



## (Liquid + liquid) equilibria of polymer-salt aqueous two-phase systems for laccase partitioning: UCON 50-HB-5100 with potassium citrate and (sodium or potassium) formate at 23 °C

Estela Lladosa<sup>a,1</sup>, Sara C. Silvério<sup>a,b</sup>, Oscar Rodríguez<sup>a</sup>, José A. Teixeira<sup>b</sup>, Eugénia A. Macedo<sup>a,\*</sup>

<sup>a</sup> LSRE – Laboratory of Separation and Reaction Engineering, Associate Laboratory LSRE/LCM, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal

<sup>b</sup> IBB – Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

### ARTICLE INFO

#### Article history:

Received 8 February 2012

Received in revised form 23 May 2012

Accepted 1 June 2012

Available online 12 June 2012

#### Keywords:

Aqueous two-phase systems

Phase equilibria

UCON

Partition coefficient

Laccase

### ABSTRACT

Aqueous two-phase systems (ATPS) are recognized as very suitable techniques for the recovery of target solutes in biological applications. Three new phase diagrams of (UCON 50-HB-5100 + potassium citrate + water), (UCON 50-HB-5100 + sodium formate + water), and (UCON 50-HB-5100 + potassium formate + water) systems were measured at 23 °C. The binodal curves were successfully described using the empirical equation suggested by Merchuk and co-workers. The reliability of the tie-line data experimentally determined was evaluated using the equations reported by Othmer–Tobias and Bancroft and satisfactory linearity was obtained for all ATPS. Among the salts studied, potassium citrate proved to be the most effective in ATPS formation, providing the largest heterogeneous region. Besides, the effect of both anions and cations in the size of the heterogeneous region and in the slope of the tie-lines has been compared. For the same salts and conditions, the heterogeneous region using UCON as the phase-forming polymer is larger than using polyethylene glycol. Furthermore, laccase partition in the UCON-salt ATPS was studied and it was found that in all cases enzyme partition occurred preferably to the bottom phase (salt-rich phase). Laccase concentration in the salt-rich phase was approximately 2-fold that in the top phase, thus UCON-salt ATPS can be a suitable biphasic system for laccase extraction.

© 2012 Elsevier Ltd. All rights reserved.

### 1. Introduction

Organic solvents are generally not suitable for bioprocesses due to the low solubility of proteins in these systems and the risk of protein denaturation. Aqueous two-phase systems (ATPS) have been used to develop bioprocesses for the recovery and purification of many biological products including proteins, genetic material, bionanoparticles, cells and organelles [1–4]. Aqueous two-phase partitioning is a very mild method of protein purification, and denaturation or loss of biological activity are not likely to happen. This is due to the high water content and low interfacial tension of the systems [5] which will protect the proteins. The polymers themselves may also have a stabilizing effect.

Three classes of ATPS have been defined: polymer + polymer + water, polymer + salt + water, and other alternative biphasic systems (obtained using surfactants, micellar compounds or ionic liquids). Polymer-salt ATPS have additional advantages over

two-polymers ATPS: The chemicals are lower priced (salts are in general cheaper than polymers) and the phases have a lower viscosity (so that shorter times are required for phase separation). Therefore, polymer-salt ATPS are widely used for large-scale and laboratory-scale liquid extraction [4,6,7].

The major drawback of ATPS is the difficult regeneration of the phase-forming components, and thus their large consumption. This gives rise to environmental as well as economical problems, forcing to find a solution that makes extraction with ATPS (economically) feasible on a large scale.

Although a number of different water-soluble polymers may be utilized to form aqueous two-phase systems, polyethylene glycols (PEGs) are probably the most used in combination with inorganic salts. Because PEGs are nontoxic, nonflammable and nonvolatile, PEG-based ATPS cause less environmental problems compared to conventional solvent extraction systems utilizing water-immiscible organic solvents.

The use of thermo-separating polymers has been introduced in ATPS to facilitate its recovery. Random copolymers of ethylene oxide (EO) and propylene oxide (PO) can be used as substitutes for polyethylene glycol (PEG). The advantage of EOPO copolymers is that the polymer can be easily recovered after use due to its

\* Corresponding author. Tel.: +351 22 508 1653; fax: +351 22 508 1674.

E-mail address: [eamacedo@fe.up.pt](mailto:eamacedo@fe.up.pt) (E.A. Macedo).

<sup>1</sup> Permanent address: Department of Chemical Engineering, University of Valencia, 46100 Burjassot, Spain.

thermo-separating properties. When heated above a lower critical solution temperature (LCST) (about 50 °C for Ucon [8], used here) these polymers separate from the aqueous solution, thus an easy polymer recovery and reutilization is possible. PEG is also a thermo-separating polymer, but its LCST is too high (above 100 °C) [9] for applications involving labile molecules.

In early studies, salting out of different polymers has been accomplished by the use of either phosphates or sulfates. Salts, like phosphates or sulfates, also need to be recycled; otherwise, they increase the load on wastewater treatment. Salt recycling has been shown to be possible; however, it has not proved more economical than processes without salt recycling [10]. This has motivated the use of salts that do not create wastewater treatment problems, e. g., citrate salts, which are easily degraded [11], and ammonium carbamate, which is volatile [12]. Formate is also biodegradable and nontoxic and could be discharge into biological wastewater treatment plants. In this respect, the liquid–liquid aqueous two-phase systems containing PEG and (potassium or sodium) citrate and (potassium or sodium) formate has been studied [13–16]. However, only a very limited amount of research has been reported using EOPO copolymers with citrate or formate salts [17].

