Liquid-Liquid Equilibria of Aqueous Two-Phase Systems Containing PEG + Sodium Citrate + Water at various pH

LLE of PEG+SC+H₂O at various pH

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Abstract- Liquid-liquid equilibrium data of aqueous two-phase systems (ATPS) formed by sodium citrate (SC) and poly (ethylene glycol) (PEG) of different molecular weights (600, 1000, 2000, 4000, and 6000) with water was determined at different pH (6, 7, and 8). Binodal data was described using a third order polynomial equation and statistical significance of the parameters was done by regression analysis. The tie-lines were correlated using the Othmer-Tobias and Bancroft equations. An increase in molecular weight of PEG shifted the binodal curve to lower PEG and salt concentrations. An increase in pH showed similar shape and tend to merge at high concentration of PEG and salt.

Keywords- Aqueous Two-Phase Systems; Liquid-Liquid Equilibrium; Binodal Curve; Titration Method; Tie-Line Length; Purification; Downstream Processing

I. INTRODUCTION

It has been shown by many researchers that ATPS, a liquid–liquid extraction method formed by polymer/salt/water is a very efficient and cheap method for the separation and partial purification of biomolecules as compared to polymer/polymer/water systems [1]. High percentage (>80%) of water in top and bottom phases of ATPS makes them suitable for the partitioning of biomolecules and thus minimizes the denaturation [1]. The affordable price, biocompatibility and non-toxicity make PEG one of the commonest phase forming polymers in ATPS.

The commonly used salts in ATPS like phosphates and sulfates lead to water pollution and therefore, currently biodegradable salts such as citrate salts are used to form ATPS with PEG [2].

For the effective separation and purification of a biomolecule by ATPS, an understanding of the LLE and the factors responsible for the partitioning is vital. There are a lot of reports available for PEG + Sodium Citrate system [3-5]. However, binodal data for PEG + Sodium Citrate+ Water at 6, 7 and 8 pH values are not available in detail. In the present study, an attempt was made to prepare ATPSs composed of various MW PEG (600, 1000, 2000, 4000 and 6000) + Sodium citrate + Water systems at different pH (6, 7, 8) and constant temperature (25 °C). Since the data of ATPS depends on temperature, we have performed all the experiments at a constant temperature. These data would be helpful for the separation and purification of biomolecules if the target biomolecule is hydrophilic and high molecular weight (>10,000 Da) and the isoelectric point (pI) is less than 8 [6].

II. EXPERIMENTAL METHODS

A. Materials

PEGs of the average molecular masses 600 (PEG600), 1000 (PEG1000), 2000 (PEG2000), 4000 (PEG4000), and 6000 (PEG 6000), Tri-sodium citrate and citric acid were purchased from Merck and used without further purification. Millipore-Milli-Q water was used in all the experiments.

B. Construction of Binodal Curve

Binodal curves were obtained by the titration method, as described by [1, 7]. Stock solutions of 50% (w/w) of different MW PEGs were prepared. Stock solutions of 30% (w/w) of tri-sodium citrate and 30% (w/w) citric acid were prepared and the pH of the stock was maintained at 6, 7 and 8. The pH of the salt solution was adjusted by mixing appropriate ratio of sodium citrate and citric acid. The pH values of the solutions were measured precisely with a pH meter (EUTECH pH 510 model) with ± 0.01 accuracy. All the experiments were carried out at 25 °C.

A known amount of PEG stock solution was taken in a conical flask and it was weighed. To this flask, a stock solution of tri-sodium citrate was added till the first sign of turbidity and the weight of the salt solution needed to make this turbidity was noted. A known weight of water was added to below the cloud point and repeated to get other binodal points. The composition of the mixture was determined by mass using analytical balance with a precision of ± 0.1 mg. All the experiments were done in triplicates and average values were recorded.

C. Determination of TLL

In order to determine the tie line composition, a series of ATPSs of known total compositions were prepared in graduated 15 ml test tubes and placed in a thermostatic water bath.

