Experimental determination and thermodynamic modeling of phase equilibrium and protein partitioning in aqueous two-phase systems containing biodegradable salts

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ARTICLE INFO

ABSTRACT

Phase diagrams of sustainable aqueous two-phase systems (ATPSs) formed by polyethyleneglycols (PEGs) of different average molar masses (4000, 6000, and 8000) and sodium succinate are reported in this work. Partition coefficients (Kps) of seven model proteins: bovine serum albumin, catalase, beta-lactoglobulin, alpha-amylase, lysozyme, pepsin, urease and trypsin were experimentally determined in these systems and in ATPSs formed by the former PEGs and other biodegradable sodium salts: citrate and tartrate.

An extension of Pitzer model comprising long and short-range term contributions to the excess Gibbs free energy was used to describe the (liquid + liquid) equilibrium. Comparison between experimental and calculated tie line data showed mean deviations always lower than 3%, thus indicating a good correlation. The partition coefficients were modeled by using the same thermodynamic approach. Predicted and experimental partition coefficients correlated quite successfully. Mean deviations were found to be lower than the experimental uncertainty for most of the assayed proteins.

1. Introduction

Aqueous two-phase systems (ATPSs) are usually formed by combining either two flexible chain polymers or a polymer and a kosmotropic salt in water above a certain critical concentration [1,2]. Generally, the former is comprised of polyethylenglycol (PEG) and another polymer like dextran, starch, polyvinylalcohol, *etc.* In contrast, the later is composed of PEG and phosphate or sulfate salts. Liquid–liquid extraction with ATPSs constitutes a powerful tool for purifying bio-materials such as cells, organelles, proteins, *etc.* [3,4]. Since the pioneer works of Albertson in 1956, ATPSs have found applications in diverse areas such as extractive fermentations [5], determination of solute surface properties [6], and characterization of ligand–nucleic acid binding [7]. The relevance of these systems is supported by the following unique features: the high water content (above 0.85 wt/wt) in each phase, which provides a gentle environment for the preservation of labile biomaterials; the low interfacial tension, which favours the

migration of biomolecules to the interface; the possibility of linear scalability up to 10^4 times [8], which facilitates its industrial application. In addition, complex mixtures like crude homogenate and fermentation broth containing particulate materials can be directly loaded into the ATPSs without a previous centrifugation or filtration step. This allows reducing the number of initial downstream steps, since clarification, concentration and partial purification are integrated in one unit operation [9]. Despite these multiple advantages, ATPS extraction has not been widely adopted commercially for the large-scale recovery of biological products. Its further applicability has been hampered by several reasons, including the environmental problem associated with the disposal of phase chemicals after recovery processes [10] and the lack of a simple theory based on fundamental principles capable of explaining and predicting the experimental trends observed. In this context, the use of ATPSs formed by PEG and sodium succinate, tartrate and citrate salts becomes attractive due to the biodegradability of these anions and therefore, their improved eco-friendly characteristics when compared to the traditional ATPSs. Previous works from other authors focused on the experimental description, modeling and application of certain PEG/citrate [11–13] and PEG/tartrate ATPSs [14]. In our laboratory, we further explored these systems in a systematic manner and reported complete (liquid + liquid) equilibrium data corresponding to ATPSs formed by sodium

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citrate/tartrate and PEGs of different molecular mass (600, 1000, 2000, 4000, 6000, and 8000) $g \cdot mol^{-1}$ [15,16].

In this article, we report (liquid + liquid) equilibrium data for aqueous system containing sodium succinate and PEGs of different molecular masses (2000, 4000, and 6000) $g \cdot mol^{-1}$ that have not yet been published. We also include a comparative study of these systems with those containing sodium citrate/tartrate, previously reported. Besides, a particular effort is focused in applying an extension of Pitzer model [14] for the excess Gibbs free energy of aqueous electrolyte solutions to describe and predict binodal curves and partitioning behaviour of model proteins, in an attempt to aid a rational design and optimization of aqueous two-phase extraction using these biodegradable salts.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (mass fraction purity $\geqslant 0.98$), catalase (mass fraction purity 0.98), beta-lactoglobulin (mass fraction purity 0.97), alpha-amylase (purity 0.97), lysozyme (mass fraction purity 0.97), pepsin (mass fraction purity 0.98), urease (mass fraction purity 0.98) and trypsin (mass fraction purity 0.98) were purchased from Sigma–Aldrich Co. and used without further purification. Stock protein solutions (1000 to 1500 µM) were prepared by dissolving the solid in an adequate buffer solution and stored until using.

Polyethyleneglycols of average molar masses (4000, 6000, and 8000) $g \cdot mol^{-1}$ (PEG4000; PEG6000 and PEG8000) with mass fraction purities greater than 0.98 were also supplied by Sigma–Aldrich Co. Tartaric acid (mass fraction purity $\geqslant 0.99$), citric acid (mass fraction purity $\geqslant 0.99$), succinic acid (mass fraction purity $\geqslant 0.99$) and sodium hydroxide (mass fraction purity $\geqslant 0.98$) were supplied by Sigma–Aldrich Co. Table 1 summarizes relevant information on the provenance and mass fraction purity of all the materials used in this work.

2.2. Determination of equilibrium diagrams of PEG/succinate ATPSs

The experimental methodology employed to determine the binodal data was essentially similar to that used at previous works for

TABLE 1
Provenance and mass fraction purity of the materials used in this work.

Chemical name	Source	Mass fraction purity
PEG4000[a]	Aldrich	$\geqslant 0.98$[b]
PEG6000[c]	Aldrich	$\geqslant 0.98$[b]
PEG8000[d]	Aldrich	$\geqslant 0.98$[b]
Citric acid[e]	Sigma–Aldrich	$\geqslant 0.99$[b]
Tartaric acid[f]	Sigma–Aldrich	$\geqslant 0.99$[b]
Succinic acid[g]	Aldrich	$\geqslant 0.99$[b]
Sodium hydroxide	Aldrich	$\geqslant 0.98$[b]
Bovine serum albumin	Sigma	$\geqslant 0.98$[b]
Catalase	Sigma	0.98[h]
Beta-lactoglobulin	Sigma	0.97[h]
Alpha-amylase	Sigma	0.97[h]
Lysozyme	Sigma	0.97[h]
Pepsin	Sigma	0.98[h]
Urease	Sigma	0.98[h]
Trypsin	Sigma	0.98[h]