In this work, binodal curves and tie-lines were obtained experimentally for three UCON-salt ATPSs at 23 °C. The polymer used in this work, UCON, is an example of a temperature-responsive polymer. The salts used (potassium citrate, sodium formate and potassium formate) allow a study of the effects of both cations and anions in the size of the heterogeneous region and in the slope of the tie-lines. Besides, the partition coefficient of laccase (an oxidative enzyme of wide application) in each UCON-salt ATPS was determined in order to investigate their suitability for laccase extraction.

## 2. Experimental

### 2.1. Chemicals

UCON 50-HB-5100, a random copolymer (average molecular weight  $M_r = 3900$ ) of 50% ethylene oxide and 50% propylene oxide, was obtained from Union Carbide (NY, USA). Potassium citrate tri-basic monohydrate ( $K_3C_6H_5O_7 \cdot H_2O$ , purum p.a., >99.0 wt.%) was purchased from Fluka Analytical. Sodium formate ( $NaCHO_2$ , puriss. p.a. ACS > 99.0 wt.%), potassium formate ( $KCHO_2$ , purum p. a., >99.0 wt.%) and laccase (from *Trametes versicolor*) were supplied by Sigma-Aldrich Ltd. All products were used as received without further purification. Stock solutions of each chemical were prepared in deionized water (ca. 50 wt.% for UCON, 40 wt.% for potassium citrate, 40 wt.% for sodium formate and 30 wt.% for potassium formate), and all concentrations were obtained gravimetrically after evaporation on a heating plate (Stuart hot plate SB300) for salts or after lyophilization (Scan Vac, model CoolSafe 55-4) for UCON. Deionized water was used for all diluting purposes. All weighing was carried out on an Adam Equipment balance model AAA250L, precise to within  $\pm 0.2$  mg.

### 2.2. Phase diagram determination

The binodal curves were obtained experimentally using the cloud-point method. Known masses of polymer and salt stock solutions were added to assay tubes to obtain several different heterogeneous systems. The tubes were vigorously shaken in a vortex mixer (VWR, model VV3) and placed into a thermostatic bath (Techne, Tempette TE-8D) at  $(23 \pm 0.2)$  °C. Known amounts of water were successively added to the tubes until one homogeneous phase was obtained. The binodal curves were determined for each system using different initial masses of polymer and salt

stock solutions. Each experimental curve was adjusted to the empirical equation suggested by Merchuk and co-workers [18]:

$$y = a \cdot \exp(b \cdot x^{0.5} - c \cdot x^3) \quad (1)$$

where  $y$  and  $x$  are the polymer and salt compositions in mass fraction, respectively, and  $a$ ,  $b$  and  $c$  are adjustable parameters. These parameters were obtained by nonlinear regression (least-squares) using the SigmaPlot v5.0 (SPSS Inc.) software.

Four different tie-lines were determined for UCON-potassium citrate system and three different tie-lines for each UCON-formate salt systems. Biphasic systems with known compositions of UCON and salt (total weight of 15 g) were prepared by weight in decanting ampoules, vigorously shaken, and then allowed to equilibrate for at least 48 h, in a thermostatic bath (Grant, LTC1, GD120) at  $23 \pm 0.1$  °C. Samples of both top and bottom phases were withdrawn (top phase using a pipette, bottom phase through the valve of the ampoule). Salt concentration was measured in both phases, at 23 °C, by electrical conductivity using a WTW LF538 Conductivity Meter with a standard conductivity cell TetraCon 325 (a 4-electrode conductivity cell) precise to within  $\pm 0.5\%$ . For each salt, a calibration curve was obtained after measuring the electrical conductivity of several stock solutions with known salt concentrations within the range 0.01–0.2% (w/w). Polymer interference in the electrical conductivity measurements was tested in the range 0.01–0.4% (w/w), and no interferences were observed. Average errors in salt concentration calculated using the calibration lines were below 1% for all salts used. Three samples from the top and bottom phases were withdrawn and conveniently diluted, and the electrical conductivity was measured. Final salt concentration of each phase was obtained from the average of the three measurements (standard deviations less than 0.15%), after correction with the dilution factor. The UCON composition in the top phase was obtained after lyophilization: three samples of the top phase were taken, diluted with water (1/5), and placed in the freezer at  $-18$  °C for 24 h. After being frozen, the samples were freeze-dried, and the average dry weight was determined (standard deviation less than 0.31%). The final polymer concentration for the top phase was calculated by subtraction of the corresponding salt concentration from the average dry weight. Due to the low concentration of polymer in the bottom phase and the errors associated to salt quantification by electrical conductivity, the values obtained for UCON concentration by lyophilization were not satisfactory in this phase. For this reason, the UCON concentration in bottom phases was calculated using equation (1).

### 2.3. Laccase partition

Partition coefficients were determined with the same procedure used in previous works [19,20]. A laccase stock solution was prepared in deionized water with concentration 4 mg/mL. Partitioning studies were performed by adding different amounts of laccase stock solution to six ATPS replicates, in order to get a final concentration ranging from 0 up to 200  $\mu$ g/mL. Each system has the same ATPS composition but different laccase concentrations, to ensure that protein aggregation effects were avoided. The components were vortex mixed (VWR, model VV3) for 2 min and then centrifuged (minispin, Eppendorf) at  $10^4$  r.p.m. for 15 min to ensure complete phase separation. After that, the biphasic systems were placed into a thermostatic bath (Techne, Tempette TE-8D) at  $23 \pm 0.2$  °C during approximately 2 h to allow interface clarification. Samples of each phase were withdrawn, conveniently diluted with water, and their absorbance at 280 nm was measured in a UV-Vis spectrophotometer (Thermo Scientific, Genesys 10S). Partition coefficients ( $K$ ) for laccase were determined as the slope of the straight line obtained when comparing laccase absorbance in the

**TABLE 1**

Compositions (in mass fraction) determined for the feed, top, and bottom phases, and the corresponding tie-line length (TLL) and slope (STL).