The solution was mixed by rigorous vortexing and was centrifuged at 2000 rpm for 10 min to speed up the separation process. The concentrations of sodium ions in the top and bottom phases were determined by flame photometry (Systronics128 flame photometer). The equilibrium concentration of PEG in both phases was determined by refractive index measurements performed using an Abbe-type refractometer (Advance Research Instruments Co., New Delhi, Model R-4). Since the refractive index depends on both PEG and sodium citrate concentration calibration charts were drawn between refractive index versus different MW of PEG (10–50%) for the different concentration of sodium citrate (1–10%). One such calibration chart was shown in Figure 1 for refractive index and PEG 6000 (%w/w) with different concentration of sodium citrate. For all PEG fractions the curves are linear and have similar slopes for the salt concentrations investigated.



Fig. 1 Refractive index calibration curves for PEG 6000 + Sodium Citrate + water

The relation between the refractive index, nD, and the weight fraction of PEG, WP, and salt, WSC, is given by Cheluget et al., and Regupathi et al., 2009.

$$n_D = a_0 + a_1 W_{SC} + a_2 W_P \tag{1}$$

The values of the coefficients a_0 , a_1 and a_2 for the PEG + Sodium citrate + Water system were determined and given in the Table 1 along with the corresponding average absolute relative deviation (AARD).

CABLE 1 REFRACTIVE INDEX CALIBRATION CONSTANT

Component	ao	\mathbf{a}_1	\mathbf{a}_2	AARD / % *
Water	1.3322			
Sodium Citrate		0.1466		
PEG 600			0.1357	0.0283
PEG 1000			0.1374	0.01803
PEG 2000			0.1387	0.04316
PEG 4000			0.1396	0.05395
PEG 6000			0.1409	0.01854

* AARD = $\frac{1}{N} \sum_{i=1}^{n} \left[\frac{|n_{D} - n_{D}^{our}|}{n_{D}^{exp}} \right]$, where N = number of experimental data points

III. RESULTS AND DISCUSSIONS

A. Effect of PEG Molecular Weight

The binodal data for the systems with varying PEG molecular weight at three different pH values were performed and the data for PEG 600 + Sodium citrate + Water is shown in Table 2. Binodal curves for different MW PEG + Sodium Citrate + Water System at constant pH 8 is shown in the Figure 2. As the PEG MW increases, the hydrophobicity of the PEG increases and therefore a lesser amount of PEG is needed to form two phases [3, 10]. The same kind of observation was observed in all the investigated systems. It is evident from the figure that the binodal curves tend to shift towards the origin with the increase in PEG MW. Similar trend was also observed for other pH (data not shown).

TABLE 2 BINODAL DATA FOR THE PEG 600(1) + SODIUM CITRATE (2) + WATER (3) SYSTEM AT PH 6, 7 and 8

pH 6		pH 7		pH8		
W _P / %	W _{SC} / %	W _P / %	W _{SC} / %	W _P / %	W _{SC} / %	
47.17	6.39	47.68	6.73	48.54	6.34	
46.35	7.42	44.80	7.36	37.12	8.18	
44.41	7.53	40.77	7.86	31.82	10.78	
42.32	8.72	37.96	8.90	25.51	12.27	
40.73	8.57	36.28	9.68	24.96	12.41	
38.56	9.44	33.14	10.45	21.56	13.99	
31.37	11.55	28.17	11.98	17.52	16.53	
28.47	12.23	25.02	13.11	15.99	17.50	
27.11	12.47	23.18	13.98	14.87	18.22	
25.68	13.27	21.60	14.90	12.90	19.44	
23.35	14.52	19.67	16.01	12.07	19.96	
19.49	16.61	17.18	17.42	10.86	20.85	
18.24	17.33	16.03	18.09	9.40	21.93	
15.44	18.47	14.17	18.96	8.92	22.29	
13.72	20.06	12.90	20.01	8.01	23.02	
11.28	21.59	11.38	20.98	7.13	23.73	
9.33	23.48	9.65	22.51	5.75	25.13	
8.74	24.77	9.07	23.35	3.99	27.67	
7.22	25.29	8.07	23.79	3.12	29.44	
6.59	26.83	7.54	24.74	3.05	30.02	
5.34	27.54	6.67	25.28			
4.47	28.11	6.02	25.73			
3.38	29.29	5.26	26.51			
3.19	30.05	5.01	27.02			



