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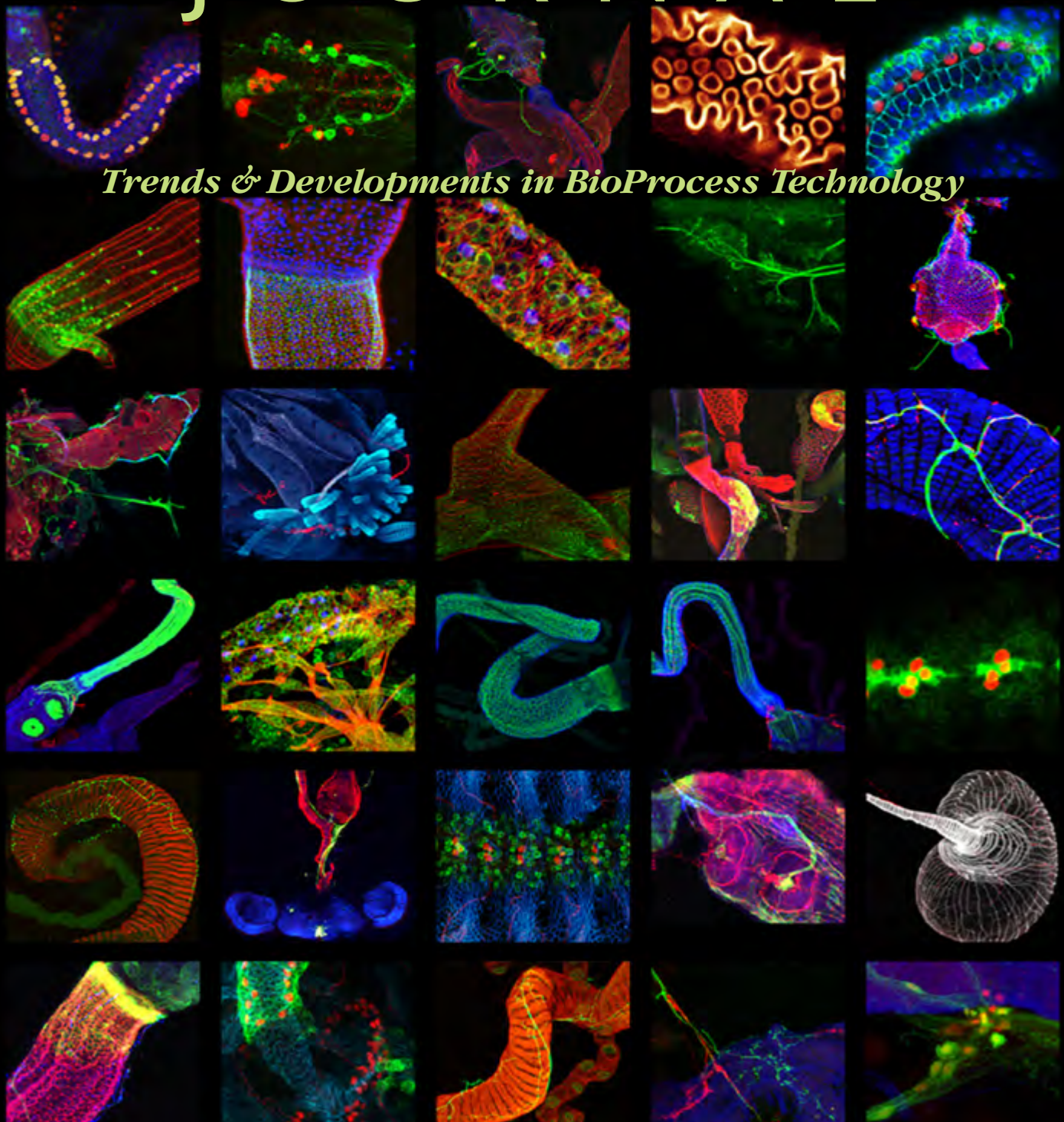

Trends & Developments in BioProcess Technology

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TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

Bovine Serum Albumin Partitioning in Aqueous Two-Phase Systems: *Effects of Variables and Optimization*

By NANGARTHODY SINDHU, SIVAKUMAR KALAIVANI, and IYYASWAMI REGUPATHI

Abstract

The objective of this study was to optimize process conditions for the effective partitioning of bovine serum albumin (BSA) using response surface methodology (RSM). Initially, four different salts (tripotassium citrate, tripotassium phosphate, sodium carbonate, and sodium sulphate) were tested for the ability to partition BSA. Among the salts chosen, tripotassium citrate was observed to yield a high partition coefficient.

The effect of phase-forming components: concentration, PEG molecular weight, and pH were studied for a PEG/tripotassium citrate system and the information obtained was utilized to fix the ranges in RSM studies. Four different independent variables (PEG 2000, tripotassium citrate, NaCl concentrations, and pH) were considered for RSM studies and the responses generated were partition coefficient (*k*) and *percentage yield*. A statistical model was developed and the values obtained were 99 % within the confidence level. Optimal conditions of the system were found as: 0.25 M sodium chloride, 32 % PEG 2000 (w/w), 16 % tripotassium citrate (w/w), pH 6.0, a partition coefficient of 6.03, a recovery of 91.76 %, and a controlled operating temperature of 303.15 K.

Introduction

The challenges which the biotechnology industry faces today are ever more demanding. In the case of therapeutic protein production for human use, extremely high purity standards of 99.9 % or higher are required. Proteins must be separated from a very large number of production contaminants such as host cell proteins, nucleic acids, and polysaccharides present in the cell culture or cell lysate. Separation techniques used for downstream purification should be relatively simple, provide high resolution, speed, ease of scale-up, and if needed, operated continuously. In this regard, aqueous two-phase systems (ATPS) are gaining in importance and researchers are focusing in this area to develop more environmentally friendly and economical systems to optimize product recovery.

