



# LIQUID-LIQUID EQUILIBRIUM DATA, VISCOSITIES, DENSITIES, CONDUCTIVITIES, AND REFRACTIVE INDEXES OF POLY (VINYL PYRROLIDONE)+ TRI-SODIUM CITRATE + UREA AQUEOUS TWO-PHASE SYSTEMS AT DIFFERENT PH

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**Abstract** – The present study investigated liquid–liquid equilibria of polyvinylpyrrolidone (PVP) K30 + tri-sodium citrate + urea (5% and 10% mass) aqueous two-phase systems at 25°C and pH values of 6.2, 7.4, and 10.0. Results showed that the binodal was displaced toward higher concentrations as the urea concentration increased. The effect of pH, polymer/salt (w/w) ratio, urea concentration, tie line length, and slope of tie line on the partition behavior of urea was examined. The viscosity, density, electrical conductivity, and refractive index of PVP (K30) + tri-sodium citrate + urea + water two-phase systems were measured versus pH value. The relation between tie line length and the density and viscosity of the aqueous two-phase systems was also examined.

**Keywords:** Aqueous two-phase systems; Phase diagram; Urea; Viscosity; Polyvinylpyrrolidone

## INTRODUCTION

An aqueous two-phase system (ATPS) is a liquid–liquid extraction strategy that can efficiently separate biological materials such as recombinant protein and enzymes (Albertsson, 1986; Hatti-Kaul, 2000). Polyethylene glycol (PEG) is an important component of two-phase partitioning. PVP is a water soluble polymer that can be applied with suitable salts to form ATPSs. This polymer is used in industrial production, medicine, and pharmacology

(Bühler, 2005; Wang et al.2000; Zafarani-Moattar et al. 2015). PVP is a biocompatible alternative that is an inexpensive and stable polymer with good potential for aqueous two-phase extraction (Zafarani-Moattar and Zafaranloo, 2009).

Citrates have applications in biochemical and chemical reactions. They are used as substitutes for inorganic salts to form ATPSs along with PVP for protein extraction. They are biodegradable, nontoxic, and can be directly discharged into biological wastewater treatment plants (Sadeghiet al.2006). Table 1 lists some ATPS containing PVP.

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**Table 1.** Parameters of ATPSs containing PVP that effect LLE

PVP (Mw)	Salt/Polymer	Effective parameter(s)	Reference
PVPK15 (10000)	Sodium di-hydrogen phosphate	Temperature	Zafarani-Moattar and Sadeghi (2002)
PVPK15 (10000)	Di-sodium hydrogen phosphate	Temperature; type of salt	Zafarani-Moattar and Sadeghi (2005)
	Tri-sodium phosphate		
PVP K15 (10000)	Magnesium sulfate	Temperature	Salabat et al. (2006)
PVPK15 (10000)	Potassium citrate	Temperature	Sadeghi (2006)
PVPK15 (10000)	Sodium citrate	Temperature	Sadeghi et al. (2006)
	Tri-potassium phosphate	Temperature	Zafarani-Moattar and Seifi-Aghjekohal (2007)
PVP K12 (3500)	Di-potassium hydrogen phosphate		
PVPK17 (3882)	Sodium sulfate	Molecular mass of PVP	Fedicheva et al. (2007)
PVPK30 (17750)			
PVPK90 (138600)			
PVP (24000)	Di-ammonium hydrogen phosphate/ ammonium di-hydrogen phosphate buffer	Temperature; pH	Foroutan and Zarrabi (2008)
PVPK12 (3500)	Di-potassium oxalate or di-potassium tartrate	Temperature	Zafarani-Moattar and Zaferanloo (2009)
	Ammonium phosphate	Type of ammonium salt; temperature	Wang et al. (2012)
	Ammonium sulfate		
PVPK30 (60000)	Di-ammonium carbonate		
	Ammonium tartrate		
	Ammonium citrate		

Inclusion body refolding is a vital step in the production of recombinant proteins. Solubilization of aggregated protein with denaturants such as guanidine hydrochloride and urea has been reported to be essential to recovery of active protein from the inclusion bodies (Clark, 2001). Urea is a powerful protein denaturant as it disrupts the noncovalent bonds in the proteins (Parnica and Antalika, 2014). Several articles have been written about the application of urea in ATPS for the initial recovery step (Rahimpour et al., 2010; Rämisch et al., 1999), but little has been published about the complex problem of how the urea affects the phase diagram behavior or about determining its partition coefficient in ATPS.

