a thermostatic bath (Techne, Tempette TE-8D), for the total interface clarification. Samples from top and bottom phases were withdrawn and to avoid interferences from phase forming components, they were conveniently diluted with water. Solute quantification in each phase was performed by absorbance measurements at 362 nm, using UV–Vis spectrophotometer (Thermo Scientific Varioskan Flash), as reported in the literature [17,27,35].

Partition coefficients (K_i) for the four DNP-amino acids were determined as the slope of the straight lines from the representation of the absorbance in the top phase (PEG-rich) versus the absorbance in the bottom phase (salt-rich). They were corrected with the respective dilution factors (DF, volume fraction of final volume to initial volume) [27]:

$$K_i = \frac{Abs(top) \cdot DF_{top}}{Abs(bottom) \cdot DF_{bottom}} \quad (1)$$

The K_i values presented in this work are weight fraction-based.

3 Results and discussion

The feed composition and the partition coefficients obtained for four DNP-amino acids using the previously reported tie-lines [9,12] are presented in Table 2. The partition coefficients, K_i , were described in the previous section and calculated according to equation (1). Coefficients of determination, r^2 , for each set of six replicates with different solute concentration, were higher than

0.990 (average $r^2 = 0.999$). Additionally, there are no interactions that interfere on the partitioning process, as all constant b values (intercept), obtained after linear regression for each partition coefficient versus TLL (defined in equation (2)), are close to zero (average $b = 0.07$), as it can be observed from Fig. 1(a–f).

Table 2 also contains the values of the tie-line lengths, TLL, which are obtained from the phase composition:

$$TLL = \sqrt{(w_{PEG}^T - w_{PEG}^B)^2 + (w_{salt}^T - w_{salt}^B)^2} \quad (2)$$

where w_{salt} and w_{PEG} are respectively salt and polymer mass fractions.

Since the distribution coefficient strongly depends on the ATPS composition, Fig. 1 presents the linear function of its logarithm on TLL, which can be expressed as:

$$\ln K_i = \alpha \cdot TLL \quad (3)$$

where α is a constant value that has influence on the composition at equilibrium. It can be seen that the highest distribution coefficients are obtained for the ATPS composed by sodium citrate, if comparing the salt effect. The partition coefficients follow the order: $K_{LEU} > K_{VAL} > K_{ALA} > K_{GLY}$, which is in agreement with previous studies for different systems [17,27,28,35] and can be explained using a hydrophobicity scale for amino acids. The more hydrophobic an amino acid the larger the affinity for the more hydrophobic PEG-rich phase, which consequently leads to higher K_i