[a] PEG4000 = polyethyleneglycol Average M = 4000 $g \cdot mol^{-1}$.
[b] Informed by supplier.
[c] PEG6000 = polyethyleneglycol Average M = 6000 $g \cdot mol^{-1}$.
[d] PEG8000 = polyethyleneglycol Average M = 8000 $g \cdot mol^{-1}$.
[e] Citric acid = 2-hydroxypropane-1,2,3-tricarboxylic acid monohydrate.
[f] Tartaric acid = 2,3-dihydroxybutanedioic acid.
[g] Succinic acid = butanedioic acid.
[h] Determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

ATPSs formed by PEG and citrate/tartrate sodium salts [15,16]. PEG stock solutions of known concentration (0.40 wt/wt) were prepared by weighing solid PEG of different molar masses (4000, 6000, and 8000) $g \cdot mol^{-1}$ and water. Sodium succinate stock solution was prepared from a succinic solution (0.24 wt/wt) whose pH was adjusted to 5.00 by the addition of the appropriate quantities of sodium hydroxide. The methodology to determine the binodal curve consisted of a turbidimetric titration [17] of the sodium succinate stock solution contained in a glass tube with small aliquots of the PEG stock solution until the system turned turbid (cloud point), thus indicating the formation of two liquid phases. The starting and added solution masses were measured on an analytical balance with a precision of ±0.0001 g. In accordance with the amount of titrant added until the turbidity appearance, the composition of the mixture for each point on the binodal curve was calculated. The system temperature was maintained constant (295 K) and controlled to within ±0.1 K by immersing the glass tube and the stock solutions in a thermostatic bath. Binodal points corresponding to PEG 4000 ATPSs at high salt concentration (above 0.20 wt/wt) could not be obtained by this methodology. In that situation, each point on the binodal curve was obtained by preparing a series of systems with a given total succinate concentration and the PEG total concentration varying from (0.00050 to 0.00250) wt/wt (by weighing and adding solid PEG into the succinate solution). Composition of the turbid system with the lowest PEG concentration in the assayed series was considered to belong to the binodal curve. Each system was prepared in triplicate.

Tie lines (TLs) were determined by the method described by Merchuk et al. [18]. A four equation system, whose resolution yields the equilibrium compositions, was started from the application of the lever-arm rule on binodal diagram. The knowledge of both a function describing the binodal curve and the top/bottom mass ratio were required. Systems of different total compositions were prepared into graduate tubes by mixing appropriate masses of solid PEG, sodium succinate stock solution and water, stirred vigorously and left to allow equilibrium for 24 h. The top/bottom mass ratio was calculated from volume and density measurements. Volumes were obtained by visual estimation and densities were determined using an Anton Paar model DMA 35 N densimeter (±0.0001 $g \cdot cm^{-3}$ uncertainty). Binodal data were fitted with different expressions, previously used by other authors. The best results were obtained with the Mistry empirical equation [19]:

$$\ln w_{PEG} = a + b w_{Succinate}^{0.5} + c w_{Succinate}^3, \tag{1}$$

where w_{PEG} and $w_{Succinate}$ are the mass fractions of PEG and sodium succinate, respectively; a, b and c are the fitting parameters.

2.3. Determination of the partition coefficient (Kp) of model proteins

To prepare mother biphasic aqueous systems, stock solutions of the phase components: PEG of different molar mass 0.30 (wt/wt) and tartaric/citric/succinic acid 0.22 (wt/wt) were mixed according to the equilibrium data reported either in this article or in previous works [15,16]. Low-speed centrifugation was used to speed up phase separation after a thorough gentle mixing of the system components and then 1 cm^3 of each phase was mixed to reconstitute several working two-phase systems in which the protein partition was assayed. Partitioning behaviour of assayed proteins was analysed by dissolving a given amount of protein (1.5 to 2.1 µM total system concentration) in the working two-phase systems. Small aliquots of the protein stock solution (1000 µM) were added to the systems (0.010 to 0.020) cm^3 in order to make the change of the total volume of each phase negligible. After mixing by inversion for 20 min and leaving it to settle for at least 60 min, the system was centrifuged at low speed for the two phase separation.

Samples were withdrawn from separated phases and diluted appropriately with the equilibrated phase free from protein. The protein content in each phase was determined by measuring the absorbance at 280 nm. Equally diluted samples from identical phase systems without protein were used as blanks. The partition coefficient was calculated with the following expression:

$$Kp = \frac{C_{Prot,top}}{C_{Prot,bottom}}, \tag{2}$$

where $C_{Prot,top}$ and $C_{Prot,bottom}$ are the equilibrium concentrations of the partitioned protein in the PEG- and salt-enriched phases, respectively. Temperature was maintained constant and controlled to within ±0.1 K by immersing the glass tubes in a thermostatic bath. All the measurements were carried out in triplicate.

3. Thermodynamic framework

Several theoretical and empirical models have been offered for predicting thermodynamic properties of aqueous two-phase systems. The complete reviews of Walter *et al.* [20], Abbott *et al.* [21] and Cabezas [22] provide critical analyses of the different mathematical approaches.

When modelling polymer/salt ATPSs, it must be kept in mind that coexisting phases are intrinsically non-ideal solutions due to (i) the high solute size (polymer) with regard to that of the solvent (water) and (ii) the strong electrostatic interactions present between phase components. Due to the presence of salts, the excess Gibbs free energy (G^E) is considered as a sum of terms related to long- and short-range interactions ($G^{E,LR}$; $G^{E,SR}$):

$$\frac{G^E}{n_w RT} = \frac{G^{E,LR}}{n_w RT} + \frac{G^{E,SR}}{n_w RT} \tag{3}$$

which implies that the activity coefficient of a given solute i is also split into two related terms:

$$\ln \gamma_i = \ln \gamma_i^{LR} + \ln \gamma_i^{SR}. \tag{4}$$

A widely used approach to the modelling of both phase equilibrium and protein partitioning the so-called virial equations, in which the short-range term in the previous equation (6) is a virial-like expansion. Among all models based on virial expansions, the most widely employed is the Pitzer model [23]. According to this model, the term for the long-range interactions, which accounts for the Coulomb forces, is a derivation of that initially proposed by Debye–Hückel and can be presented as:

$$\frac{G^{E,LR}}{n_w RT} = -A_j n \left(\frac{M_w}{1000} \right) \frac{4I}{b} \ln(1 + b(I)^{1/2}), \tag{5}$$