Despite these favorable features, ATPSs have not been extensively adopted for either industrial or commercial applications. The main reason is lack of knowledge about the mechanisms involved in partitioning equilibria of macromolecules, the lack of a comprehensive theory that can predict experimental trends, and the empirical nature of the method. This lack of knowledge has motivated researchers to study ATPSs (Yan-Min et al., 2010; Rocha and Nerli, 2013).

The present study obtained phase equilibrium data for PVP (K30) + tri-sodium citrate (pH = 6.2, 7.4, and 10.0) + urea (5% and 10% (mass) + water at 25°C. The effect of pH, PVP/salt ratio, urea concentration, tie line length (TLL), and slope of tie line (STL) on the partition behaviors of urea were also studied. The viscosity, density, electrical conductivity, and refractive index of the PVP + tri-sodium citrate + urea aqueous two-phase system and the top and bottom phases of the two-phase system were determined.

The relation between TLL and the density and viscosity of the ATPSs was also examined.

## EXPERIMENTAL

### Materials

PVP (K30; average MW = 60000; moisture content < 5.0% by mass fraction) was obtained from Aldrich. Tri-sodium citrate (anhydrous GR for analysis > 99%), sodium hydroxide (NaOH; mass purity > 0.99%) and sulfuric acid (95% to 97% H<sub>2</sub>SO<sub>4</sub>; GR for analysis > 95.0%) were obtained from Merck (Germany) and used without further purification. Urea was purchased from Sigma-Aldrich. Distilled, deionized water was used for the preparation of all solutions. All other materials were of analytical grade.

### Apparatus and Procedure

The biphasic systems were prepared by mixing PVP (K30), tri-sodium citrate, and urea at the required pH. The composition of the mixture was determined by mass. Feed samples (10 g) were prepared by mixing appropriate amounts of polymer, salt, urea, and water in 15 ml graduated tubes using an analytical balance (A&D model GF300; Japan) with a precision of  $\pm 10^{-4}$  g at 25°C. To maintain a constant temperature (25°C) with an uncertainty of 0.05°C, the tubes were placed in a thermostatic bath (Memert model INE400; Germany). The pH values of the salt solutions were adjusted by mixing the appropriate ratio

of tri-sodium citrate, sodium hydroxide, and sulfuric acid. The pH values of the solutions were measured precisely with a Metrohm 827pH lab meter (Switzerland). For each mentioned system, 5% and 10% (w/w) samples of urea were produced.

The contents of the test tube were rigorously vortexed for 10 min before being placed in a 25°C thermostatic bath for 2 h. To separate the resulting phases, the tubes were centrifuged (Hermle Z206A; Germany) at 6000 rpm for 5 min. The phases showed no turbidity and the top and bottom samples were easily separated. The electrical conductivity and refractive index for each sample were measured at 298.15 K using a JENWAY 4510 model with a precision of  $0.01\mu\text{S}^{-1}\text{mS}$  and a refractometer (Ceti; Belgium) with a precision of 0.0001 nD, respectively. All data measurements were conducted in duplicate and the average values were reported. The salt concentrations ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ ) were determined using atomic absorption spectroscopy (Shimatsu model AA-6300; Japan). The calibration plots of the refractive index and conductivity were prepared for the known polymer and urea compositions at the individual salt concentrations at 25°C. The measured values were then interpolated. The average relative deviation of the urea and polymer concentrations using this method was about 0.1% (wt).

## RESULTS AND DISCUSSION

The viscosity, density, electrical conductivity, and refractive index of the PVPK30 + tri-sodium citrate + urea + water systems are shown in Table 2. Because the electrical conductivity and the refractive index of the phase samples depend on PVP (K30), urea, and salt concentration, calibration plots of the refractive index versus polymer concentration were prepared for different concentrations of salt and urea. Sample calibration plots for aqueous solutions of PVP (K30) + sodium citrate + urea + water are shown in Figure 1.

To increase the knowledge about ATPSs containing urea, phase diagrams of a range of systems based on PVP (K30) + sodium citrate + water at 298.15 K and pH values of 6.2, 7.4, and 10.0 in the presence of urea (5% and 10% mass) were determined. The experimental data of the binodal, tie line data, and STL for the PVP (K30) + sodium citrate + urea + water system at 25°C and the physical properties of the top and bottom phases are reported in Table 3. All concentrations are expressed as mass percentages.