Feed		Top phase		Bottom phase		STL	TLL
Salt	Polymer	Salt	Polymer	Salt	Polymer		
<i>UCON-potassium citrate</i>							
0.0701	0.1610	0.0247	0.3064	0.1163	0.0073	-3.27	0.313
0.0804	0.1711	0.0206	0.3543	0.1361	0.0013	-3.06	0.371
0.0900	0.1823	0.0166	0.3964	0.1543	0.0002	-2.88	0.419
0.1013	0.1899	0.0134	0.4356	0.1728	0.00001	-2.73	0.464
<i>UCON-sodium formate</i>							
0.0802	0.2185	0.0392	0.4222	0.1110	0.0175	-5.60	0.411
0.0902	0.2278	0.0259	0.5239	0.1296	0.0040	-4.97	0.530
0.0999	0.2378	0.0182	0.6120	0.1457	0.0008	-4.77	0.624
<i>UCON-potassium formate</i>							
0.1091	0.1990	0.0465	0.4733	0.1413	0.0201	-4.72	0.463
0.1194	0.2086	0.0407	0.5359	0.1619	0.0086	-4.33	0.541
0.1296	0.2180	0.0387	0.5998	0.1765	0.0044	-4.30	0.611

Uncertainty:  $\pm 0.0002 \pm 0.0004 \pm 0.0012 \pm 0.0025$ .

top phase against that in that bottom phase, for the six replicates prepared, corrected with the corresponding dilution factors (DF, the final volume divided by the initial volume):

$$K = \frac{Abs_{Top} \cdot DF_{Top}}{Abs_{Bottom} \cdot DF_{Bottom}} \quad (2)$$

### 3. Results and discussion

#### 3.1. UCON + salt + water ATPS

The (liquid + liquid) equilibrium data of assayed UCON-salt ATPS are given in table 1. The tie-line lengths (TLL) were calculated using the equation:

$$TLL = \sqrt{(W_{UCON}^T - W_{UCON}^B)^2 + (W_{Salt}^T - W_{Salt}^B)^2} \quad (3)$$

Where  $W_{UCON}^T$ ,  $W_{Salt}^T$ ,  $W_{UCON}^B$ ,  $W_{Salt}^B$  are the top (T) and bottom (B) equilibrium mass fractions of UCON and salts. TLL is expressed in mass fraction. The slopes of the tie-lines (STL) were obtained from the linear regression of the top, bottom, and feed compositions of each tie-line. The corresponding values for the TLL and STL are also presented in table 1.

The experimental binodal curves for the three UCON-salt ATPS were successfully adjusted to the equation of Merchuk and co-workers. The values obtained for the parameters  $a$ ,  $b$ , and  $c$  in equation 1 are shown in table 2, together with the coefficients of determination ( $r^2$ ) and  $F$ -test results.

Several equations have been proposed to evaluate the reliability of tie-line data in (liquid + liquid) equilibrium. The equations suggested by Othmer-Tobias (equation 4) and Bancroft (equation 5) are the most widely used [21]:

$$\text{Log} \left( \frac{1 - W_{UCON}^T}{W_{UCON}^T} \right) = k_1 + n \cdot \text{Log} \left( \frac{1 - W_{Salt}^B}{W_{Salt}^B} \right) \quad (4)$$

$$\text{Log} \left( \frac{W_{water}^B}{W_{Salt}^B} \right) = k_2 + r \cdot \text{Log} \left( \frac{W_{water}^T}{W_{UCON}^T} \right) \quad (5)$$

**TABLE 2**

Number of solubility points used to determine each binodal curve ( $N$ ), together with the adjustable parameters ( $a$ ,  $b$  and  $c$ ) obtained from Merchuk equation (equation 1) and respective coefficients of determination ( $r^2$ ) and  $F$ -test.

ATPS	$N$	$a$	$b$	$c$	$r^2$	$F$
UCON-potassium citrate	12	1.09 $\pm$ 0.04	-7.3 $\pm$ 0.3	1595 $\pm$ 128	0.9995	9711
UCON-sodium formate	21	1.66 $\pm$ 0.09	-7.2 $\pm$ 0.4	1570 $\pm$ 174	0.9963	2396
UCON-potassium formate	15	8.6 $\pm$ 1.1	-13.5 $\pm$ 0.6	343 $\pm$ 105	0.9981	3229

where  $W_{water}^T$  and  $W_{water}^B$  are the equilibrium mass fractions of water in the top and bottom phases, respectively. All other compositions were defined above. Both equations were applied to the (liquid + liquid) equilibrium data determined in this work (see table 1) and a satisfactory linearization was found (figure 1). The values obtained for the fit parameters and the respective coefficients of determination ( $r^2$ ) are presented in table 3. For both equations, the  $r^2$  are better than 0.98.