where the molecular mass of solvent (water) $M_w = 0.018$ kg · mol^{-1}; the solvent-dependent constant $b = 1.2$ kg$^{1/2}$ · mol$^{-1/2}$ [24] and the ionic strength I is defined as:

$$I = \frac{1}{2} \sum_i m_i z_i^2 \tag{6}$$

z_i being the charge number of species i; the summation is carried out over all charged species. The factor A_φ is the Debye–Hückel parameter expressed as:

$$A_\phi = \frac{1}{3} \left(\frac{e^2}{4\pi \varepsilon_o \varepsilon_r RT} \right)^{3/2} (2\pi N_{Av} \rho)^{1/2} \tag{7}$$

e is the electron charge; N_{Av}, the Avogadro number; ρ, the solvent density; ε_o and ε_r, the vacuum and the solvent relative permittivity respectively. For aqueous solutions at 298 K, A_φ has a constant value of 0.3914 kg$^{1/2}$ · mol$^{-1/2}$ irrespective of the solute considered. The

long-range activity coefficient γ_i^{LR} can be obtained from proper derivation of equation (9):

$$\ln \gamma_i^{LR} = -A_j z_i^2 \left[\frac{2}{b} \ln(1 + b(I)^{1/2}) + \frac{(I)^{1/2}}{1 + b(I)^{1/2}} \right]. \tag{8}$$

The short-range interaction contribution to G^E proposed by Pitzer is a virial-type expression which contemplates binary (λ_{ij}) and ternary (Λ_{ijk}) interaction parameters:

$$\frac{g^{E,SR}}{n_w M_w RT} = \sum_{i \neq w} \sum_{j \neq w} \lambda_{i,j}(I) m_i m_j + \sum_{i \neq w} \sum_{\phi \neq \omega} \sum_{k \neq w} \wedge_{ijk} m_i m_j m_k. \tag{9}$$

The matrices of λ_{ij} are symmetric, i.e. $\lambda_{ij} = \lambda_{ji}$. The quantity $\lambda_{ij}(I)$ represents the short-range interactions in presence of the solvent between solute particles i and j. This binary interaction parameter or second virial coefficient does not itself have any composition dependence for neutral species, but for ions it can be expressed as:

$$\lambda_{ij}(I) = \lambda_{ij}^{(0)} + \lambda_{ij}^{(1)} \frac{2}{\alpha^2 I} \left[1 - (1 + \alpha(I)^{1/2}) exp(-\alpha(I)^{1/2}) \right]. \tag{10}$$

The second term stands for the ionic strength influence on λ_{ij}. The value of $\lambda_{ij}^{(1)}$ parameter is an indicator of the mentioned influence and α is a constant (2.0 kg$^{1/2}$ · mol$^{-1/2}$).

By neglecting all ternary interactions ($\Lambda_{ijk} = 0$) in equation (15) and assuming the $\lambda_{ij}(I)$ function in equation (17), we obtain the following expression for $\ln \gamma_i^{SR}$:

$$\ln \gamma_i^{SR} = 2 \sum_{j \neq w} \lambda_{ij}(I) m_j - z_i^2 M_w \sum_{j \neq w} \sum_{k \neq w} \lambda_{ij}^{(1)}$$
$$\times \frac{1}{\alpha^2 I^2} \left[1 - \left(1 + \alpha(I)^{1/2} + \frac{\alpha^2 I}{2} \right) exp(\alpha(I)^{1/2}) \right] m_j m_k. \tag{11}$$

Then, γ_w^{LR} and γ_w^{SR} (long- and short-range activity coefficients for water) can be obtained by applying Gibbs–Duhem relationship:

$$\ln \gamma_w^{LR} = 2A_\phi M_w \frac{I^{3/2}}{1 + b(I)^{1/2}}, \tag{12}$$

$$\ln \gamma_w^{SR} = -M_w \left[\sum_{i \neq w} \sum_{j \neq w} (\lambda_{ij}^{(0)} + \lambda_{ij}^{(1)} exp(-\alpha(I)^{1/2})) m_i m_j \right]. \tag{13}$$

In this work, when applying Pitzer equations for the modelling of ATPS, two major modifications were introduced, which accounted for: (i) the polymer presence like a second solvent that affects the density and dielectric constants on the long-range interaction term and (ii) the dependency of binary interaction parameters corresponding to polymer/polymer and polymer/salt short-range interactions on the polymer chain length.

The influence of polymer presence on solvent properties was taken into consideration through the replacement of the water ρ and ε_r constants in the A_φ Debye–Hückel parameter (equation (7)) by ρ and ε_r average quantities, defined as:

$$\rho_{av} = \rho_w \phi_w + \rho_{PEG} \phi_{PEG}, \tag{14}$$

$$\epsilon_{r(av)} = \epsilon_{r(w)} \phi_w + \epsilon_{rPEG} \phi_{PEG}. \tag{15}$$

These expressions arise from the application of mixture rules [25] and allow to obtain mean values of ρ and ε_r that depend on water and PEG volume fractions (ϕ_w, ϕ_{PEG}). According to this, the A_φ is not a constant parameter at a given temperature, since it is affected by the PEG phase concentration. The values for PEG were obtained from literature [25].

The effect of polymer chain length on binary parameters $\lambda_{PEG-PEG}$ and $\lambda_{PEG-salt}$ corresponding to PEG–PEG and PEG–salt short-range interactions was also considered in this extension of

Pitzer model by introducing appropriate relationships between them ($\lambda_{PEG-salt}$; $\lambda_{PEG-PEG}$) and the PEG molar mass (see section 4).