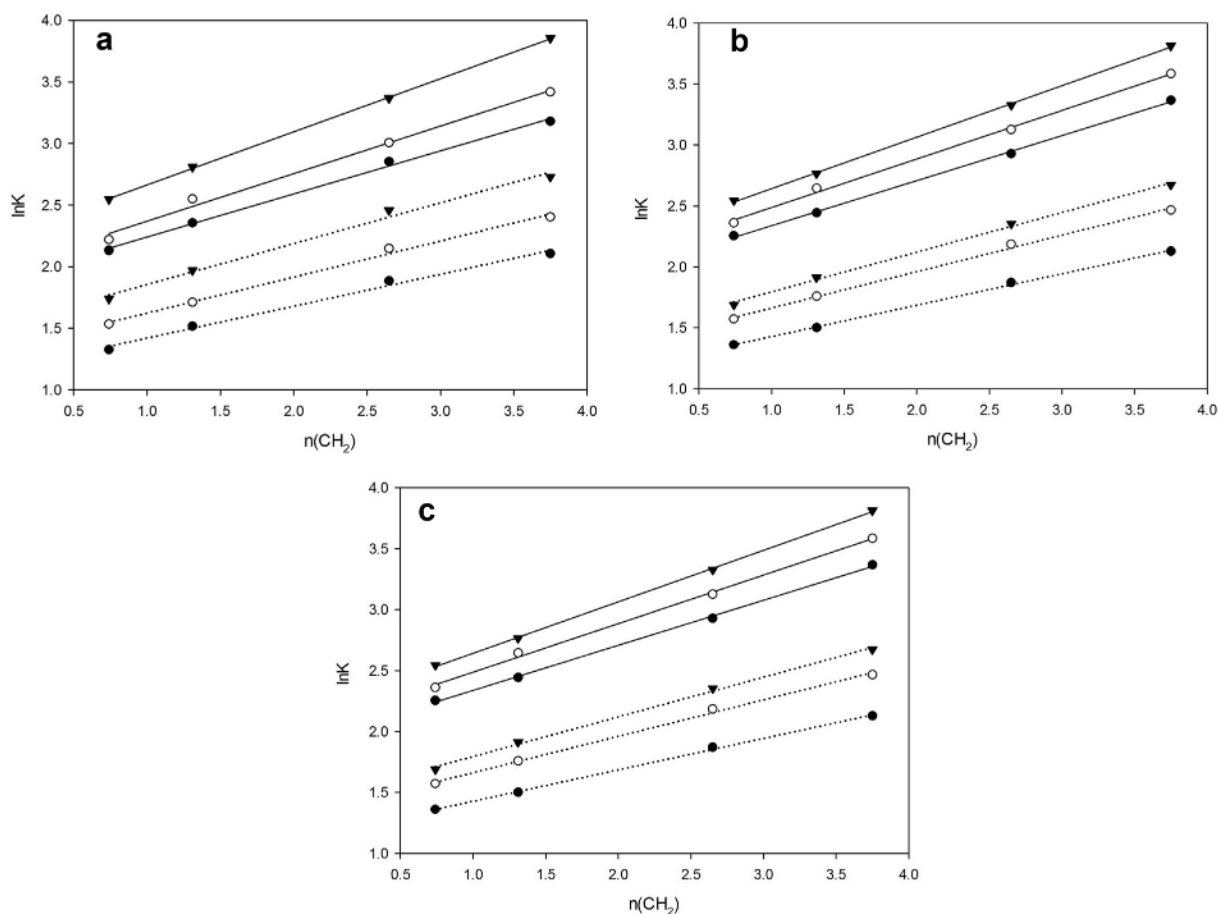


Fig. 2 Logarithm of the distribution coefficients of the DNP-amino acids, in polymer: PEG 4000 (a), PEG 6000 (b), PEG 8000 (c)–organic salt: potassium citrate (—), potassium sodium tartrate (---) ATPS, as a function of the average number of methylene groups. Tie lines: ● (1), ○ (2), ▼ (3).

Table 3
Values of $G^*(CH_2)$ and parameters C and E determined for the systems studied.

System	TLL	C	E	r^2	$G^*(CH_2)$ [kJ/mol]
PEG4000 K₃Citrate					
	0.337	1.890	0.350	0.9968	−0.868 ±0.035
	0.363	1.981	0.386	0.9921	−0.958 ±0.061
	0.403	2.233	0.431	0.9998	−1.070 ±0.012
PEG6000 K₃Citrate					
	0.381	1.965	0.370	0.9990	−0.918 ±0.021
	0.399	2.087	0.398	0.9977	−0.987 ±0.033
	0.417	2.220	0.422	0.9995	−1.047 ±0.016
PEG8000 K₃Citrate					
	0.310	1.651	0.317	0.9909	−0.786 ±0.053
	0.381	2.051	0.370	0.9926	−0.917 ±0.056
	0.443	2.440	0.422	0.9952	−1.047 ±0.051
PEG4000 KNaTartrate					
	0.246	1.160	0.259	0.9916	−0.642 ±0.042
	0.288	1.330	0.292	0.9948	−0.725 ±0.037
	0.333	1.523	0.332	0.9907	−0.822 ±0.056
PEG6000 KNaTartrate					
	0.248	1.170	0.258	0.9989	−0.639 ±0.015
	0.301	1.363	0.298	0.9973	−0.740 ±0.027
	0.332	1.470	0.325	0.9975	−0.807 ±0.029
PEG8000 KNaTartrate					
	0.240	1.108	0.236	0.9895	−0.585 ±0.043
	0.290	1.263	0.278	0.9931	−0.689 ±0.041
	0.335	1.508	0.316	0.9953	−0.784 ±0.038
PEG4000 Na₃Citrate^a					
	0.260	1.467	0.253	0.9993	−0.627 ±0.011
	0.298	1.682	0.292	0.9992	−0.724 ±0.015
	0.334	1.822	0.333	0.9996	−0.825 ±0.011
PEG6000 Na₃Citrate^a					
	0.253	1.552	0.282	0.9934	−0.699 ±0.040
	0.290	1.711	0.312	0.9951	−0.773 ±0.038
	0.341	2.006	0.365	0.9999	−0.905 ±0.054
PEG8000 Na₃Citrate					
	0.266	1.762	0.298	0.9934	−0.740 ±0.043
	0.320	2.056	0.337	0.9946	−0.836 ±0.043
	0.361	2.287	0.371	0.9949	−0.921 ±0.047