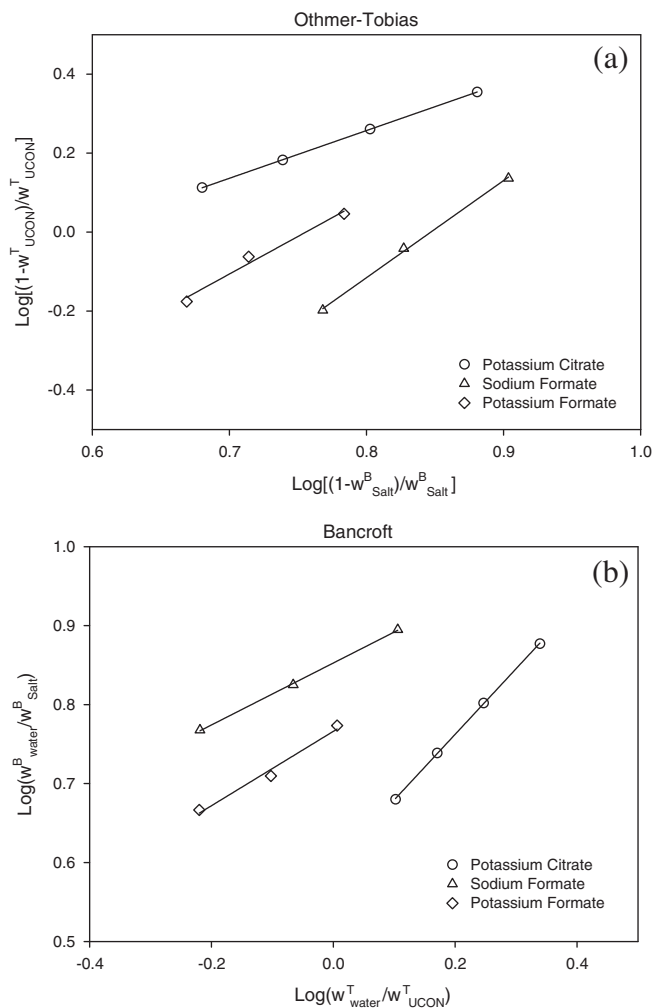
Figures 2 and 3 present the phase diagrams corresponding to the UCON-salt ATPS at 23 °C. They include the binodal curve (which represents the borderline between the one-phase and the two-phase regions) and the tie-lines (which relate the compositions of the two equilibrium phases). Moreover, literature data of the UCON-sodium citrate ATPS at 20 °C [17] were added to figure 2 for the comparison of UCON-citrate ATPS. According to these figures, and considering only the ATPS obtained in this work, UCON-potassium citrate ATPS provided the largest heterogeneous region and UCON-potassium formate the smallest one. However, considering all four salts present in figures 2 and 3, sodium citrate produced the largest heterogeneous region.

#### 3.2. Effect of salt type on the binodal curves

Several researchers have studied the influence of the type of inorganic salts on the phase separation of ATPS [22,23], suggesting that the stronger is the hydration of the ion, the lower is the concentration required for phase splitting. Therefore, salts having the same anion can be easily compared through the free energy of hydration ( $\Delta G_{hyd}$ ) of the cations: The salt whose cation has more negative  $\Delta G_{hyd}$  is better (less salt concentration is needed) at salting-out UCON. A comparison of the influence of the cation (Figures 2 and 3) deserves further attention.  $Na^+$  is clearly more effective than  $K^+$  in ATPS formation, and the biphasic regions obtained with sodium salts are larger than the corresponding potassium salts in all cases. An equivalent effect on the size of the heterogeneous region was reported in a previous work for UCON-(Sodium or Potassium) phosphate [24]. This behavior directly follows the cation's  $\Delta G_{hyd}$  values: -295 kJ/mol for  $K^+$  and -365 kJ/mol for  $Na^+$ . These  $\Delta G_{hyd}$  values were taken from Marcus [25].

The salting-out effects are additive and the anions have a contribution as well (with the same arguments as offered for cations). When comparing salts having the same cation, more negative  $\Delta G_{hyd}$  indicates a larger hydration shell and consequently a better salting-out of the polymer. As can be seen in figures 2 and 3, UCON-formate salts ATPS present smaller heterogeneous regions than the corresponding UCON-citrate salts. This behavior can be explained if the  $\Delta G_{hyd}$  values for the anions are compared: -2793 kJ/mol for citrate [26] and -395 kJ/mol for formate [25].

Nevertheless, when compared with other anions, citrate fails to follow such tendency imposed by  $\Delta G_{hyd}$ . Figure 4 compares the results obtained in this paper for UCON- $Na_2HCO_2$  with those obtained in previous works [17,20,24] using different salts (sharing a common cation:  $Na^+$ ). Ananthapadmanabham and Goddard [23] observed that the higher the valence of the anion, the lower the concentration required to form ATPS, using PEG combined with up to eight different sodium salts (including phosphate, sulfate,



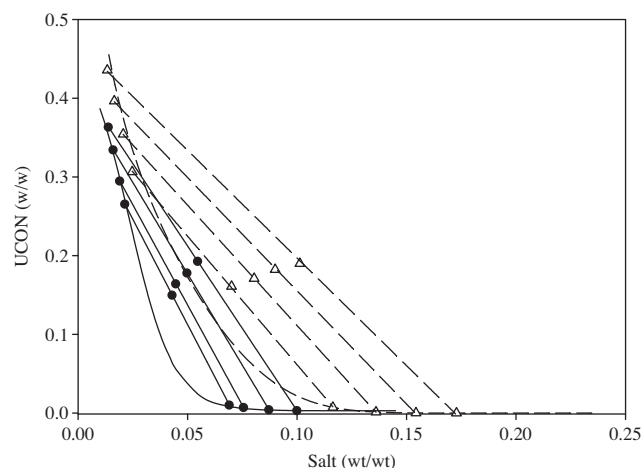
**FIGURE 1.** Linearization of the liquid-liquid equilibrium data determined for the UCON-salt ATPS using: (a) Othmer–Tobias equation and (b) Bancroft equation.