ATPS are formed when two mutually incompatible polymers, or a polymer and salt, are mixed above a critical concentration.[1] Mixtures of proteins added to this ATPS tend to partition unequally between the phases, thus allowing for the extraction of a particular protein. Several researchers were employed to study the purification characteristics of proteins using bovine serum albumin as a model protein through ATPS. Alves *et al.*[2] used polyethylene glycol (PEG)/maltodextrin (MD) ATPS for the partitioning of BSA where BSA prefers the MD-rich phase over the PEG-rich phase. However, they did not assess the pH and salt concentration effects on protein partitioning. Conversely, the partitioning characteristics of the ATPS were dependant on many operating variables like pH, salt and PEG concentrations, the addition of neutral salt (sodium chloride [NaCl]) and the concentrations, tie line length (TLL), temperature, *etc*. The effects of these parameters on the partitioning coefficients of a variety of proteins for different ATPS have been reported in literature in recent years.

Haghtalab *et al.*[3] investigated the partitioning of lysozyme, BSA, and α-amylase in ATPS of: (1) PEG/dipotassium hydrogen phosphate (K_2HPO_4)/water (H_2O); and (2) PEG/sodium sulphate (Na_2SO_4)/H_2O; both at 298.15 K. They found that pH and salt concentrations also affected protein partitioning. Capezio *et al.*[4] analysed the influence of PEG molecular mass, pH, and NaCl concentration on the partitioning of bovine whey proteins (BSA, α-lactalbumin [α-LA] and β-lactoglobulin [β-LG]) and α-1 antitrypsin (α1AT) using PEG (1000, 1500, and 3350)/potassium phosphate (K_3PO_4). BSA and α-LA concentrated in the PEG-rich phase, while β-LG and α1AT showed affinity for the phosphate-rich phase. They also found that an increase in

the medium's pH induced the partition coefficient of all these proteins while the increase in PEG molecular mass induced the negative behaviour. Boaglio *et al.*[5] studied the influence of PEG concentration, TLL, molecular mass, and pH on the partitioning of bovine whey proteins (BSA, α-LA, and β-LG) and α1AT for PEG (1000, 1450, and 3350)/sodium citrate systems and found that the partition coefficient decreased with increasing TLL for all of these proteins. They demonstrated that the change in TLL affects the free volume available for a different solute to accommodate in a given phase. Perumalsamy *et al.*[6] investigated the effect of phase-forming components, pH, and neutral salt additions on BSA partitioning in PEG 2000/sodium citrate-based ATPS at 298.15, 308.15, and 318.15 K. They found that the partition coefficient of BSA decreased with an increase in PEG 2000 concentration and temperature. Further partition behaviour of BSA was investigated by Salabat and Batcha[7] in a PEG 4000, 6000, and 8000/sodium citrate ATPS. The effect of different polymers (polypropylene glycol [PPG 425] and PEG 6000), and salts (magnesium sulphate [$MgSO_4$], ammonium sulphate [$(NH_4)_2SO_4$], and Na_2SO_4) on the partition behaviour of different proteins (BSA, β-LG, and zein) as model proteins have been studied in detail by Salabat *et al.*[8] The recovery results showed a trend in the salting-out power of these salts (Na_2SO_4, $MgSO_4$, and $(NH_4)_2SO_4$).

Literature has shown that the process of partitioning in ATPS is influenced by many factors. Varied results for a particular protein in different ATPS is to be expected because of the interactions that exist between the factors inherent in the system itself. Such factors responsible for the interactions include: (1) the choice of system components (polymers and salts); (2) polymer molecular weights and concentrations; (3) the types and concentrations of phase-forming salts, ionic strength, concentration of neutral salt; (4) phase volume ratios, temperatures, and pH; and (5) those of the target protein, such as surface hydrophobicity, charge, structure, specific bonding sites, and molecular weight.[9,10] Thus, the partitioning of a particular protein is highly dynamic with respect to an ATPS. Although the available literature provides some information, no comprehensive theory currently exists to guide the design of systems for the separation of specific proteins and particle mixtures. Therefore, applying the ATPS technique requires extensive experimentation to design an adequate phase system for optimal partitioning of a particular protein.

This study attempts to investigate the influence and optimization of such process variables like the type and concentration of salt, PEG molecular weight and concentration, pH, and how the addition of neutral salt at 303.15 K influence the partitioning behaviour of the well-established model protein, BSA, in ATPS.

Materials and Methods

Materials

Bovine Albumin Fraction V was purchased from HiMedia Laboratories. Polyethylene glycol of different average molecular weights of 2000, 3000, 4000, and 6000 were purchased from Merck Specialities Pvt. Ltd. Sodium sulphate, sodium carbonate, dipotassium hydrogen phosphate, tripotassium citrate, and sodium chloride were also procured from Merck Specialties Pvt. Ltd. All salts were of analytical grade with a purity of more than 99 %, and used without further purification. Bradford Reagent from Sigma-Aldrich was used for protein analysis. Double-distilled water (ddH_2O) was used for all purposes.

Binodal Curve

The cloud point method was used to determine the phase equilibrium concentrations for establishing binodal curves.[10] Each experiment was carried out in a jacketed glass vessel. The temperature of the working vessel, a refrigerating bath (JEIO Tech, model RW-0525G), was maintained at 303.14 ± 0.1 K by circulating water through the external jacket. Aqueous stock solutions of 50 % PEG (w/w) and 30 % salt (w/w) were prepared and used for the experiments. To ensure uniform concentration of the ATPS constituents in the jacketed vessel, constant stirring was applied using a magnetic stirrer. A salt solution of known concentration was titrated against the polymer solution (or *vice versa*) until the clear solution turned turbid and then water was added until turbidity was eliminated. The procedure was repeated to get the second binodal point. An analytical balance with a precision of ± 0.1 mg (Ohaus-Essae-Teraoka, model AR2140) was used to determine the composition of the mixture.