Fig. 2 Binodal curves for different MW PEG + Sodium Citrate + Water System at pH 8

There are several correlations available to fit the binodal data in literature for PEG/Sodium citrate systems [3, 4, 11, 12]. Saravanan et al., (2006) had successfully fitted the binodal data of PEG + Poly Acrylic Acid + Water system with a third order polynomial equation.

For our investigation, a similar type of polynomial equation was correlated for binodal curve

$$W_P = B_0 + B_1 W_{SC} + B_2 W_{SC}^2 + B_3 W_{SC}^3$$
⁽²⁾

where W_P and W_{SC} are the weight percentages of PEG and sodium citrate respectively. The values of the coefficients B_0 , B_1 , B_2 and B_3 are obtained by regression analysis. These values along with the standard deviation, regression coefficient and P value are given in the Table 3. The regression analysis was done by Microsoft Excel 2010 and a method called the analysis of variance (ANOVA) was used to test the significance of regression and an ANOVA table for the PEG 600 + Sodium citrate + water data is shown in the Table 4.

TABLE 3 BINODAL CURVE COEFFICIENTS OF POLYNOMIAL EQUATION (1) FOR AQUEOUS TWO-PHASE PEG 600 + SODIUM CITRATE + WATER SYSTEM

pН	\mathbf{B}_{0}	B ₁	B ₂	B ₃	SD	R ²	P value
6	82.61187	-6.30164	0.184123	-0.00208	0.906221	0.9967	1.68x10 ⁻²⁷
7	82.75167	-6.73739	0.218075	-0.00279	0.449017	0.9989	4.31x10 ⁻³³
8	81.85877	-6.76298	0.213526	-0.00254	0.916391	0.9948	9.05x10 ⁻²¹

TABLE 4 THE ANALYSIS OF VARIANCE (ANOVA) OF THE MODEL THAT IS REPORTED IN TABLE III FOR THE BINODAL DATA OF PEG 600+ sodium citrate + water system at PH 8

ANOVA						
	df	SS	MS	F	P value	
Regression	3	2916.71	972.2365	1157.739	9.05E-21	
Residual	18	15.11589	0.839772			
Total	21	2931.825				

In the ANOVA table, the sum of the squares is used to estimate the significance of regression. It is done by calculating F ratio [4] defined as the ratio of the mean square to the residual mean square. Since $F_{0.05,3,18} = 3.16$, and the calculated F ratio value was greater than this value, the regression model was significant. Furthermore, the *P* value which is defined as the smallest level of significance leading to rejection of the null hypothesis was less than 0.05 implied that the model was significant and the variation explained by the model is not due to chance. In addition, the high values of regression coefficient, R^2 showed that the model can be used to explain most of the variations in the data.

Tie-Line Length (TLL) and the slope of the tie-line (STL) can be related to the equilibrium phase composition as follows:

$$TLL = \sqrt{\left[W_{SC}^{b} - W_{SC}^{t}\right]^{2} + \left[W_{P}^{t} - W_{P}^{b}\right]^{2}}$$
(3)

Tie lines are commonly parallel and hence the STL can be calculated by the following formula thus facilitating the construction of further tie lines.

$$STL = \frac{\left[w_P^t - w_P^b\right]}{\left[w_{SC}^b - w_{SC}^t\right]} \tag{4}$$

The TLL data and STL are given in the Table 5. The total phase composition did not have any significant effect on the slope of the tie lines (STL) for all the systems studied and all tie lines were parallel to each other.