The experimental data of the binodal for PVP (K30) + sodium citrate + denaturant + water systems at 25°C and pH 6.2 at urea concentrations of 5% and 10% (w/w) are plotted in Figure 2.

**Table 2.** Density( $\rho$ ), viscosity, refractive index ( $n_D$ ), and electrical conductivity ( $k$ ) for aqueous single-phase system (PVP K30 (p) + tri-sodium citrate (s) + urea (u) + water system) at 298.15 K

$w_p$	$w_s$	$w_u$	$\rho$ kg.m <sup>-3</sup>	$\eta$ mPa.s	$n_D$	$k$ mS.cm <sup>-1</sup>
0	0	0	0.9970	0.894	1.3327	0.125
0	0	5	1.0137	0.987	1.3391	6.43
0	0	10	1.0215	1.104	1.3450	14.10
5	0	0	1.0061	1.046	1.3401	0.125
5	0	5	1.0203	1.215	1.3456	5.82
5	0	10	1.0348	1.426	1.3552	10.9
10	0	0	1.0115	1.210	1.3450	0.240
10	0	5	1.0340	1.460	1.3521	2.12
10	0	10	1.0468	1.751	1.3640	7.73
15	0	0	1.0210	1.460	1.3517	0.321
15	0	5	1.0470	1.798	1.3600	1.04
15	0	10	1.0559	2.213	1.3719	6.30
0	5	0	1.0266	2.362	1.3406	27.1
0	5	5	1.0425	2.693	1.3463	33.5
0	5	10	1.0538	2.941	1.3538	38.5
5	5	0	1.0390	2.706	1.3475	23.1
5	5	5	1.0524	2.973	1.3565	28.3
5	5	10	1.0649	3.201	1.3631	33.7
10	5	0	1.0500	2.992	1.3551	18.9
10	5	5	1.0644	3.231	1.3653	24.7
10	5	10	1.0776	3.449	1.3728	30.1
15	5	0	1.0605	3.294	1.3634	13.5
15	5	5	1.0761	3.478	1.3738	19.4
15	5	10	1.0916	3.686	1.3821	25.2
0	10	0	1.0540	5.655	1.3480	40.6

Table 2. Cont.

$w_p$	$w_s$	$w_u$	$\rho$		$\eta$		$n_D$		$k$	
			$\text{kg.m}^{-3}$		$\text{mPa.s}$				$\text{mS.cm}^{-1}$	
0	10	5	1.0711		6.205		1.3557		46.1	
0	10	10	1.0832		8.184		1.3602		51.1	
5	10	0	1.0708		6.284		1.3573		36.3	
5	10	5	1.0853		8.502		1.3656		41.3	
5	10	10	1.0950		10.35		1.3716		46.9	
10	10	0	1.0838		8.635		1.3687		31.8	
10	10	5	1.0935		10.52		1.3755		36.9	
10	10	10	1.1102		11.34		1.3817		42.1	
15	10	0	1.0967		10.59		1.3767		26.9	
15	10	5	1.1061		11.41		1.3855		32.3	
15	10	10	1.1218		11.76		1.3913		36.6	
0	15	0	1.0912		9.360		1.3563		46.8	
0	15	5	1.1038		16.36		1.3623		53.3	
0	15	10	1.1167		25.60		1.3679		55.4	
5	15	0	1.1032		16.80		1.3669		43.1	
5	15	5	1.1163		27.13		1.3731		49.1	
5	15	10	1.1313		38.08		1.3798		50.1	
10	15	0	1.1161		27.83		1.3775		38.6	
10	15	5	1.1295		39.09		1.3837		43.8	
10	15	10	1.1443		50.89		1.3897		45.7	
15	15	0	1.1338		40.59		1.3862		32.8	
15	15	5	1.1471		52.57		1.3937		38.2	
15	15	10	1.1591		65.29		1.4017		41.1	