Tie Line and Partition Coefficient

For the determination of tie lines and the partition coefficient, 10 g of systems (total weight) were prepared by mixing appropriate amounts of PEG, salt, protein, and water in graduated centrifuge tubes. The BSA concentration was maintained at 0.5 mg/mL as a feed for each of the experiments. The samples were thoroughly combined with a vortex mixer at lower rpm for 10 min. The solution was allowed to settle for 24 h in a constant temperature bath. Top and bottom phase aliquots were taken using a pipette. While withdrawing the sample from bottom phase, a small positive pressure was maintained to avoid contamination from the top phase.[11,12] Thus, the top and bottom phases were separated, diluted, and analyzed for PEG, salt, and protein concentrations.

PEG Concentration Estimation

Determining PEG concentration was done through a measuring refractive index using a digital refractometer (Atago Co. Ltd., model RX-5000α) with a precision of ± 0.00004 (range 1.3–1.7 ηD). A correlation was developed to measure the refractive index at various known compositions of PEG and salt. For dilute aqueous solutions containing polymer and salt, the relation between the refractive index (ηD) and the mass fractions of polymer (w_p) and salt (w_s) is given by:

$$\eta D = a_0 + a_1 w_P + a_2 w_S \quad (1)$$

where a_0, a_1, and a_2 are the fitting parameters and are tabulated in Table 1 for different ATPS used in the present study. For PEG analysis, Equation 1 was used successfully in literature.[13-15] The correlation is valid in the concentration range of PEG < 10 % and salt < 5 %. Further, the salt concentration was quantified by the estimation of sodium and potassium concentration in the solution through a Flamephotometer (Elico Ltd., model CL 378, range 1–100 ppm).

Protein Concentration

The determination of protein in both phases was done through the Bradford Protein Assay methodology. To avoid interference of PEG and salt in the sample, extensive calibration was done by varying the concentrations of PEG, salt, and protein, and the absorbance was measured using the Bradford Assay at 595 nm in a Spectro UV-Vis double beam spectrophotometer (Labomed, Inc., model UVD-3500). The following correlation was determined through regression analysis:

$$Absorbance = a_0 w_{BSA} + a_1 w_p^{b1} + a_2 w_s^{b2} \quad (2)$$

where a_0, a_1, and a_2 are the fitting parameters for different systems and are tabulated in Table 2. This is applicable in the concentration range of PEG < 10 % and salt < 5 %. For the determination of protein concentration, samples withdrawn from each phase were diluted and the absorbance was measured in the spectrophotometer at 595 nm using a Bradford Assay.

Partition Coefficient (*k*)

Partitioning between the two phases is a complex phenomenon, guided mainly by the interaction of the partitioned substance and the phase components through hydrogen bonds (*e.g.*, van der Waals, hydrophobic, and electrostatic interactions, and steric and conformational effects, *etc.*). The partition coefficient is defined as the ratio of protein concentration in the top phase to that in the

TABLE 1. Coefficients of Equation 1 for different systems.

Systems	a_0	a_1	a_2	R^2
PEG 4000/Sodium Sulphate	1.3325	0.1509	0.1578	0.9968
PEG 4000/Sodium Carbonate	1.3325	0.1409	0.1827	0.9992
PEG 4000/Dipotassium Hydrogen Phosphate	1.3325	0.1106	0.1392	0.9944
PEG 2000/Sodium Citrate/Water	1.3325	0.1314	0.2430	0.9949
PEG 3000/Potassium Citrate/Water	1.3325	0.1416	0.2290	0.9901
PEG 4000/Potassium Citrate/Water	1.3325	0.1079	0.1596	0.9955
PEG 6000/Potassium Citrate/Water	1.3325	0.1383	0.1227	0.9970

R^2: regression coefficient

TABLE 2. Coefficients of Equation 2 for different systems.

Systems	a_0	a_1	a_2	b_1	b_2	AARD %
PEG 4000/Sodium Sulphate	1965.903	0.5116	-0.1599	0.9968	0.4611	1.9286
PEG 4000/Sodium Carbonate	500.3408	0.0484	-0.0499	0.9992	2.0199	1.0072
PEG 4000/Dipotassium Hydrogen Phosphate	1296.198	3.4429	23.227	0.9944	2.7122	2.5921
PEG 2000/Sodium Citrate/Water	1056.873	0.6702	0.1511	0.9949	0.5873	4.0862
PEG 3000/Potassium Citrate/Water	425.0001	0.1048	0.0303	0.9901	0.1239	1.6408
PEG 4000/Potassium Citrate/Water	1036.741	0.2164	0.2736	0.9955	0.9497	1.5434
PEG 6000/Potassium Citrate/Water	215.2484	0.1096	0.4005	0.9970	0.9664	1.9361

AARD %: average arithmetic relative deviation (AARD) = (Σ| (Exptl – Cal)/ (Exptl)|)/ (N) x 100.

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bottom phase, as shown in this equation:[16]

$$k = C_p^{top}/C_p^{bot} \quad (3)$$

where k is the partition coefficient, C_p^{top} and C_p^{bot} are protein concentrations (mg/mL) in top and bottom phases, respectively.

Volume Ratio

Volume ratio (*VR*) is the ratio of top phase volume (V^T in mL) to bottom phase volume (V^B in mL) that is measured in all the experiments as follows:

$$VR = V^T/V^B \quad (4)$$

The volume ratio of a phase system can be modified by shifting system initial feed compositions along a particular tie line to get the larger top phase volume or larger bottom phase volume.

Protein Recovery

The percentage recovery of protein in the top phase is calculated as follows:

$$\%\ recovery = \frac{C_p^{top}\ V^{top}}{C_p^0\ V^0} \quad (5)$$

where $C_p^0$and C_p^{top} are the concentration of protein (mg/mL) in feed mixture and the top phase, respectively. V^0 and V^{top} are volume (mL) of feed sample and top phase, respectively.