4. Results and discussion

4.1. (Liquid + liquid) equilibrium

Figure 1 shows the effect of PEG molecular mass on (liquid + liquid) equilibrium for PEG/succinate systems. Slight displacements of binodal curves to the left can be observed when increasing the size of PEG molecule. This trend, observed in other PEG/salt systems [15,16], suggests an increase in the incompatibility between the phase components due to the increase of hydrophobic character of PEGs of higher molecular mass. The experimental results for the equilibrium compositions of these ATPSs in absence of biomolecules are presented in Table 2. When compared with the formerly obtained equilibrium compositions of systems formed by PEG and either tartrate or citrate [15,16], it can be seen that, while tartrate or citrate yield similar phase diagrams, the two-phase region for systems containing succinate is considerably smaller, which means that the amounts of constituents (either PEG or salt) necessary to induce phase separation are higher in this case. Moreover, the tie-line slopes are similar for these three salts. A more comprehensive comparison of these results can be carried out when coupled with the modelling of (liquid + liquid) equilibrium data. A wide range of systems was considered in order to test the reliability of the model, comprising 36 ATPSs formed by PEGs

of different chain length and sodium salts of succinate, tartrate and citrate. Once more, equilibrium data of PEG/citrate and PEG/tartrate ATPSs were taken from two previous works of our group [15,16] while those corresponding to PEG/succinate ATPSs were obtained in this work. Systems with PEGs of lower molecular mass (under 4000) were not modelled since when mixing with sodium succinate too high concentrations of both components were required to form the biphasic systems. Working pH and dissociation constants of citric, succinic and tartaric acids were needed to estimate the charge (z_i) and concentration (m_i) of each anionic species from acid dissociation and therefore to obtain the phase ionic strength (equation (6)).

The first step in the calculation was to group equilibrium data according to the PEG size and salt type. The following four model parameters were fitted to experimental data:

(i) $\lambda_{Na^+-An}^{(0)}$ and $\lambda_{Na^+-An}^{(1)}$ for interactions between Na$^+$ and biodegradable anion (citrate, tartrate, succinate).

(ii) $\lambda_{PEG-salt}^{(0)}$ and $\lambda_{PEG-PEG}^{(0)}$ for PEG interactions with salt and another PEG molecule respectively. As PEG is a neutral polymer, the binary parameters $\lambda_{PEG-salt}^{(1)}$ and $\lambda_{PEG-PEG}^{(1)}$ were neglected ($\lambda_{PEG-salt}^{(1)} = \lambda_{PEG-PEG}^{(1)} = 0$).

The optimization procedure was carried out by minimization of the following objective function (OF1):

$$OF_1 = \left(\frac{\sum_{t=1}^{t}\sum_{p=1}^{2}\sum_{i=1}^{2}(w_i^{p,cal} - w_i^{p,exp})}{4t} \right)^{1/2} \quad (16)$$

in which t corresponds to the number of assayed tie lines (4/3 in this first step); p represents the phase (top and bottom) and i indicates a phase component, the summation being carried out over the number of independent phase components (PEG and salt). The difference $w_i^{p,cal} - w_i^{p,exp}$ indicates the deviations between calculated and experimental mass fraction of phase components at equilibrium condition. The calculated compositions are obtained by an equilibrium flash-type calculation using a global composition that corresponds to the middle point of the experimental tie-line, and the phase experimental equilibrium compositions as initial guesses. Low OF_1 values (<0.0028) were obtained in all the system groups explored, thus indicating a good agreement between calculated and experimental data. Moreover, for a given salt, the obtained $\lambda_{PEG-salt}$ and $\lambda_{PEG-PEG}$ binary parameters showed to be dependent on PEG molar mass (M_{PEG}) according to the following equations:

$$\lambda_{PEG-salt}^{(0)} = m_1 M_{PEG} = m_1 n_{Mon} M_{Mon}, \quad (17)$$

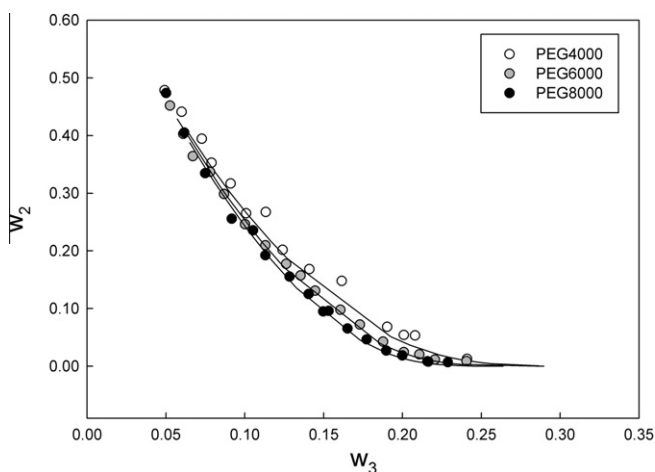$$\lambda_{PEG-PEG}^{(0)} = m_2 (M_{PEG})^2 = m_2 (n_{Mon}M_{Mon})^2, \quad (18)$$

FIGURE 1. Binodal curves for the systems {water (1) + polyethyleneglycol (2) + sodium succinate (3)} at pH 5.20 at $T = 295.1$ K. Standard uncertainty of each point is $u(w) = 0.0003$.

TABLE 2
Total and experimental (liquid + liquid) equilibrium data for the system {water (1) + polyethyleneglycol (2) + sodium succinate (3)} at $T = 295.1$ K.[a]

PEG mass	Tie line n°	Total composition		Top phase composition		Bottom phase composition	
		$100\,w_3$	$100\,w_2$	$100\,w_3$	$100\,w_2$	$100\,w_3$	$100\,w_2$
4000	1	12.56	23.92	9.35	30.78	22.29	3.12
	2	12.97	24.42	8.58	33.42	24.50	0.70
	3	13.24	24.66	8.03	35.44	25.07	0.14
6000	1	13.13	17.57	11.47	21.15	20.70	2.41
	2	13.57	20.15	8.23	31.55	22.60	0.84
	3	14.01	23.73	6.35	39.22	25.50	0.45
8000	1	12.81	19.10	9.00	26.88	22.17	0.03
	2	13.00	20.00	8.50	28.90	22.73	0.01
	3	13.17	21.43	8.31	30.61	23.87	0.01

[a] Standard uncertainties u are $u(T) = 0.1$ K and $u(w) = 0.0003$.

TABLE 3
Dependence of Pitzer binary parameters on PEG molecular mass.

ATPS	$m_1/10^{-3}$ (kg · g^{-1})	R_1	$m_2/10^{-6}$ (kg · mol · g^{-2})	R_2
PEG/citrate	1.224	0.99	1.174	0.99
PEG/tartrate	1.002	0.91	2.293	0.98
PEG/succinate	1.745	0.95	2.449	0.97

R_1, R_2 regression coefficients.

TABLE 4
Results for the correlation of (liquid + liquid) equilibrium of systems containing PEG and salts of biodegradable anions.