citrate, formate and others). They postulated that anions with a higher valence are better salting-out agents because they hydrate more water than lower valence anions, thus decreasing the amount of water available to hydrate the polymer. According to figure 4, the anion ability to form ATPS with UCON follows the tendency:  $C_6H_5O_7^{3-} \approx HPO_4^{2-} \approx SO_4^{2-} > H_2PO_4^- > HCO_2^-$ . Thus, although  $C_6H_5O_7^{3-}$  has the highest valence, the effects produced in the binodal curve are very similar to those obtained with the bivalent anions  $HPO_4^{2-}$  and  $SO_4^{2-}$ . As expected,  $H_2PO_4^-$  and  $HCO_2^-$  presented the smallest heterogeneous regions. Another way to explain the anion effect in the binodal curve is by means of the  $\Delta G_{hyd}$ , as discussed above. Considering the  $\Delta G_{hyd}$  values for the anions represented in figure 4, the following order is obtained:  $C_6H_5O_7^{3-}$  ( $\Delta G_{hyd} = -2793$  kJ/mol) [26] <  $HPO_4^{2-}$  ( $\Delta G_{hyd} = -1125$  kJ/mol) <  $SO_4^{2-}$  ( $\Delta G_{hyd} = -1080$  kJ/mol) [25] <  $H_2PO_4^-$  ( $\Delta G_{hyd} = -465$  kJ/mol) [25] <  $HCO_2^-$  ( $\Delta G_{hyd} = -395$  kJ/mol) [25]. The  $\Delta G_{hyd}$  value for  $HPO_4^{2-}$  is not available, thus it was calculated using the method

**TABLE 3**

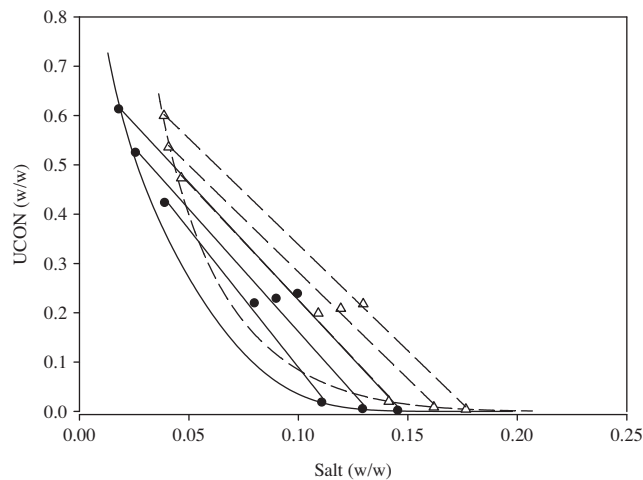
Fit parameters obtained for Othmer–Tobias equation (equation 4) and Bancroft equation (equation 5) and the corresponding coefficients of determination ( $r^2$ ).

ATPS	Othmer–Tobias			Bancroft		
	$n$	$k_1$	$r^2$	$r$	$k_2$	$r^2$
UCON-potassium citrate	1.209	0.195	>0.999	0.831	3.946	>0.999
UCON-sodium formate	2.461	0.008	0.999	0.392	7.124	>0.999
UCON-potassium formate	1.904	0.036	0.982	0.471	5.836	0.982

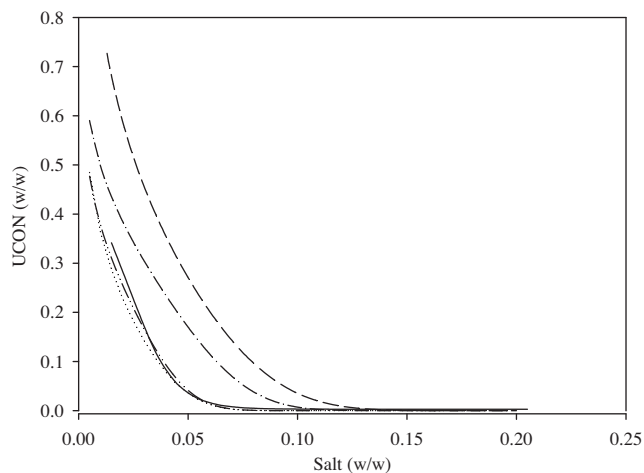


**FIGURE 2.** Binodal curve (correlation with equation 1) and tie-lines (experimental) for the systems: UCON-sodium citrate (—) obtained at 20 °C [17] and UCON-potassium citrate (---) obtained at 23 °C.