Partitioning Optimization

The common method of experimental design and optimization, where operating conditions are optimized by changing one variable at a time, gives no guarantee for the optimal parameter determination.[17, 18] RSM consists of a group of mathematical and statistical techniques for seeking the optimum conditions in a system which had multi-variable and complex interactions existing between the parameters. It can be used to define the relationships between the response variables (*% recovery*, partition coefficient) and the independent variables (% PEG [w/w], % tripotassium citrate [w/w], NaCl concentration, and the pH) and also generates a mathematical model that describes the process.

A central composite rotatable design (CCRD) coupled with a full quadratic polynomial model is a powerful combination that efficiently provides an adequate representation of most continuous responses without expending many resources. In this present study, design of experiments (DoE) was performed based on the central composite rotatable design at a temperature of 303.15 K with four independent variables: (1) % PEG (w/w); (2) % tripotassium citrate (w/w); (3) pH; and (4) NaCl (M). The ranges of variables in the form of coded and noncoded values are shown in Table 3. For each of the four factors, high (coded value: +2) and low (coded value: –2) set points were determined at an axial distance (= $[2k]^{1/4}$) dependent on the number of factors (k). The experimental design consisted of 31 experiments, including 16 factorial points, eight axial points, and seven replications at the center points to evaluate the pure error. The experiments at the design points were performed and responses such as partition coefficient and *% recovery* were obtained for the selected system. Further, to obtain the optimum values of the independent variables for response, a full quadratic equation (or the diminished form of this equation) represented (as follows) was developed by fitting the experimental values:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{i<j}^{k} \beta_{ij} x_i x_j \quad (6)$$

where y is the response, x_i and x_j are noncoded independent variables, k is the number of independent variables, and β_0, β_i, β_{ii}, and β_{ij} are intercept, linear, quadratic, and interaction coefficients, respectively. Response surface analysis, used to determine the conditions that maximize the *% yield* of the protein, experimental data fitting of quadratic polynomial equation through least square technique, analysis of variance (ANOVA), and the lack-of-fit test for the final predictive equation were done using statistical software (Minitab, Inc., version 16).

Results and Discussion

The Effects of Variables on BSA Partitioning

In the present study, partitioning of BSA was carried out in a series of PEG/salt/water-based ATPS. There are many factors influencing the partitioning in ATPS. Differences in

TABLE 3. Independent variables and levels used for experimental design.

Factors	Variables	Levels				
		-2	-1	0	+1	+2
% PEG	1965.903	24	28	32	36	40
% Tripotassium Citrate	500.3408	12	14	16	18	20
pH	1296.198	4.0	5.0	6.0	7.0	8.0
Salt Molality	1056.873	0.05	0.15	0.25	0.35	0.45

partitioning result from the interaction of factors inherent in the system itself such as choice of system components (polymer/salt), type and concentration of phase-forming salts, polymer molecular weight and concentration, pH, concentration of neutral salt, *etc*. The effects are analyzed in detail at a temperature of 303.15 K. Initially, the following ATP combinations with salts like sodium sulphate, sodium carbonate, dipotassium hydrogen phosphate, and tripotassium citrate; and polymers PEG 2000, 3000, 4000, and 6000 were considered for partitioning of BSA at 303.15 K.

Salts

When the anion and cation of a salt have different relative affinities for different phase-forming polymers, and consequently, for each phase, the requirement of electro-neutrality in each phase results in a Donnan-type electrostatic potential difference between the phases. This potential difference appears to have large effects on partitioning of charged solutes (such as proteins). Therefore, the choice of salt is an easy way to influence the target biomolecule partitioning. The most hydrophobic anions or cations will drive the partitioning of their counter-ions to the most hydrophobic phase. Co-ions, which are less hydrophobic, will partition to the hydrophilic phase.

The variation of partition coefficient *k* against PEG 2000 concentration for different salts (sodium sulphate, sodium carbonate, dipotassium hydrogen phosphate, and tripotassium citrate) with a salt concentration of 8 % (w/w) at 303.15 K is shown in Figure 1. BSA, being hydrophilic in nature, was observed to be attracted to the bottom phase, in the case of sodium carbonate and sodium sulphate, due to the lessened hydrophobicity of the salt-rich phase. Because the phosphate salt phase is more hydrophobic than the polymer phase, water molecules were expelled to the top phase resulting in increased partitioning of BSA to that phase. Citrate also has similar properties to phosphate in the PEG/phosphate system.[19] The PEG/citrate system extracted the highest amount of BSA to the top phase suggesting that the extraction of BSA in ATPS was influenced by hydrophobicity. Increasing the difference in hydrophobicity between the phases would increase the strength of hydrophobic interaction between the protein and PEG molecules, thus improving extraction. As the PEG 2000/tripotassium citrate system demonstrated a better partitioning behaviour of BSA, this was chosen as the system for further investigation.