^a Data reported in the literature [17].

values [29]. The relative hydrophobicity of the amino acids residues was estimated by Zaslavsky et al. [30], as an average equivalent number of methylene units, $n(CH_2)$, and is different from real alkyl chain length. The positive value of $n(CH_2)$ means that a solute is hydrophobic and its relative hydrophobicity is equal to the one with n units of CH_2 groups. Values of $n(CH_2)$ are plotted in Fig. 2 together with the logarithms of partition coefficients, $\ln K$. Linearity observed in Fig. 2 can be explained using the following equation [31]:

$$\ln K_i = C + E n(CH_2) \quad (4)$$

where $\ln K_i$ is the distribution coefficient obtained for DNP-amino acid, $n(CH_2)$ is the equivalent number of methylene units of a solute. Constants C, E, which are listed in Table 3, represent the total contribution of the non-alkyl part of the DNP-amino acid structure and the contribution of CH_2 to the partition coefficient, respectively. The values of C, E against the TLL are plotted in Fig. 3. They are obtained by linear regression and represent the intercept (C) and the slope (E) of each linear curve. Coefficient E is a measure of the difference between the hydrophobic characters of the two phases. As the distribution coefficient was determined as a ratio of the absorbance in the top phase, versus the absorbance in the bottom phase, positive values of the constant E confirm the partitioning of DNP-amino acids to the upper phase. It can be also seen from Fig. 3 that salt type has higher contribution to coefficient C, than PEG molecular weight (within the range of molecular weight studied in this work).

The Gibbs free energy of transfer of a solute i , between the coexisting phases can be described as follows [32]:

$$G = -RT \ln K_i \quad (5)$$

where R is the universal gas constant and T is the absolute temperature [K].

By introducing parameter E into equation (5), the Gibbs energy of transfer of a methylene group from one phase to another, $G^*(CH_2)$, can be calculated:

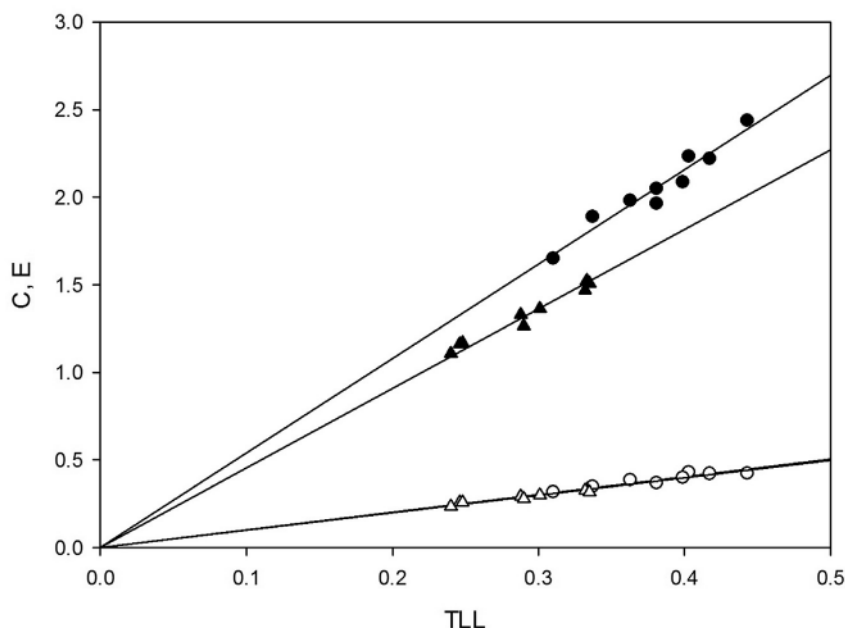


Fig. 3 C, E coefficients obtained in this work at $T = 298.15$ K for ATPS; Systems of PEG (4000, 6000, 8000) and organic salt: potassium citrate (● C, ○ E), potassium sodium tartrate (▲ C, ▲ E).