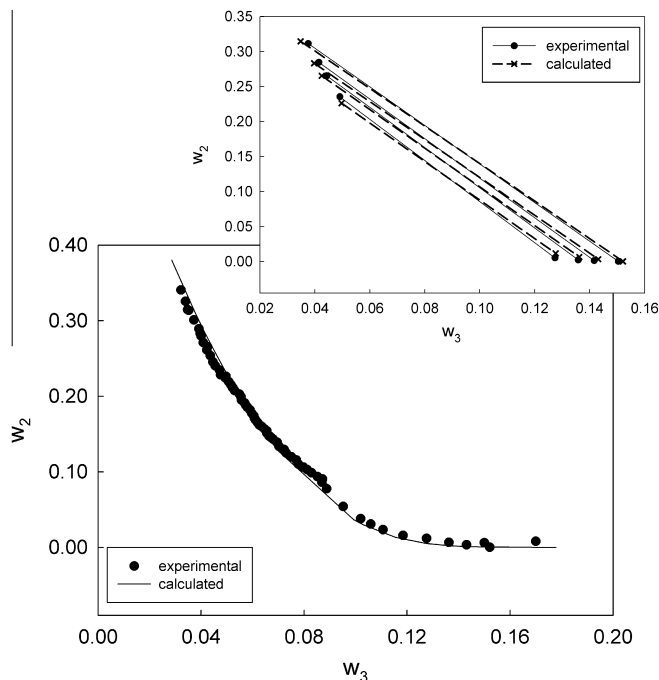| ATPS	$\lambda_{Na^+-An}^{(0)}/$ (kg · mol^{-1})	$\lambda_{Na^+-An}^{(1)}/$ (kg · mol^{-1})	$\lambda_{Mon-salt}^{(0)}/$ (kg · mol^{-1})	$\lambda_{Mon-Mon}^{(0)}/10^{-6}$ (kg·mol^{-1})	OF_1
PEG/citrate	0.0299	−0.0089	0.0016	1.7272	0.0039
PEG/tartrate	0.0500	−0.0100	0.0022	2.6961	0.0013
PEG/succinate	0.0009	−0.0001	0.0010	2.0373	0.0032

OF_1, objective function calculated according to equation (18).
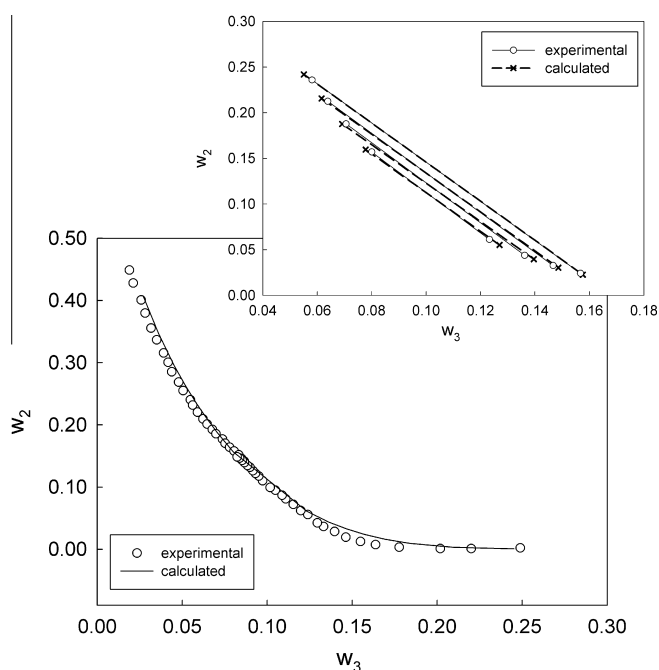
FIGURE 2. Comparison between experimental and correlated data corresponding to systems {water (1) + PEG4000 (2) + sodium citrate (3)} at pH 5.20. T = 295.1 K. Standard uncertainty of each point is $u(w)$ = 0.0003.

FIGURE 3. Comparison between experimental and correlated data corresponding to systems {water (1) + PEG8000 (2) + sodium tartrate (3)} at pH 5.00. T = 295.1 K. Standard uncertainty of each point is $u(w)$ = 0.0003.
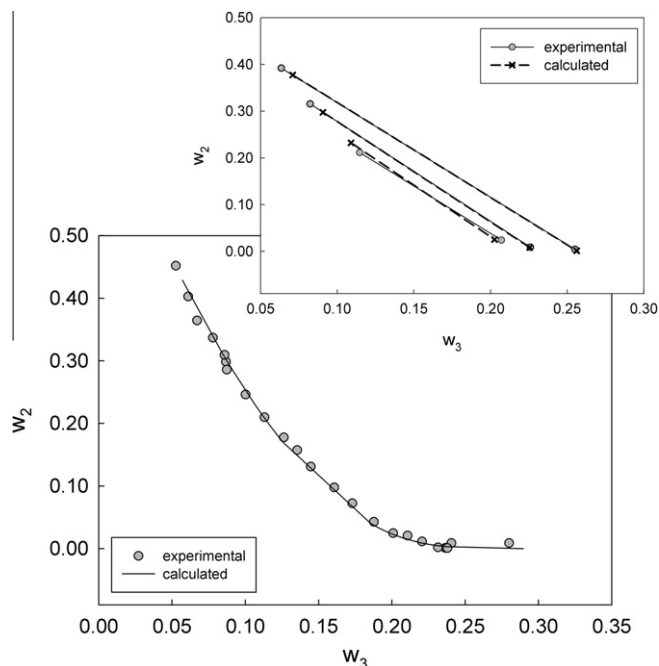
FIGURE 4. Comparison between experimental and correlated data corresponding to systems {water (1) + PEG6000 (2) + sodium succinate (3)} at pH 5.00. T = 295.1 K. Standard uncertainty of each point is $u(w)$ = 0.0003.

where n_{Mon} and M_{Mon} are the number and molecular mass of monomer units in PEG molecule (0.044 kg · mol^{-1}) respectively. m_1 and m_2 are proportional constants that were determined from the slope of $\lambda_{PEG-salt}$ vs. M_{PEG} and $\lambda_{PEG-PEG}$ vs. M_{PEG}^2 plots. Their values are summarized in table 3. These findings agree with Pitzer model assumptions and allow us to define new binary parameters: $\lambda_{Mon-salt}$ and $\lambda_{Mon-Mon}$, both of them PEG molecular weight-independent:

$$\lambda_{Mon-salt}^{(0)} = \frac{\lambda_{PEG-salt}^0}{n_{Mon}} = m_1 M_{Mon}, \qquad (19)$$

$$\lambda_{Mon-Mon}^{(0)} = \frac{\lambda_{PEG-PEG}^{(0)}}{n_{Mon}^2} = m_2 (M_{Mon})^2. \qquad (20)$$