PEG Molecular Weight in Tripotassium Citrate System

For this study, partitioning of BSA was conducted in the PEG/tripotassium citrate system for different molecular weights of PEG (2000, 3000, 4000, and 6000) and the variation of partition coefficient is shown in Figure 2. It was observed that as the molecular weight of the PEG increased, the partition coefficient decreased, which may be

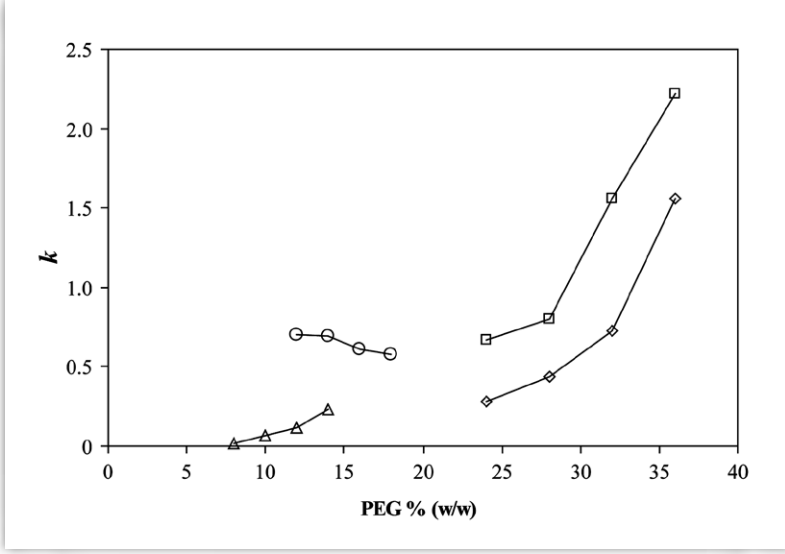

FIGURE 1. Variation of partition coefficient *k* against PEG concentration for different salts (8 % salt concentration) at a temperature of 303.15 K: ◇ K_2HPO_4; □ $K_3C_6H_5O_7$; △ Na_2CO_3; o Na_2SO_4.

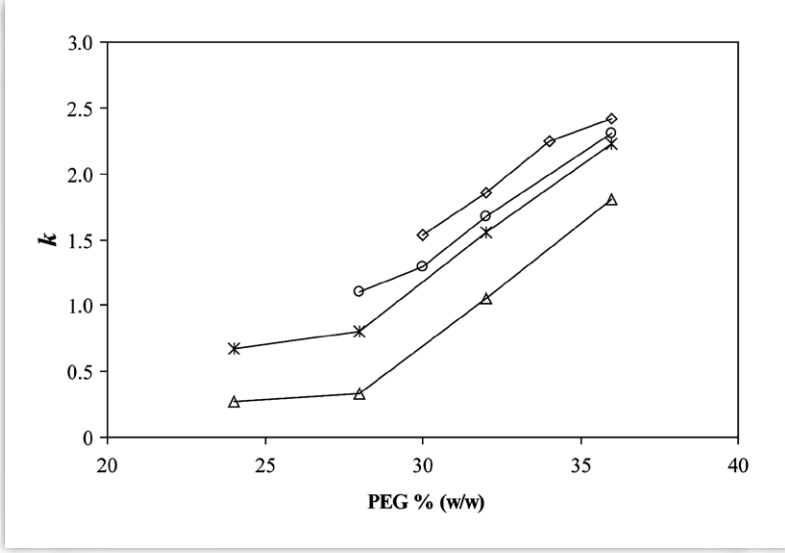

FIGURE 2. Partition coefficient *k* against PEG concentration for different molecular weights of PEG with an 8 % $C_6H_5K_3O_7$ concentration at 303.15 K: ◇ PEG 2000; o PEG 3000; * PEG 4000; △ PEG 6000.

due to the excluded volume effects. Johansson[20] observed that in general, the protein partition coefficient could be increased by reducing the molecular weight of the polymer in the top phase. A decrease in the partition coefficient while increasing the PEG molecular mass was found for other proteins (reported earlier).[5, 7, 21] This behaviour was in agreement with an exclusion effect owing to the diminution of the free volume available in the top phase. Published literature has shown that the exclusion volume effect[22, 23] can lead to the protein's movement from the top phase to the bottom phase and then lessened as the molecular weight of PEG is lowered. BSA showed a high affinity for the top phase in PEG 2000/tripotassium citrate due to the lower molecular weight of PEG. Per Huddleston *et al.*,[24] because the hydrophobic character of PEG increases with the chain length, the BSA is also liable to transfer to the top PEG-rich phase with the decrease of PEG molecular weight, substantiating the hydrophilic character of the BSA. The maximum partition was obtained using PEG 2000 suggesting that the lower PEG molecular weight was favorable for the partitioning of BSA (Figure 2).

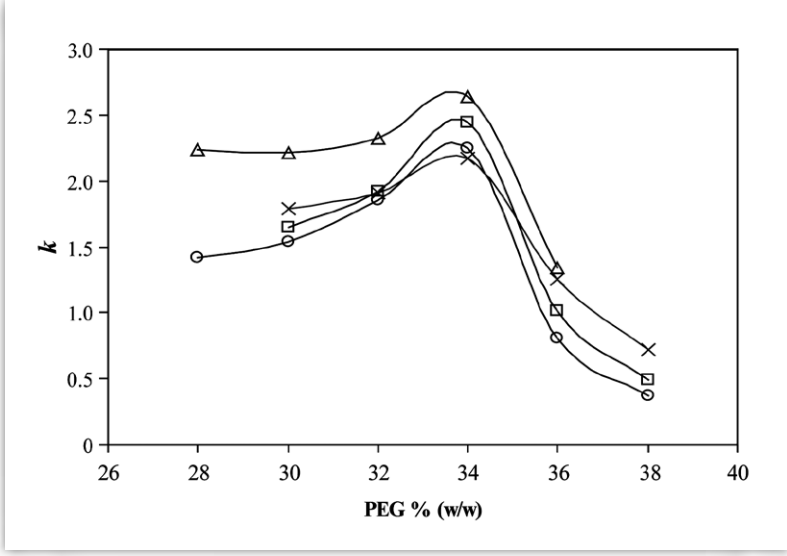

FIGURE 3. The effect of PEG 2000 concentration on the partitioning of BSA in PEG 2000/$C_6H_5K_3O_7$ at 303.15 K: o 8 % salt; □ 12 % salt; △ 16 % salt; × 20 % salt.