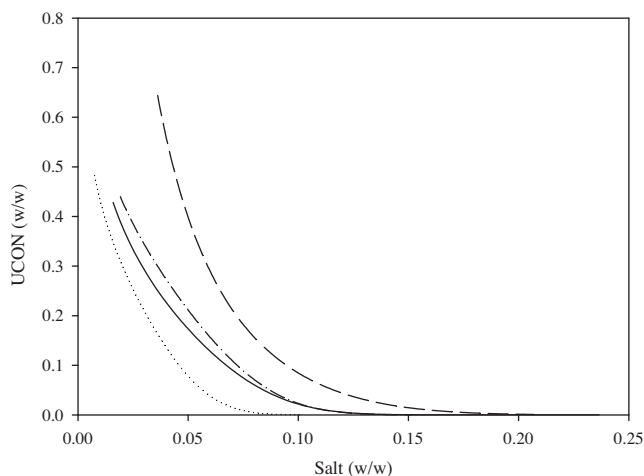
proposed by Marcus [25] with the ionic radius taken from literature [27]. According to these  $\Delta G_{hyd}$  values, the  $C_6H_5O_7^{3-}$  anion was expected to be the most effective in ATPS formation, but this is not observed in figure 4. The similar  $\Delta G_{hyd}$  values for  $HPO_4^{2-}$  and  $SO_4^{2-}$  are in agreement with the tendency obtained ( $HPO_4^{2-} \approx SO_4^{2-}$ ). For the other anions ( $HCO_2^-$  and  $H_2PO_4^-$ ), the effectiveness of the anion in forming an aqueous two-phase system with UCON follows the  $\Delta G_{hyd}$  values or the valence number. In figure 5, the results obtained in this work for UCON-potassium salt are compared with those obtained from literature for UCON-potassium phosphate salts [24]. According to this figure, the anion ability to form ATPS with UCON follows the tendency:  $HPO_4^{2-} > C_6H_5O_7^{3-} > H_2PO_4^- > HCO_2^-$ . Once more, although the highest valence and most negative  $\Delta G_{hyd}$  of  $C_6H_5O_7^{3-}$ , this anion was not the most effective in ATPS formation, providing a binodal curve close that obtained with  $H_2PO_4^-$ . In fact, the largest heterogeneous region was obtained with the bivalent anion  $HPO_4^{2-}$ . A similar behavior was found when UCON is replaced by PEG8000 (or PEG6000, found as similar to PEG8000 for several PEG-salt ATPS [16,28,29]) and combined with  $Na_3C_6H_5O_7$  [16],  $Na_2SO_4$  [30],  $Na_2HPO_4$  [31],  $NaH_2PO_4$  [31], and  $NaHCO_2$  [16] (Figure 6). In this case, the tendency found was:  $HPO_4^{2-} \approx SO_4^{2-} > C_6H_5O_7^{3-} > H_2PO_4^- > HCO_2^-$ . Therefore, the most effective anions in ATPS formation were  $HPO_4^{2-}$  and  $SO_4^{2-}$  and not



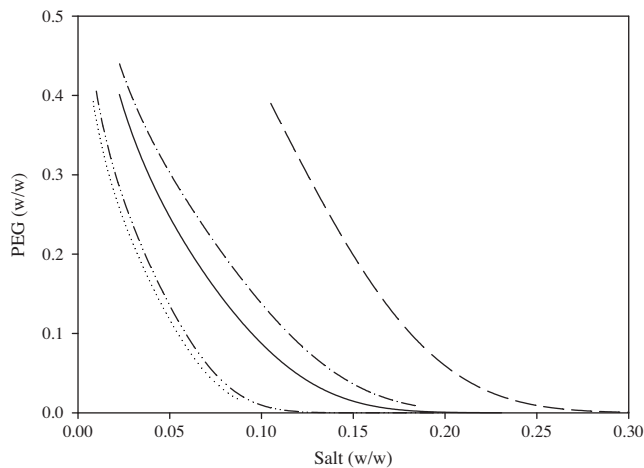
**FIGURE 3.** Binodal curve (correlation with equation 1) and tie-lines (experimental) obtained at 23 °C for the systems: UCON-sodium formate (—) and UCON-potassium formate (---).



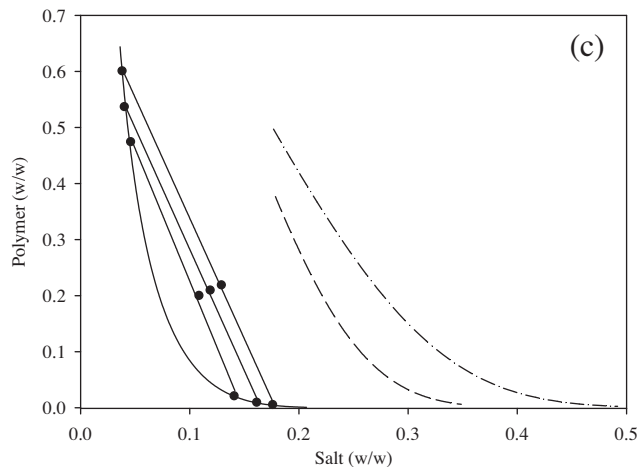
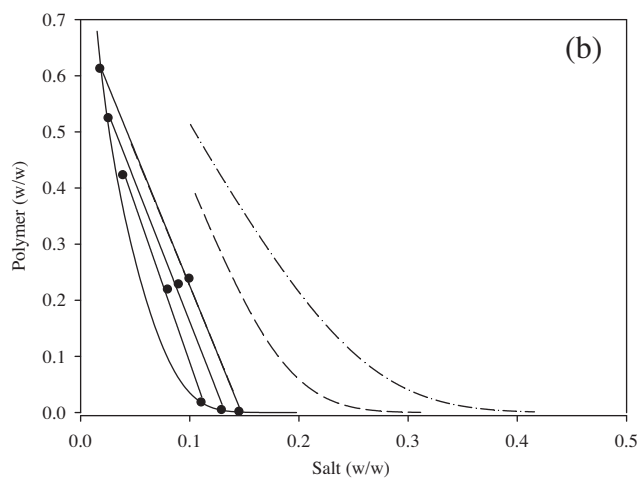
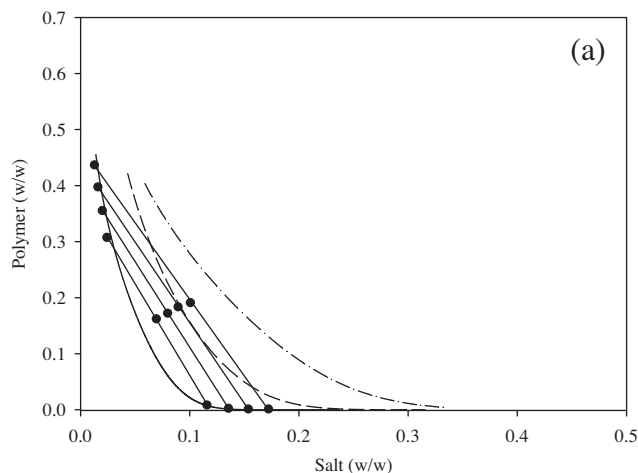
**FIGURE 4.** Effect of salt type on the binodal curves of UCON-sodium salt ATPS:  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$  (—) [17];  $\text{Na}_2\text{SO}_4$  (-·-·-) [20];  $\text{Na}_2\text{HPO}_4$  (·) [24];  $\text{NaH}_2\text{PO}_4$  (- - -) [24] and  $\text{NaHCO}_2$  (- -) , this work;