Table 3. Phase composition, tie line data, STL, and physical properties for PVP (K30) + sodium citrate + urea (5% w/w) + water system at 25°C

pH	Total System (%mass)		Top phase (%mass)				Bottom phase (%mass)				Urea	TLL (%)	STL (%)	
	$100w_p$	$100w_s$	$100w_p$	$100w_s$	$\rho$	$\eta$	$100w_p$	$100w_s$	$\rho$	$\eta$				$100w_u$
					$\text{kg.m}^{-3}$	$\text{mPa.s}$			$\text{kg.m}^{-3}$	$\text{mPa.s}$				
6.2	13.60	16.00	23.40	9.04	1.1254	181.0	2.20	24.08	1.1678	3.589	5	25.99	1.41	
	13.00	16.00	22.52	9.47	1.1256	161.4	2.45	23.29	1.1636	3.585	5	24.37	1.45	
	12.30	15.20	20.79	9.75	1.1232	124.4	2.88	21.26	1.1515	3.497	5	21.29	1.56	
	12.00	15.10	19.78	10.18	1.1232	106.7	3.02	20.62	1.1481	3.483	5	19.75	1.61	
	11.00	14.50	17.65	10.65	1.1202	72.73	3.37	19.01	1.1391	3.450	5	16.55	1.71	
	10.50	14.10	16.76	10.73	1.1187	61.16	4.10	17.53	1.1312	3.486	5	14.37	1.86	
	13.60	16.00	21.11	10.41	1.1296	139.6	5.20	22.10	1.1614	3.944	10	19.74	1.36	
	13.00	16.00	20.11	10.72	1.1292	120.3	5.30	21.00	1.1543	3.874	10	18.03	1.44	
	12.30	15.20	19.16	10.96	1.1282	102.6	5.50	19.80	1.1473	3.823	10	16.27	1.55	
	12.00	15.10	18.01	11.15	1.1265	83.42	5.70	19.00	1.1427	3.799	10	14.60	1.57	
	11.00	14.50	17.48	11.31	1.1264	75.95	6.50	18.00	1.1380	3.836	10	12.85	1.64	
	10.50	14.10	16.56	11.60	1.1257	63.68	7.20	17.00	1.1334	3.861	10	10.81	1.73	
7.4	15.00	11.50	24.51	6.79	1.1131	180.9	1.17	18.41	1.1285	2.660	5	26.08	2.01	
	14.00	11.50	23.44	7.05	1.1115	155.3	1.30	17.33	1.1224	2.643	5	24.41	2.16	
	13.00	11.10	21.28	7.47	1.1091	114.9	1.81	15.96	1.1146	2.728	5	21.24	2.29	
	12.50	11.00	19.22	8.01	1.1072	83.16	2.33	15.48	1.1131	2.877	5	18.46	2.26	
	12.00	10.80	17.73	8.36	1.1052	63.57	3.09	14.61	1.1100	3.057	5	15.92	2.34	
	11.50	10.70	15.64	8.96	1.1039	42.74	4.39	13.69	1.1073	3.293	5	12.20	2.38	
	12.00	13.60	23.57	7.62	1.1170	170.8	3.50	17.93	1.1304	3.338	10	22.56	1.95	
	11.50	13.45	22.02	7.97	1.1155	138.0	3.91	17.22	1.1267	3.377	10	20.33	1.96	
	10.65	13.00	20.17	8.29	1.1129	103.8	4.62	15.90	1.1200	3.428	10	17.31	2.04	
	10.00	13.00	19.00	8.63	1.1121	86.06	4.96	15.36	1.1173	3.455	10	15.57	2.09	
	9.00	13.00	17.46	9.06	1.1112	65.93	5.45	14.56	1.1134	3.490	10	13.20	2.18	
	8.00	13.00	15.26	9.64	1.1094	42.74	6.38	13.75	1.1105	3.582	10	9.78	2.16	

Table 3. Cont.