TABLE 5	TIE LINE	COMPOSI	TIONS PEO	G 600 +	SODIUM	CITRATE +	WATER	SYSTEM	at 30	19

Tie Line	Total System		Top phase		Bottom phase		Slope of Tie Line
	W _P / %	W _{SC} / %	W_P / %	W _{SC} / %	W_P / %	W _{SC} / %	-
PEG 600 + Sodium Citrate at pH 6							
TL1	22.77	16.12	31.24	11.53	9.57	23.86	-1.758
TL2	24.17	17.33	35.19	10.28	7.22	26.58	-1.716
TL3	25.89	17.99	42.47	7.77	4.91	28.89	-1.778
PEG 600 + Sodiu	um Citrate at	рН 7					
TL1	25.31	14.79	33.76	10.43	10.32	24.09	-1.716
TL2	27.43	15.93	41.08	8.09	6.33	26.54	-1.883
TL3	28.22	16.84	43.89	7.58	5.19	28.62	-1.839
PEG 600 + Sodiu	um Citrate at	рН 8					
TL1	23.59	14.82	32.22	10.17	9.22	23.55	-1.719
TL2	25.22	16.19	39.73	7.44	4.33	27.84	-1.735
TL3	27.41	17.13	44.42	6.72	3.88	29.95	-1.745

To fit the tie line data, Othmer-Tobias Equation (5) and Bancroft Equation (6) were used as given by [15, 16]

$$\left(\frac{1-W_P^b}{W_P^t}\right) = K_{OT} \left(\frac{1-W_{SC}^b}{W_{SC}^b}\right)^n \tag{5}$$

$$\left(\frac{W_W^b}{W_{SC}^b}\right) = K_B \left(\frac{W_W^t}{W_F^b}\right)' \tag{6}$$

The values of estimated parameters (K_{OT} , n, K_B , r) for PEG + Sodium Citrate + Water System at 30 °C and pH 6 are given in the Table 6. All the lines were linear with a high value of correlation coefficient ($\mathbb{R}^2 > 95$).

PEG	Кот	Ν	\mathbf{R}^2	K _B	r	\mathbf{R}^2	
600	0.2663	1.8434	0.9538	1.98	0.4927	0.9657	
1000	0.1905	2.0801	0.9759	2.3189	0.4244	0.9869	
2000	0.0743	2.6325	0.9682	2.7363	0.3699	0.9683	
4000	0.1621	1.6326	0.9742	3.1443	0.5241	0.9858	
6000	0.0736	1.9667	0.9992	3.8114	0.4634	0.9990	

TABLE 6 VALUES OF PARAMETERS OF OTHMER – TOBIAS AND BANCROFT EQUATIONS, $K_{ot},$ N, K_{b} and R for PEG + sodium citrate + water system at 30 $^{\circ}{\rm C}$ and PH 6

B. Effect of pH

Figure 3 summarizes the phase diagrams corresponding to ATPSs of PEG MW 600 at different pH values. It is clear from the figure that change in pH does not significantly alter the phase diagram. However, changes in pH alter the divalent to trivalent cations in the phases. As a result a relatively lesser concentration of salt is needed to form two phases at higher pH values. Similarly, volume exclusion plays a major role in phase formation as the MW of PEG increases. These results agree with the literature for similar systems [3, 17]. For other MW PEGs also similar kind of results was observed (data not shown).



Fig. 3 Binodal curves for PEG 600 + Sodium Citrate + Water System at pH 6, 7 and 8

IV. CONCLUSIONS

Binodal curve for different MW PEG (600, 1000, 2000, 4000 & 6000) at pH 6, 7 and 8 at a constant temperature 25 $^{\circ}$ C were developed by titration method. An increase in PEG molecular weight and pH tend to increase the binodal region and all the binodal curves were satisfactorily described using a polynomial equation and the statistical significance was analysed by regression analysis. A low value of P was observed which indicated that the polynomial equations were valid for the binodal data. The tie-line compositions were correlated and the parameters of Othmer-Tobias and Bancroft equations were calculated. The high value of R^2 suggests a good correlation for the data developed.

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