**FIGURE 5.** Effect of salt type on the binodal curves of UCON-potassium salt ATPS:  $\text{K}_3\text{C}_6\text{H}_5\text{O}_7$  (—), this work;  $\text{K}_2\text{HPO}_4$  (·) [24];  $\text{KH}_2\text{PO}_4$  (- - -) [24] and  $\text{KHCO}_2$  (- -), this work.



**FIGURE 6.** Effect of salt type on the binodal curves of PEG (8000 or 6000, see text)-sodium salt ATPS:  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$  (—) [16];  $\text{Na}_2\text{SO}_4$  (-·-·-) [30];  $\text{Na}_2\text{HPO}_4$  (·) [31];  $\text{NaH}_2\text{PO}_4$  (- - -) [31] and  $\text{NaHCO}_2$  (- -) [16].



**FIGURE 7.** Binodal curves at 23 °C for (a) polymer-potassium citrate, (b) polymer-sodium formate and (c) Polymer-potassium formate. UCON (—), this work; PEG1500 (-·-·-) [16] and PEG8000 (- - -) [16].

$\text{C}_6\text{H}_5\text{O}_7^{3-}$ , as expected from anion valence or  $\Delta G_{\text{hyd}}$ . The same trend was also observed by Zafarani-Moattar [15] for citrate. From the above mentioned, it is clear that the empirical rule for ATPS formation based on ion valence or  $\Delta G_{\text{hyd}}$  is not followed by citrates.

### 3.3. Effect of polymer type on the binodal curves

The phase diagrams for the ATPS obtained in this work can be compared with previous results for PEG-salt ATPS [16], as presented



**TABLE 4**

Laccase partition coefficients obtained from equation 6 and the corresponding coefficient of determination ( $r^2$ ), together with the volume ratio (VR) and ATPS composition (in mass fraction).

ATPS	Composition			Partition coefficient	
	Salt	UCON	VR <sup>a</sup>	K	r <sup>2</sup>
UCON-Sodium Citrate[17]	0.0432	0.1488	0.68	0.559 ± 0.006	0.999
UCON-Potassium Citrate	0.0701	0.1610	0.82	0.515 ± 0.005	0.999
UCON-Sodium Formate	0.0802	0.2185	0.35	0.501 ± 0.008	0.998
UCON-Potassium Formate	0.1091	0.1990	0.51	0.480 ± 0.007	0.998

<sup>a</sup> VR =  $V_{\text{Top}}/V_{\text{Bottom}}$ .

in figure 7. Inspection of this figure shows that less salt and polymer are required for phase splitting in the UCON-salt ATPS compared to ATPS with PEG. This is because UCON is more hydrophobic than PEG (as indicated above, UCON is a random co-polymer of ethylene glycol and propylene glycol monomers). This makes the process more economical and reduces the environmental problems. Tubio et al. [17] and Silvério et al. [20] observed the same trend in UCON- or PEG-salt ATPS, with the salts:  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ ,  $\text{Li}_2\text{SO}_4$ ,  $\text{Na}_2\text{SO}_4$ , and  $(\text{NH}_4)_2\text{SO}_4$ .

### 3.4. Laccase partition

Laccase partition coefficients were determined at 23 °C in UCON- $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ , UCON- $\text{K}_3\text{C}_6\text{H}_5\text{O}_7$ , UCON- $\text{NaHCO}_2$  and UCON- $\text{KHCO}_2$  ATPS. For each biphasic system, six replicates with different laccase concentrations were prepared. A straight line was obtained when laccase absorbance in the top phase (UCON-rich phase) was plotted against laccase absorbance in the bottom phase (salt-rich phase), for the six replicates (data not shown). Laccase partition coefficient ( $K$ ) was obtained, after linear regression, from the slope of the straight line:

$$\text{Abs}_{\text{Top}} = K \cdot \text{Abs}_{\text{Bottom}} + b \quad (6)$$

where  $b$  corresponds to the intercept and it was found to be very close to zero in all cases (average  $b = 0.006$ ). The linearity observed in all cases indicates that all partition coefficients calculated using this procedure have thermodynamic meaning, since they are independent of the solute concentration. Moreover, this linearity confirms that there are no interactions affecting solute behaviour, like solute self-association or dissociation [5]. Laccase partition coefficients and the corresponding coefficient of determination ( $r^2$ ) found for each straight line are presented in table 4. This table also includes the volume ratio (VR, top phase volume divided by bottom phase volume) and the composition of the ATPS where laccase partition was performed. The smallest tie-lines of each ATPS were chosen for the partition studies because less salt and polymer are required and they present lower viscosities.  $K$  values lower than unity (from 0.480 up to 0.559) were obtained for all the ATPS. This means that laccase concentration in the salt-rich phase was approximately 2-fold higher than that in the UCON-rich phase. UCON-Potassium Formate ATPS presented the best partition coefficient, but differences are small and all UCON-salt ATPS can be of interest for laccase extraction.