$$G(\text{CH}_2) = -\text{RTE} \quad (6)$$

The coefficient E represents an average $\ln K$ increment per CH_2 group [30].

$$\frac{\ln K}{E} = \frac{G}{G(\text{CH}_2)} = n(\text{CH}_2) \quad (7)$$

The values for $G^*(\text{CH}_2)$, which are presented in Table 3, are negative, proving that the PEG-rich phase has higher hydrophobicity.

The Gibbs free energy of transfer of ATPS studied in this work

and also reported in the literature [17,35] is plotted against the TLL in Fig. 4 (a, b, c). Regarding the cation effect, larger relative hydrophobicity is observed for ATPS formed by salts with sodium cation compared to potassium. A double salt of tartaric acid, which contains both cations, has the lowest relative hydrophobicity, what can be explained by a stronger anion effect. That is, in terms of hydrophobic effect, systems composed by PEG and citrate salt can be considered to be a better media for amino acids separation, in comparison to PEG–tartrate salt ATPS.

In order to support these conclusions, Fig. 4 (d, e, f) presents the binodal curves for the systems studied in this work and published in the literature [12,17,36]. All the figures present consistent

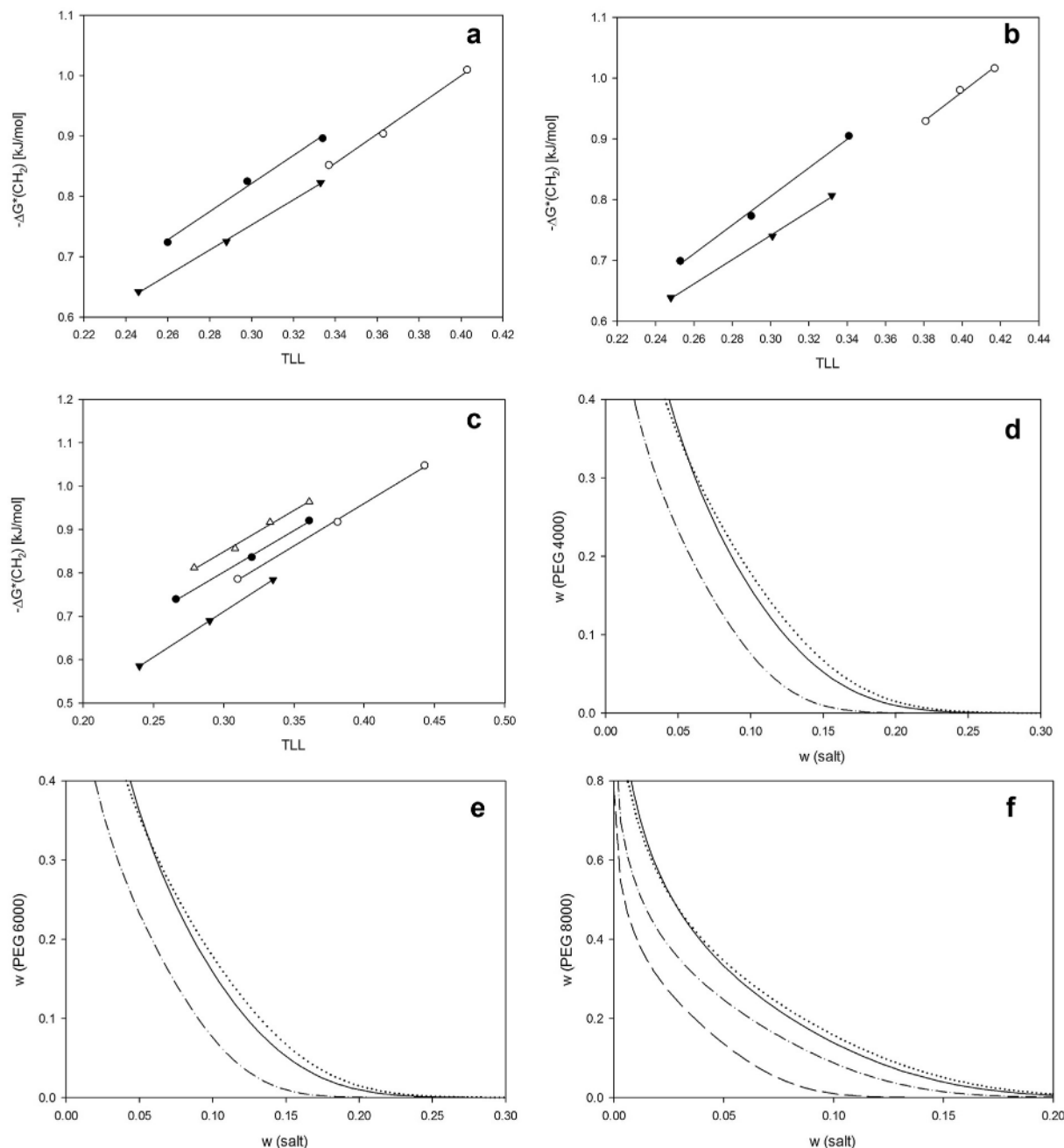


Fig. 4 Values of $G(\text{CH}_2)$ as a function of the TLL for systems: PEG 4000 (a), PEG 6000 (b), PEG 8000 (c) and potassium citrate (\circ), potassium sodium tartrate (\blacktriangleright), this work, sodium citrate (\bullet), sodium sulfate (\circ), at $T = 298.15$ K, data obtained within this work and [17,35]; corresponding binodal curves for ATPS formed by PEG 4000 (d), PEG 6000 (e), PEG 8000 (f) and potassium citrate (—), potassium sodium tartrate (---), sodium citrate (— · —), at $T = 298.15$ K [12,17] and PEG 8000 + sodium sulfate (—), at $T = 296.15$ K [36].

tendency with the biphasic diagrams and follow the same order (from the least hydrophobic to the most hydrophobic): PEG–potassium sodium tartrate, PEG–potassium citrate, PEG–sodium citrate. Larger two-phase regions are observed for ATPS formed by salt with sodium cation and citrate anion. The cation effect is related to the salting-out effect and follows the Hofmeister series [33]. Sodium cation presents better ability to form ATPS than potassium cation.