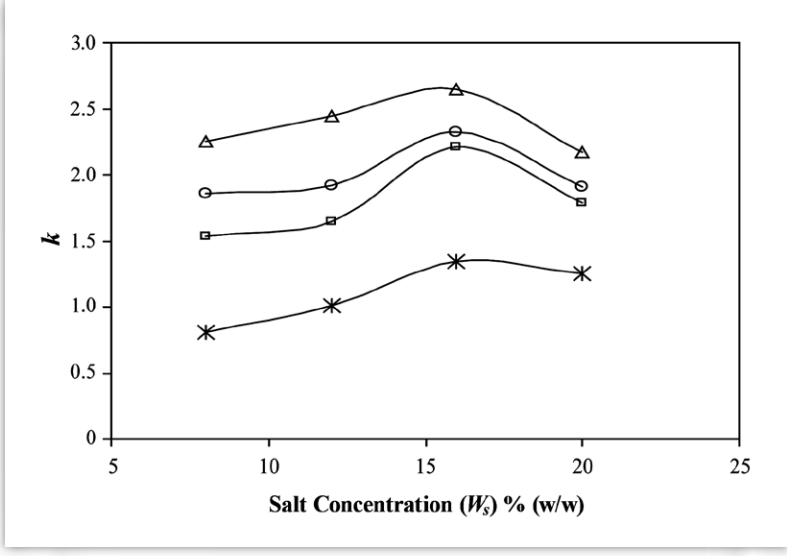

FIGURE 4. The effect of $C_6H_5K_3O_7$ concentration on the partitioning of BSA in PEG 2000/$C_6H_5K_3O_7$ at 303.15 K: □ 30 % PEG; o 32 % PEG; △ 34 % PEG; * 36 % PEG.

PEG 2000 Concentration

The concentration of the phase-forming polymer would greatly influence the partitioning behaviour of target products. Generally, the partition coefficient tends to become exceedingly higher or lower, beyond the critical polymer concentration. The concentration of PEG 2000 was varied for different tripotassium citrate concentrations and the partition coefficient was analyzed and plotted in Figure 3. It was found that the partition coefficient would increase at first, reach a peak value, and then decrease with the increased concentration of phase-forming polymer. The same trend was also reported by Wu *et al.*[25] As salt becomes more hydrophobic than PEG 2000 (at lesser PEG 2000 concentrations), when the PEG 2000 concentration is increased, salt will expel the water molecules to the top phase providing more water volume for the protein, thus increasing the *k* value. Beyond a particular composition, the protein transfer to the salt-rich phase may be due to a decrease in the free volume available in the top phase as a consequence of the increased PEG 2000 concentration.[5]

Tripotassium Citrate Concentration

The effect of tripotassium citrate concentration on the partitioning of BSA was investigated and is shown in Figure 4. It was observed that the partition coefficient first increased and then decreased as the salt concentration was raised. The available free volume in bottom phase decreases and biomolecular partition occurs more fully in the top or interphase (salting-out). After the concentration of BSA in the top phase increases to a certain point, the propulsion among the biomolecules and salt ionic attraction results in a salting-in effect.[26] More BSA molecules go to the lower phase at this point, leading

to a decrease in the partition coefficient. In a secondary event, a high concentration of salt in the ATPS may damage the target molecule, resulting in lowered activity of the recovered molecules.

pH

The variation of partition coefficient with pH for constant PEG 2000 and tripotassium citrate concentration is shown in Figure 5. The change in partition coefficient can be explained by considering the protein surface charge compared to its isoelectric point (pI). The protein carries net negative charge when the pH of the system is higher than the pI of the protein. It was observed that the increase and decrease of the partition coefficient of BSA was directly correlated with the pH of the system while in the range of 4.0 to 7.0. As pH increased, so did the partition coefficient, as was the case upon decrease. The reduction in the partition coefficient beyond pH 7.0 may be due to the electrostatic force overcoming the hydrophobic force as explained in the following equation by Albertsson:

$$\ln K = \ln K_0 + (FZ\Delta\varphi/RT) \qquad (7)$$

According to Formula 7, protein partition is driven by two effects: the electrostatic component ($FZ\Delta\varphi/RT$) determined by the net electrical protein charge, and a hydrophobic component (K_0) which has a maximal effect when the medium pH is near the isoelectric point (for BSA, the pI is 4.7). When pH is increased from 4.0 to 7.0, the hydrophobic component is greater than the electrostatic forces. Therefore, the PEG tends to interact with the protein to create the increase of the partition coefficient.[27]

When pH increases beyond 7.0, the net charge of protein (Z) becomes negative and the electrostatic potential difference ($\Delta\varphi$) between the phases acquires positive value due to the accumulation of citrate anions in the bottom phase. Hence, the electrostatic term ($FZ\Delta\varphi/RT$) will be less than zero. As a result, the net negative protein charge increases with > 7.0 pH, decreasing the k value.[28]

Adding Neutral Salt

Salts have been added to systems to increase the selectivity of protein partitioning in the aqueous two-phase methodology application for biological separations. Generally, the neutral salts have the capacity to modify the water structure. The cation always decreases the partition coefficient of BSA in ATPS.[29] This behaviour can be explained on the basis that the ions of the salt have different affinities for the two phases, giving rise to an electrostatic potential difference between phases.[1, 30] The variation of the partition coefficient of BSA upon addition of NaCl is shown in Figure 6. The partition coefficient of BSA decreases with increasing NaCl concentrations in the small salt concentrations (up to 0.15 M NaCl) as has been observed with α-LA and β-LG in literature.[31] In this range of NaCl concentration, the interaction of the protein molecules with the ions and the PEG molecules in the polymer-rich phase is increased. For NaCl concentrations above 0.15 M, higher partition coefficients of