Therefore, a new more generalised fitting of experimental data was carried out by inputting the calculated m_1 and m_2 as feed parameters. In this second calculation, the experimental data were grouped according to the salt type irrespective of PEG molecular weight. The optimization procedure is similar to that described for the first step by minimizing the OF_1. However, in this case the summation was extended to 12/9 tie lines (t). Table 4 shows all the correlated interaction parameters together with the OF_1 values. Clearly, OF_1 values lower than 0.0040 suggest a quite successful
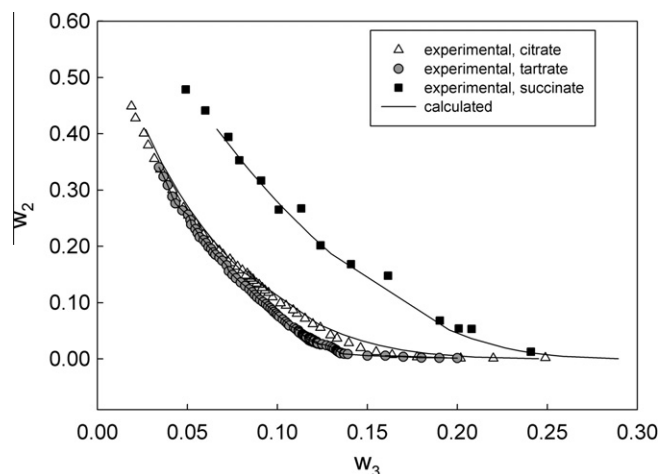
FIGURE 5. (Liquid + liquid) equilibrium in systems {water (1) + PEG4000 (2) + sodium salts (3)}. $T = 295.1$ K. Standard uncertainty of each point is $u(w) = 0.0003$.

TABLE 5
Total and equilibrium (liquid + liquid) compositions for the systems {water (1) + polyethyleneglycol (2) + sodium salt (3)} used to assess the model in protein-partitioning at $T = 295.1$ K.[a]

ATPS	Total composition		Top phase composition		Bottom phase composition	
	100 w_3	100 w_2	100 w_3	100 w_2	100 w_3	100 w_2
PEG4000/citrate	10.87	10.45	6.92	18.76	13.96	3.96
PEG6000/citrate	9.58	10.40	6.24	18.64	12.98	2.24
PEG8000/citrate	11.88	4.39	6.07	17.16	12.92	2.10
PEG4000/tartrate	13.66	9.48	4.86	26.21	14.10	1.11
PEG6000/tartrate	10.02	10.00	4.70	24.02	13.34	1.24
PEG8000/tartrate	8.79	14.00	4.27	26.53	13.62	0.66

[a] Standard uncertainties u are $u(T) = 0.1$ K and $u(w) = 0.0003$.

correlation, the best fitting being observed for PEG/tartrate ATPS. A graphical comparison between the experimental and calculated data is also shown in figures 2 to 4. A reasonable agreement can be seen between the calculated and experimental binodal curve and the tie lines, thus indicating that the Pitzer model extension applied is able to predict the phase split over the whole composition range assayed. The relative deviations between calculated and experimental equilibrium concentrations are always below the 3% for the assayed systems. Additionally, figure 5 allows a comparison of the effect of the different biodegradable anions on phase separation. The binodal curve for PEG4000/tartrate ATPS is slightly shifted to the left with regard to that of PEG4000/citrate ATPS, while that corresponding to PEG4000/succinate exhibits a drastic displacement to the right. According to this observation, the anion capability of forming biphasic systems follows the order succinate <<< citrate < tartrate, which agrees with the sequence observed for binary parameters $\lambda_{Mon-salt}^{(0)}$, positive values of these second virial coefficients indicate that PEG monomer–salt (Mon–salt) interaction is lesser favourable than Mon-water and salt-water interactions. Consequently, the c values could be considered to account for the exclusion effect (incompatibility) between PEG monomer and the different salts, and therefore, to be related with the salt ability to form biphasic systems.

4.2. Protein partitioning

Partitioning of model proteins was assessed in different PEG/biodegradable salts systems. The composition selected for PEG/tar-
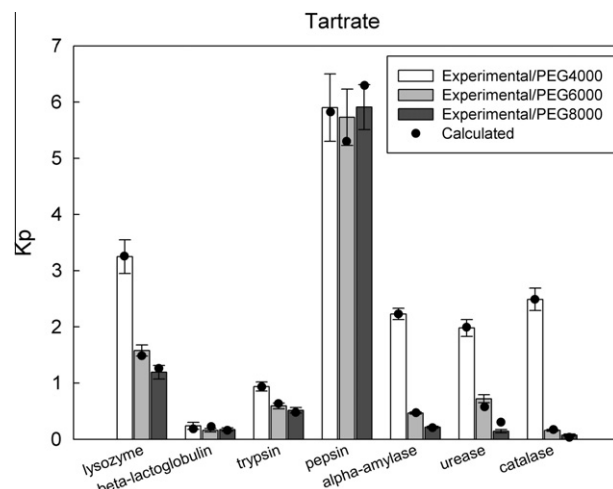

FIGURE 6. Experimental and calculated partitioning coefficients of model proteins in systems {water (1) + PEG (2) + sodium tartrate (3)} at pH 5.00. $T = 295.1$ K. The uncertainty of the Kp measurement is indicated on each bar. Compositions are those of table 5.

trate and PEG/citrate ATPSs is shown in table 5 while the composition explored for PEG/succinate is that corresponding to the tie line number 2 (table 2). Numerous experimental measurements of protein partitioning coefficient (Kp), reported since the first Albertsson works, have revealed that its value depends on both characteristics of ATPS – type of phase-forming polymer, its molecular mass, the phase component concentrations, etc., and the physicochemical properties of the molecule which is being partitioned, such as molecular size, net charge, surface hydrophobicity, and tryptophan content. The selected model proteins possess different properties. Their molar masses vary from 14 kDa in lysozyme and beta-lactoglobulin to 233 kDa in catalase and 480 kDa in urease. Besides, at working pH pepsin is negatively charged, lysozyme and trypsin are positively charged and the other proteins practically do not possess charge. In this context, different partitioning behaviours were expected. Figures 6 to 8 depict the partitioning behaviour of different model proteins in the selected PEG/salt ATPSs. Several trends are highlighted, viz.