pH	Total System (%mass)		Top phase (%mass)				Bottom phase (%mass)				Urea 100 w <sub>u</sub>	TLL (%)	STL (%)
	100wp	100ws	100wp	100ws	$\rho$ kg.m <sup>-3</sup>	$\eta$ mPa.s	100wp	100ws	$\rho$ kg.m <sup>-3</sup>	$\eta$ mPa.s			
10.0	15.00	9.30	27.19	4.13	1.1019	204.1	1.07	15.21	1.1076	2.375	5	28.38	2.36
	14.00	9.25	24.89	4.72	1.0995	156.0	1.23	14.54	1.1044	2.413	5	25.62	2.41
	13.00	9.20	23.17	5.09	1.0972	123.9	1.48	13.75	1.1003	2.471	5	23.35	2.51
	13.00	8.90	21.53	5.59	1.0967	100.8	2.71	12.98	1.0982	2.836	5	20.22	2.54
	12.50	8.90	19.80	6.08	1.0949	77.26	3.78	12.27	1.0968	3.080	5	17.17	2.59
	12.00	8.80	16.76	6.95	1.0927	45.99	5.01	11.57	1.0958	3.297	5	12.63	2.54
	14.00	10.00	24.40	5.27	1.1031	158.3	2.63	15.09	1.1102	2.922	10	23.88	2.22
	13.50	9.80	22.84	5.59	1.1013	129.2	3.37	14.39	1.1075	3.073	10	21.37	2.21
	13.20	9.70	21.69	5.98	1.1007	110.3	3.94	13.70	1.1049	3.170	10	19.35	2.3
	12.50	9.75	20.29	6.43	1.1001	90.61	5.01	12.86	1.1020	3.330	10	16.58	2.38
	12.45	9.70	18.90	6.90	1.0998	73.39	6.90	12.07	1.1014	3.572	10	13.06	2.32
	12.40	9.60	17.50	7.34	1.0991	57.97	7.89	11.52	1.1004	3.665	10	10.48	2.30

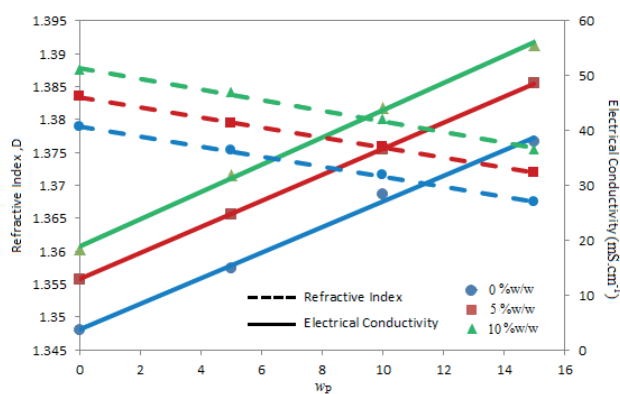


Figure 1. Refractive index and electrical conductivity calibration curves for PVP (K30) + sodium citrate + urea + water at 25°C (salt = 10% w/w)

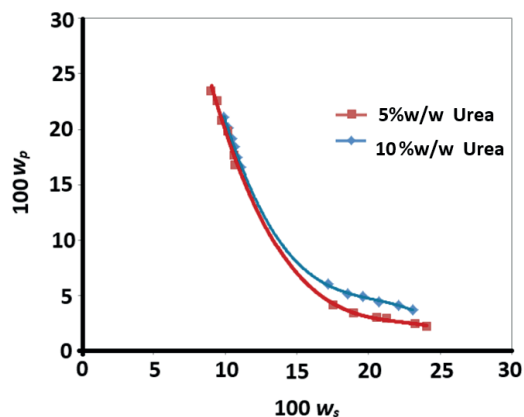


Figure 2. Binodal of PVP (K30) + sodium citrate systems at 25°C and pH 6.2 versus urea concentration.

The TLL for the different compositions with two phases were calculated as:

(1)

$$TLL = \sqrt{(C_p^{top} - C_p^{bottom})^2 + (C_s^{bottom} - C_s^{top})^2}$$

The STL is the ratio of the difference between the polymer concentration ( $C_p$ ) and the salt concentration ( $C_s$ ) in the top and bottom phases as:

$$STL = \frac{C_p^{top} - C_p^{bottom}}{C_s^{bottom} - C_s^{top}} \quad (2)$$

Figure 3 shows the effect of urea concentration on equilibrium phase composition and on STL and TLL for PVP (K30) + sodium citrate + H<sub>2</sub>O system at a pH value of 6.2. The influence of urea concentration on the binodal

curve for the PVP (K30) + sodium citrate APTS at a constant pH is shown in Tables 2 and 3 and Figures 2 and 3. It can be observed that the binodal was displaced toward higher concentrations as the urea concentration increased and that the composition of the polymer-rich phase (and TLL) behaved in a slightly shorter manner. The STL decreased as the urea concentration increased. This effect has also been reported for urea (Creighton, 1993; Rämisch et al. 1999; Silva and Meirelles, 2001) and guanidine hydrochloride (Pirdashti and Rahimpour, 2010; Pirdashti et al. 2015).