To better compare the anion effect of the salt on the partitioning process, Fig. 4 also presents $G(\text{CH}_2)$ values versus TLL (c) and binodal curve (f) for the system PEG 8000 with inorganic salt (sodium sulfate). It is important to mention that the phase diagram for system of PEG 8000–sodium sulfate, measured at $T = 296.15 \text{ K}$ and $p = 1 \text{ bar}$, was taken for the salt effect evaluation. However, the difference is not significant for this type of comparison and the binodal data fits to the LLE phase compositions measured at $T = 298.15 \text{ K}$ and $p = 1 \text{ bar}$ and reported in the literature [34]. Fig. 4 (c, f) shows that PEG 8000–sodium sulfate ATPS present the largest liquid–liquid immiscibility region and the highest values for relative hydrophobicity of all the systems studied in this work. Still, its efficiency, in terms of partitioning, is not very different from ATPS formed by PEG and sodium citrate. Thus, the inorganic potassium-based salt (which can cause harm to the environment) can be replaced with organic-based salt in ATPS without loss of efficiency.

In this work the partitioning data was evaluated using three different PEG molecular weights (4000, 6000, 8000), to compare with other results from the literature. However, the dependence of K_i values on PEG within the studied range of PEG molecular weight are within experimental uncertainty. Thus, evaluating the relative hydrophobicity of these systems in terms of PEG molecular weight does not allow conclusions.

4 Conclusions

In this work, partition coefficients for four DNP-amino acids, in six ATPS composed by PEG (4000, 6000, 8000) and organic salt (potassium citrate, potassium sodium tartrate) were determined. The results were compared with data for PEG–sodium citrate ATPS, found in the literature (PEG 4000, PEG 6000) [17] and measured in this work (PEG 8000). Cation and anion effect on the partitioning of a solute was discussed and evaluated in terms of the relative hydrophobicity. The results of $G^*(\text{CH}_2)$ calculations and the values of partition coefficients lead to the conclusion that higher extraction efficiency is observed for the systems composed by salt with sodium cation and citrate anion. These trends are in agreement with the binodal curves reported in the literature.

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