(i) Pepsin and lysozyme show a displacement to the polymer-enriched phase ($Kp > 1$) in most of the assayed systems while beta-lactoglobulin, catalase and alpha-amylase partitioned unilaterally to the salt-enriched phase ($Kp < 1$).
(ii) In PEG/succinate ATPSs, most of the assayed proteins exhibited the highest Kp values, when compared to the systems formed by either tartrate or citrate.
(iii) The increase in the PEG molecular mass (for a given salt) did not always induce the expected protein displacement to the bottom phase. This failure to obey this general trend, observed for most of partitioning systems [26], suggests the presence of an alternative mechanism different from the exclusion by size of PEG molecule.

In order to find out if these features could be accounted for the proposed Pitzer model, the correlation of partitioning data of the proposed seven proteins was evaluated. The equilibrium compositions calculated previously were assumed not to change in the biomolecule presence, since all the partitioning experiences had been carried out at low total protein concentration (1.5 to 2.1) µM. For each protein in ATPS containing a given salt with the different PEGs, new binary parameters that account for the protein interaction with monomer unit of PEG and salt ($\lambda_{Prot-Mon}$,
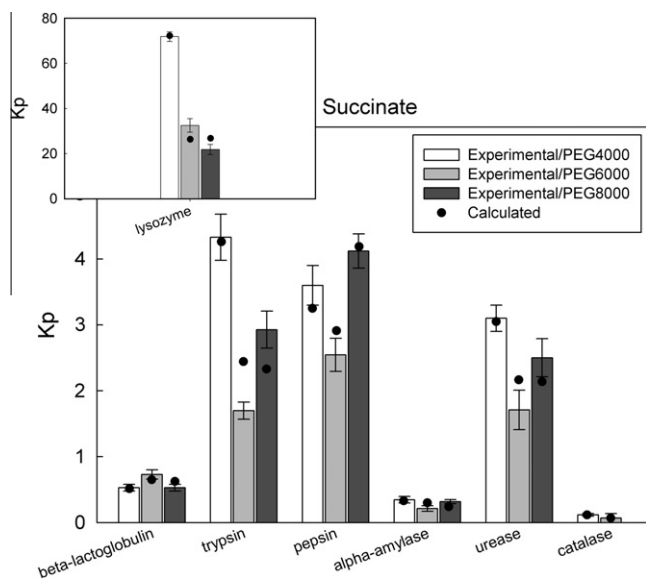
FIGURE 7. Experimental and calculated partitioning coefficients of model proteins in systems {water (1) + PEG (2) + sodium succinate (3)} at pH 5.00. T = 295.1 K. The uncertainty of the Kp measurement is indicated on each bar. Compositions correspond to the tie line number 2 in table 2.
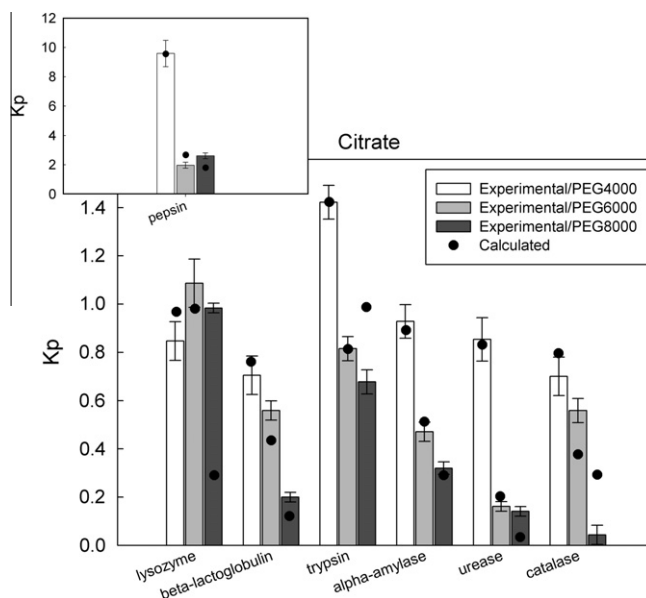

FIGURE 8. Experimental and calculated partitioning coefficients of model proteins in systems {water (1) + PEG (2) + sodium citrate (3)} at pH 5.20. T = 295.1 K. The uncertainty of the Kp measurement is indicated on each bar. Compositions are those of table 5.

TABLE 6
Results for the correlation of partitioning equilibrium of model proteins in PEG/biodegradable salt ATPS.

Binary parameter /(kg · mol^{-1})	Lysozyme	Beta-lactoglobulin	Trypsin	Pepsin	Alpha-amylase	Urease	Catalase
			PEG/citrate				
$\lambda_{Prot-salt}^{(0)}$	−0.5034	−10.2495	1.0206	1.3079	−16.1849	−19.9557	−13.3864
$\lambda_{Prot-Mon}^{(0)}$	−0.0007	0.0175	0.0017	0.0019	0.0274	0.0357	0.0233
OF_2	0.0928	0.0950	0.0012	0.6233	0.0361	0.0572	0.1483
			PEG/tartrate				
$\lambda_{Prot-salt}^{(0)}$	1.0381	−1.2660	0.9203	0.3524	1.3368	1.2579	1.5408
$\lambda_{Prot-Mon}^{(0)}$	0.0166	0.0022	0.0016	−0.0022	0.0023	0.0022	0.0027
OF_2	0.0677	0.0488	0.0337	0.3360	0.0063	0.6250	0.0321
			PEG/succinate				
$\lambda_{Prot-salt}^{(0)}$	0.2856	−0.3268	0.2635	0.1244	0.4115	0.1860	0.5409
$\lambda_{Prot-Mon}^{(0)}$	0.0014	0.0006	0.0004	−0.0326	0.0012	0.0002	0.0017
OF_2	4.5600	0.0761	0.5527	0.2955	0.0679	0.3368	0.0002

OF_2, objective function calculated according to equation (23).

$\lambda_{Prot-salt}$) were obtained through the minimization of the following objective function (OF_2):

$$OF_2 = \left(\frac{\sum_{t=1}^{t}(Kp_i^{exp} - Kp_i^{calc})^2}{t} \right)^{1/2} \qquad (21)$$

in which t is the number of tie lines considered and the difference $Kp_i^{exp} - Kp_i^{calc}$ is the deviation between measured and calculated Kp values.