The opposing components found in these systems are structure-making salts; urea is sometimes described as a structure-breaking agent. The combination of the two competing components on phase separation is interesting and cannot be predicted (Rämisch et al. 1999, Bertoluzzo et al. 2007). Urea has a similar structure-breaking effect on water and the preferential interaction with an aqueous interface (Annuziata et al. 2002). PVP is a hydrophilic polymer. The effects of urea on water structure increased the depletion force between the PVP-urea-water causing

the ratio of PVP in the two phases to change and the phase regions to shrink.

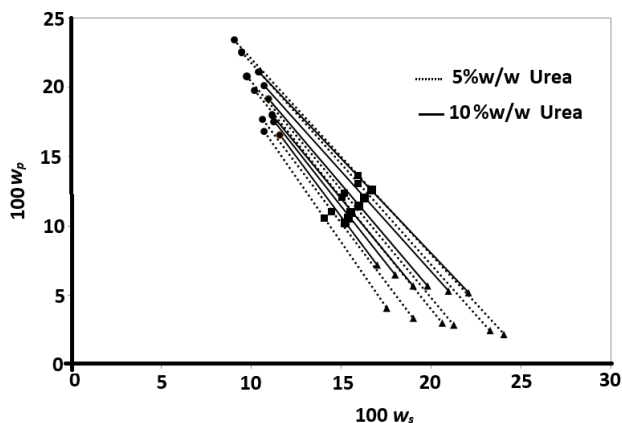
Urea added to ATPS as a solute is divided between the phases. Its partition coefficient,  $K$ , is defined as a function of the equilibrium concentrations of the solute in the upper and lower phases as:

$$K = \frac{[\text{Solute Concentration}]_{\text{top}}}{[\text{Solute Concentration}]_{\text{bottom}}} \quad (3)$$

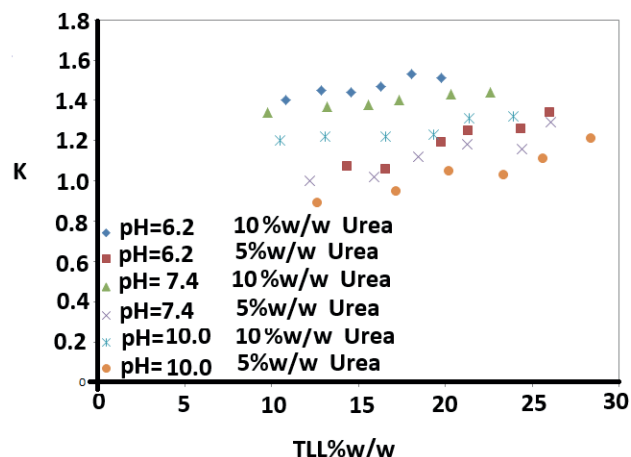
Figure 4 is a diagram of the urea partition coefficient versus TLL. It can be seen that, at a constant TLL, the partition coefficient of urea increased as the urea

concentration increased. The partitioning coefficient also increased as the TLL increased at a constant urea concentration. The slope of the partition coefficient of urea versus TLL decreased as the urea concentration increased.

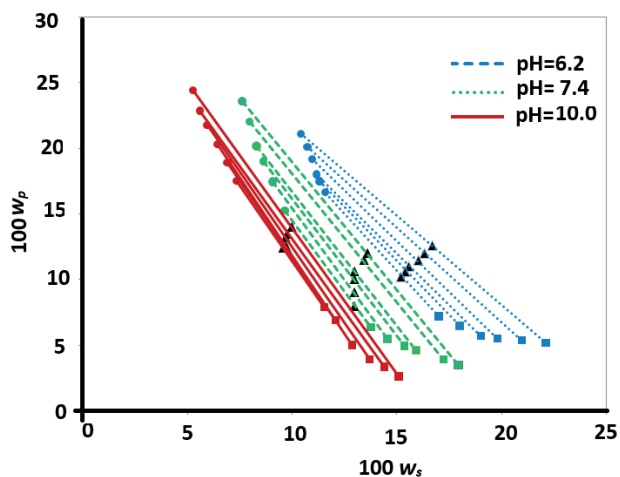
The results from Table 3 indicate that, at a constant value of pH, the partition coefficient had a direct relation with the PVP/salt ratio. At a constant pH, the partition coefficient of urea increased as the PVP/salt ratio increased. In this case, the effect of urea concentration on the urea partition coefficient was more significant than the effect of the PVP/salt ratio. At a constant concentration of urea, the partitioning coefficient of urea decreased as the pH increased. Table 2 shows that, as the pH increased, the slope and length of the equilibrium tie lines for the biphasic system increased.