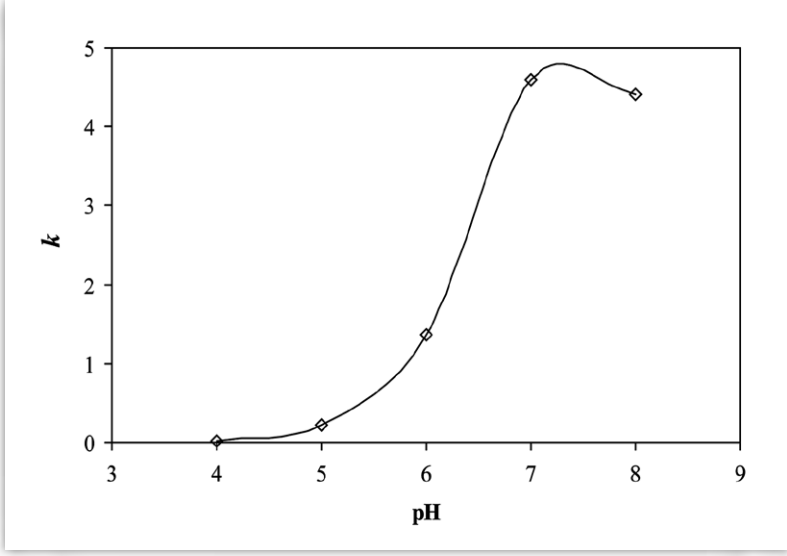

FIGURE 5. The effect of pH on the partitioning of BSA in 34 % PEG 2000/34 % tripotassium citrate at 303.15 K.

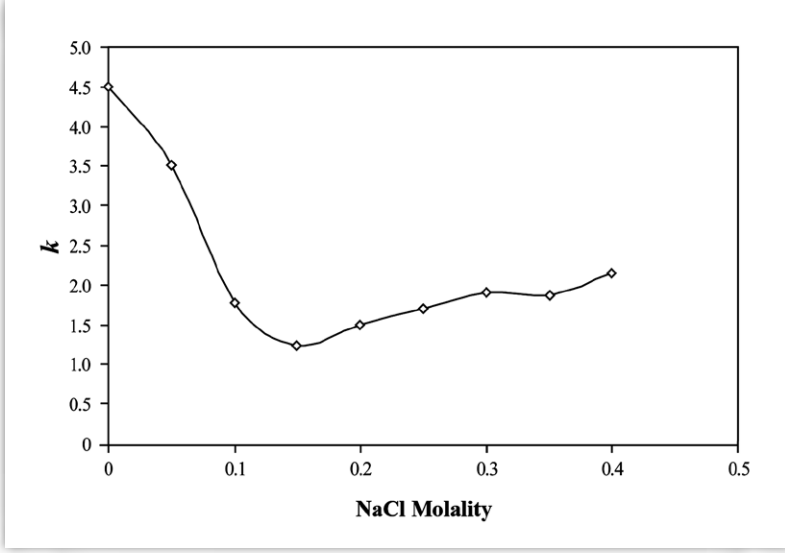

FIGURE 6. The effect of NaCl concentration on the partitioning of BSA in 34 % PEG 2000/34 % tripotassium citrate, pH 7.0, 303.15 K.

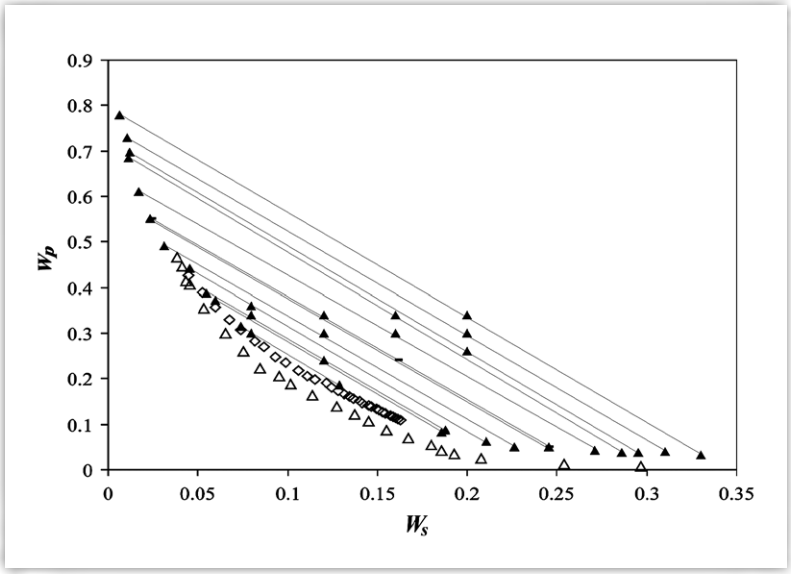

FIGURE 7. Phase diagram of △ PEG 2000/tripotassium citrate ATPS without BSA at 303.15 K: ◇ PEG 2000/tripotassium citrate ATPS with BSA at 303.15 K; ▲ tie line; ---- tie line predicted by Equations 8 and 9.

BSA have been observed at different pH values where the BSA partition is determined by its hydrophobic nature. It appears likely that the partition coefficient is totally independent of protein surface charge in combination with hydrophobic characteristics. It was confirmed that the PEG, being more hydrophobic in nature, tends to strongly interact with the non-polar regions of BSA. Thus, the partition coefficient of BSA increases beyond 0.15 M NaCl concentrations.[6]

Phase Diagram and Equilibrium of PEG 2000/Tripotassium Citrate System with BSA at 303.15 K

Binodal

In order to further understand the applicability of ATPS for any application, the phase diagram of the system is essential to explaining the equilibrium characteristics of the system components (PEG and salt). The phase diagram also explains the critical concentrations of PEG and salt required to form the ATPS. In the literature, the binodal curve for PEG 2000/tripotassium citrate system without BSA at 303.15 K was reported by Yan-Min *et al.*[20] In the present study, the binodal curve was prepared through the cloud point method with 0.05 mg/mL of BSA. The experimental binodal data and literature binodal data[20] were plotted and compared in Figure 7. With the figure, it was found that the binodal curve was shifting towards the two-phase region when protein was incorporated. The results suggested that the critical concentration of PEG/salt required to form the two phases is more, or solubility is increased with the addition of BSA.