In modelling biomolecule partitioning, proteins were considered not to possess electrical charge, in order to simplify the procedure. This consideration is correct for amylase, catalase, urease and beta-lactoglobulin since their pIs ($\cong$5.00) are close to the working pH in all the ATPSs, but not for the other proteins (pepsin, lysozyme, and trypsin) whose pIs are quite far from 5.00. Nevertheless, since the liquid phases in equilibrium have high ionic strength, one can assume that the effect of the protein net charge is screened by the presence of salt ions in solution.

Table 6 shows the binary interaction parameters of protein with PEG monomer unit and salt. It should be emphasised that the comparison of OF_2 values among the different systems does not reflect

the success of fitting procedure due to the wide range of possible Kp values. Figures 6 to 8 allow a visual evaluation of modelling performance. Clearly, the calculated partition coefficients agree with the empiric Kp values to within the experimental uncertainty of the measurements for most of the situations considered. The partitioning behaviour of alpha-amylase, pepsin and trypsin were predicted highly satisfactorily, although the pepsin and trypsin electrical charge (negative and positive respectively) at working pH had not been considered when modelling. Contrarily, trends observed for urease and catalase, proteins without net charge at working pH, were poorly described by this thermodynamic treatment. Probably, when partitioning these big proteins, interaction between each other, which is not considered in our approach, becomes significant, thus causing the observed deviations. In addition, another process such as aggregation and precipitation at the interface may take place and modify the partition behaviour.

Results from systems with different biodegradable salts show that the best model capability of predicting protein partitioning is obtained in PEG/tartrate ATPSs, which agrees with the successful results previously observed when correlating equilibrium data in absence of biomolecule.

5. Conclusions

A comparative study of ATPSs formed by polyethyleneglycols of different molecular mass and sodium salts of citrate, tartrate and succinate is reported in this work. The biodegradable character of the cited anions makes these systems become potential substitutes of traditional ATPSs for industrial purposes. The analysis of experimental data of partition coefficients, with the observed values and trends, shows that these systems can be considered as alternatives for initial steps in downstream processing.

The proposed extension of Pitzer model has demonstrated to provide a thermodynamic framework useful for describing and predicting quite satisfactorily (liquid + liquid) equilibrium of these systems. The modifications accounted for the effect of polymer presence on solvent properties, thus affecting the long-range interaction contributions and the dependence of the second virial coefficients, related to short-range interactions, on the polymer chain length. Its main feature is its simplicity, since scarce system information is needed for its application. The incorporation of those parameters neglected in our treatment such as the third Pitzer coefficients (Λ_{ijk}) or the ionic strength-dependent Pitzer binary parameters ($\lambda_{ij}^{(1)}$) could improve the results but could also increase the likelihood of over-fitting.

Acknowledgements

References

[1] H. Walter, D.E. Brooks, D. Fisher, Partitioning in Aqueous Two-Phases Systems, Academic Press Inc., Orlando, 1985.
[2] B.Y. Zaslavsky, Aqueous Two-phase Partitioning. Physical Chemistry and Bioanalytical Applications, Marcel Dekker Inc.,, New York, 1994.
[3] P.A. Albertsson, Partition of Cell Particles and Macromolecules, third ed., John Wiley-Interscience, New York, 1986.
[4] M. Rito Palomares, J. Chromatogr. B 807 (2004) 3.
[5] G.M. Zijlstra, M.J.F. Michielsen, C.D. de Gooijer, L.A. van der Pol, J. Tramper, Bioseparation 7 (1998) 117.
[6] J.A. Asenjo, B.A. Andrews, J. Chromatogr. A 1218 (2011) 8826.
[7] S. Bazile-Pham Khac, N.J. Moreau, J. Chromatogr. A 668 (1994) 241.
[8] M.R. Kula, Extraction and Purification of Enzymes, Appl. Biochem. Bioeng. in: L.B. Wingard, Jr., E. Katchalski-Katzir, L. Goldstein (Eds.), Academic Press, New York, 1979.
[9] M. Rito-Palomares, A. Lyddiatt, Chem. Eng. J. 87 (2002) 313.
[10] P. Selvakumar, T.C. Ling, S. Walker, A. Lyddiatt, J. Chromatogr. B 878 (2010) 1784.
[11] K.P. Ananthapadmanabhan, E.D. Goddard, Langmuir 3 (1987) 25.
[12] J. Vernau, M.R. Kula, Biotechnol. Appl. Biochem. 12 (1990) 397.
[13] M.T. Zafarani-Moattar, R. Sadeghi, A.A. Hamidi, Fluid Phase Equilib. 219 (2004) 149.
[14] W. Wang, X. Zhang, C. Xu, H. Cheng, Appl. Mech. Mater. 138–139 (2011) 995.
[15] G. Tubio, L. Pellegrini, G. Picó, B. Nerli, J. Chem. Eng. Data 51 (2006) 209.
[16] L. Pellegrini Malpiedi, C. Fernández, G.A. Picó, B.B. Nerli, J. Chem. Eng. Data 53 (2008) 1175.
[17] L. Pellegrini Malpiedi, G. Picó, B. Nerli, Sep. Purif. Technol. 78 (2011) 91.
[18] K.S. Pitzer, J. Phys. Chem. 77 (1973) 268.
[19] R. Hatti-Kaul, R. Ed, Aqueous Two-Phase Systems: Methods and Protocols; Methods in Biotechnology 11, Humana Press, Totowa, New Jersey, 2000.
[20] J.C. Merchuk, B.A. Andrews, J.A. Asenjo, J. Chromatogr. B 711 (1998) 285.
[21] S.L. Mistrya, A. Kaula, J.C. Merchuk, J.A. Asenjo, J. Chromatogr. A 741 (1996) 151.
[22] H. Walter, G. Johansson, D.E. Brooks, Anal. Biochem. 197 (1991) 1.
[23] N.L. Abbott, D. Blankschtein, T.A. Hatton, Bioseparations 1 (1990) 191.
[24] H. Cabezas, J. Chromatogr. B 680 (1996) 3.
[25] K.S. Pitzer, J. Am. Chem. Soc. 102 (1980) 2902.
[26] K.S. Pitzer, G. Mayorga, J. Phys. Chem. 77 (1973) 2300.

JCT-12-287