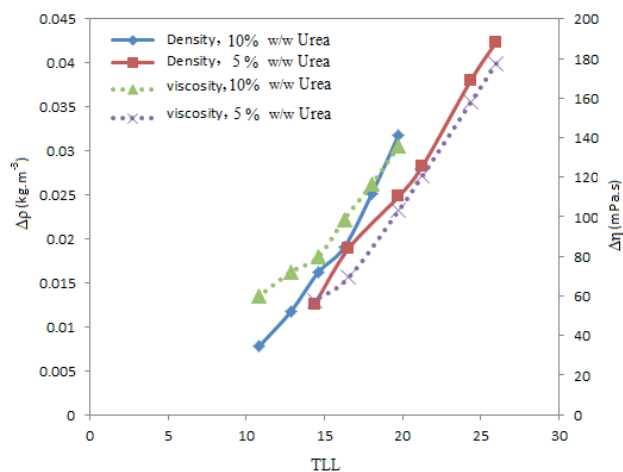
**Figure 3.** Effect of urea concentration on equilibrium phase composition, STL, and TLL for PVP (K30) + sodium citrate +  $\text{H}_2\text{O}$  system at a pH of 6.2 (■: total composition; ▲: bottom phase composition; ●: top phase composition).



**Figure 4.** Effect of TLL and urea concentration on partition coefficient of urea at different pH values for the ATPS.



**Figure 5.** Effect of pH on equilibrium phase composition, STL, and TLL for PVP (K30) + tri-sodium citrate +  $\text{H}_2\text{O}$  system (▲: total composition; ■: bottom phase composition; ●: top phase composition)



**Figure 6.** Difference in viscosity ( $\Delta\eta$ ), density ( $\Delta\rho$ ) and TLL for PVP (K30) + tri-sodium citrate + water vs. urea concentration at a pH value of 6.2.

Figure 5 shows the effect of pH on equilibrium phase composition and on the STL and TLL for the PVP (K30) + tri-sodium citrate + H<sub>2</sub>O system. The TLL and STL increased as the pH increased, which is in agreement with the results of Perumalsamy and Murugesan (2009) and Shahbazinasab and Rahimpour (2012). This could be the result of the decrease in hydrodynamic volume of the polymers in solution. Waziri et al. (2003) and Shahbazinasab and Rahimpour (2012) reported that decreasing the pH decreased the intrinsic viscosity of the polymer solution. It is known that the hydrodynamic volume of polymers in solution is proportional to their intrinsic viscosity and that a decrease in pH creates a more compact structure for the polymer chains. It has been reported that, as the pH of the aqueous polymer-salt two-phase system increases, the concentration of the polymer-rich phase increases and the concentration of the salt-rich phase decreases.

Table 3 and Figure 6 show the relation between TLL and the density and viscosity of the ATPS. The difference in density between phases ( $\Delta\rho$ ) and viscosity between phases ( $\Delta\eta$ ) increased as TLL increased.

## CONCLUSIONS

Urea was applied as a denaturant for the initial recovery steps to produce recombinant proteins in an ATPS. The liquid-liquid equilibrium, effect of denaturant on phase diagram behavior, and partition coefficient in these systems and the physical properties of ATPS are relatively unknown. The effect of urea on the phase diagram behavior of the PVP (K30) + tri-sodium citrate ATPS at 25°C was investigated at different pH values (6.2, 7.4, and 10.0). The calibration method was applied to measure the refractive index and conductivity of the phases.

The binodal was displaced toward higher concentrations as the urea concentration increased; the composition of the polymer-rich phase (and TLL) behaved in a slightly shorter manner. The STL decreased as the urea concentration increased. The density, viscosity, electrical conductivity, and refractive index of the aqueous single-phase system of PVP (K30) (p) + tri-sodium citrate (s) + urea (u) + water) at 298.15 K with the properties of ATPS were measured at 25°C. The partition coefficient of urea was shown to have a direct relation with urea concentration, pH, TLL, and PVP/salt ratio. The partition coefficient of urea increased as the pH decreased and the PVP/salt (%w/w) ratio and urea concentration increased. The relation between TLL, density, and viscosity of the ATPS was also considered.

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