## 4. Conclusions

Phase diagrams for three polymer-salt ATPS at 23 °C are presented: UCON-potassium citrate, UCON-sodium formate and UCON-potassium formate. The effect of the salts (anion and cation)

in ATPS formation was discussed. Among the salts studied here, potassium citrate proved to be the most effective for ATPS formation, while potassium formate required the largest concentration to achieve phase splitting. Due to UCON being more hydrophobic than PEG, these ATPS provided a biphasic system with a heterogeneous region larger than that of the corresponding PEG systems. In addition, advantages can be taken from the thermo-separating properties of UCON to recycle this polymer from its aqueous solution, thus reducing the economical and environmental impact. Laccase partition coefficients determined for the UCON-salt ATPS showed that in all cases laccase concentration in the salt-rich phase was approximately 2-fold higher than in the bottom phase, suggesting these ATPS can be suitable for laccase extraction.

## Acknowledgments

This work is supported by project PEst-C/EQB/LA0020/2011, financed by FEDER through COMPETE – Programa Operacional Factores de Competitividade and by FCT – Fundação para a Ciência e a Tecnologia, Portugal. SCS and OR acknowledge financial support of FCT (Ph.D. grant SFRH/BD/43439/2008) and Programme Ciência 2007 (FCT), respectively. EL acknowledges financial support of Ministerio de Educacion y Ciencia of Spain (doctoral fellowship EX2009-0404)

## References

- [1] P.A. Albertsson, Partition of Cell Particles and Macromolecules, third ed., Wiley and Sons, New York, 1986.
- [2] B.A. Andrews, J.A. Asenjo, Sep. Sci. Technol. 45 (2010) 2165–2170.
- [3] M. Rito-Palomares, J. Chromatogr. B 807 (2004) 3–11.
- [4] M.R. Kula, K.H. Kroner, H. Hustedt, Adv. Biochem. Eng. 24 (1982) 73–118.
- [5] B.Y. Zaslavsky, Aqueous Two-Phase Partitioning, Marcel Dekker Inc., New York, 1995.
- [6] A. Veide, T. Leindbäck, S.O. Enfors, Enzyme Microb. Technol. 6 (1984) 325–330.
- [7] Y.-T. Wu, M. Pereira, A. Venâncio, J. Teixeira, Bioprocess Eng. 9 (2000) 247–254.
- [8] J. Persson, A. Kaul, F. Tjerneld, J. Chromatogr. B 743 (2000) 115–126.
- [9] S. Saeki, N. Kuwahara, M. Nakata, M. Kaneko, Polymer 17 (1976) 685–689.
- [10] M.R. Kula, A. Greve, Bioproc. Eng. 4 (1991) 173–177.
- [11] J. Vernau, M.R. Kula, Biotechnol. Appl. Biochem. 12 (1990) 397–404.
- [12] M. Berlo, K.Ch.A.M. Luyben, L.A.M. der Wielen, J. Chromatogr. B 711 (1998) 61–68.
- [13] M.T. Zafarani-Moattar, R. Sadeghi, A.A. Hamidi, Fluid Phase Equilib. 219 (2004) 149–155.
- [14] T. Murugesan, M. Perumalsamy, J. Chem. Eng. Data 50 (2005) 1392–1395.
- [15] M.T. Zafarani-Moattar, Sh. Hamzehzadeh, CALPHAD 29 (2005) 1–6.
- [16] S.C. Silvério, A. Wegrzyn, E. Lladosa, O. Rodríguez, E.A. Macedo, J. Chem. Eng. Data 57 (2012) 1203–1208.
- [17] G. Tubio, B.B. Nerli, G. Picó, A. Venâncio, J. Teixeira, Sep. Purif. Technol. 65 (2009) 3–8.
- [18] J.C. Merchuk, B.A. Andrews, J.A. Asenjo, J. Chromatogr. B 711 (1998) 285–293.
- [19] O. Rodríguez, S.C. Silvério, P.P. Madeira, J.A. Teixeira, E.A. Macedo, Ind. Eng. Chem. Res. 46 (2007) 8199–8204.
- [20] S.C. Silvério, P.P. Madeira, O. Rodríguez, J.A. Teixeira, E.A. Macedo, J. Chem. Eng. Data 53 (2008) 1622–1625.
- [21] D.F. Othmer, P.E. Tobias, Ind. Eng. Chem. 34 (1942) 690–692.
- [22] E.A. Boucher, P.M. Hines, J. Polym. Sci. Polym. Phys. 14 (1976) 2241–2251.
- [23] K.P. Ananthapadmanabhan, E.D. Gobbard, Langmuir 3 (1987) 25–31.
- [24] S.C. Silvério, O. Rodríguez, J.A. Teixeira, E.A. Macedo, J. Chem. Eng. Data 55 (2010) 1285–1288.
- [25] Y. Marcus, J. Chem. Soc. Faraday Trans. 87 (1991) 2995–2999.
- [26] M.T. Zafarani-Moattar, Sh. Hamzehzadeh, Fluid Phase Equilib. 304 (2011) 110–120.
- [27] Y. Marcus, J. Chem. Soc. Faraday Trans. 89 (1993) 713–718.
- [28] C.W. Ooi, T.T. Beng, S.L. Hii, A. Ariff, H.S. Wu, J.C.W. Lan, R.S. Juang, S.M.M. Kamal, T.C. Ling, Biotechnol. Bioprocess Eng. 14 (2009) 811–818.
- [29] L.P. Malpiedi, C. Fernández, G. Picó, B. Nerli, J. Chem. Eng. Data 53 (2008) 1175–1178.
- [30] L.A. Ferreira, J.A. Teixeira, J. Chem. Eng. Data 56 (2011) 133–137.
- [31] M.T. Zafarani-Moattar, R. Sadeghi, Fluid Phase Equilib. 181 (2001) 95–112.