Tie Line

At equilibrium, the composition of phases along the TLL are especially important in the contest of the partitioning study. To obtain the equilibrium phase composition at a known feed composition of PEG and salt, the Othmer-Tobias (Equation 8) and Bancroft (Equation 9) relationships were used and the equilibrium studies were made for the current ATPS (with the presence of BSA). The two-phase systems were formed at several compositions of tripotassium citrate and PEG 2000 by mixing the individual stock solutions and allowing them to reach equilibrium. A number of tie lines were created, and the feed compositions, along with equilibrium composition of the individual phases (Figure 7) with TLL are reported in Table 4. The reliability of tie line compositions was ascertained by Equations 8 and 9.[14, 15] The regression coefficient found for the present system is 0.988 for Equation 8 and 0.9702 for Equation 9.

$$\left(\frac{1-w_{\mathrm{p}}^{\mathrm{top}}}{w_{\mathrm{p}}^{\mathrm{top}}}\right)=K\left(\frac{1-w_{\mathrm{s}}^{\mathrm{bot}}}{w_{\mathrm{s}}^{\mathrm{bot}}}\right)^{\mathrm{n}} \quad (8)$$

$$\left(\frac{w_{\mathrm{w}}^{\mathrm{bot}}}{w_{\mathrm{s}}^{\mathrm{bot}}}\right)=K_1\left(\frac{w_{\mathrm{w}}^{\mathrm{top}}}{w_{\mathrm{p}}^{\mathrm{top}}}\right)^{\mathrm{r}} \quad (9)$$

TABLE 4. Tie line data for PEG 2000/tripotassium citrate system at 303.15 K with TLL.

Feed Composition		Top Phase Composition		Bottom Phase Composition		TLL
% w_p (w/w)	% w_s (w/w)	% w_p (w/w)	% w_s (w/w)	% w_p (w/w)	% w_s (w/w)	
0.3000	0.0800	0.3150	0.0742	0.1851	0.1288	0.1409
0.3400	0.0800	0.3873	0.0551	0.0856	0.1881	0.3296
0.3600	0.0800	0.4419	0.0456	0.0608	0.2109	0.4153
0.2400	0.1200	0.3722	0.0601	0.0825	0.1856	0.3157
0.3000	0.1200	0.4904	0.0315	0.0502	0.2262	0.4814
0.3400	0.1200	0.5509	0.0232	0.0499	0.2457	0.5482
0.2400	0.1600	0.5500	0.0228	0.0495	0.2440	0.5472
0.3000	0.1600	0.6093	0.0174	0.0427	0.2709	0.6207
0.3400	0.1600	0.6845	0.0116	0.0384	0.2862	0.7021
0.2600	0.2000	0.6964	0.0124	0.0370	0.2949	0.7174
0.3000	0.2000	0.7280	0.0104	0.0385	0.3103	0.7519
0.3400	0.2000	0.7786	0.0066	0.0330	0.3302	0.8128

Where w_p^{top} represents the weight fraction of PEG 2000 in top phase, w_s^{bot} represents the weight fraction of salt in bottom phase, w_w^{bot} and w_w^{top} are weight fractions of water in bottom and top phases respectively, and K, n, K_1, and r are the fit parameters, found to be 0.1084, 1.7526, 3.3287, and 0.4909 respectively.

Tie Line Length

The % TLL provides the phase characteristics including the physical properties and chemical compositions at its equilibrium. Further, the volume ratio of the phases can be modified by altering the feed composition along a particular tie line. The TLL are estimated using the following relationship:

$$\text{TLL}\ (\%) = [(w_{p(T)} - w_{p(B)})^2 + (w_{s(T)} - w_{s(B)})^2]^{1/2} \quad (10)$$

From Figure 8, it is evident that the partition coefficient increases with an increase in TLL value for a particular salt concentration at constant temperature and molecular weight of PEG. However, for a specific TLL value, the lower salt concentration gave better partition at the same temperature and molecular weight. For the large value of TLL, the equilibrium values of the salt and PEG concentration in both phases were high (top most region of the binodal curve) where the partition behaviour was purely derived from the net force due to electrostatic force offered by the salt and hydrophobicity offered by the PEG. However, handling of these phases further for protein recovery might become difficult.

Response Surface Methodology Optimization

The central composite rotatable design was used to design the experiments by considering four independent variables: (1) % PEG 2000 (w/w); (2) % tripotassium citrate (w/w); (3) pH; and (4) NaCl (M); (shown earlier in Table 3). The range of variables was chosen according to the respective individual parameter studies in the previous sections. In total, 31 experiments were employed. The experiments were carried out in triplicate and the average values of responses (partitioning coefficient *k* and *% recovery*) are reported in Table 5 (following page). A regression analysis was carried out to fit the experimental data in the mathematical model. In order to develop a functional relationship between the responses, namely the partition coefficient *k* and *% recovery*, and the independent variables, namely the % PEG (w/w), % tripotassium citrate (w/w), pH, and NaCl concentrations, the linear model, the linear + squares model, and the quadratic model have been tested. Results determined that the quadratic model represented the data with a > 99 % confidence level for both the responses with the regression coefficient of 98.7 % and 97.4 % for Equations 11 and 12, respectively. The predicted quadratic models in terms of noncoded (actual) factors are:

$$k = -245.342 + 6.929A + 9.764B + 17.137C + 78.677D - 0.091A^2 - 0.254B^2 - 0.931C^2 - 85.464D^2 - 0.011AB - 0.123AC - 0.479AD - 0.134BC - 1.489BD + 0.260CD \quad (11)$$

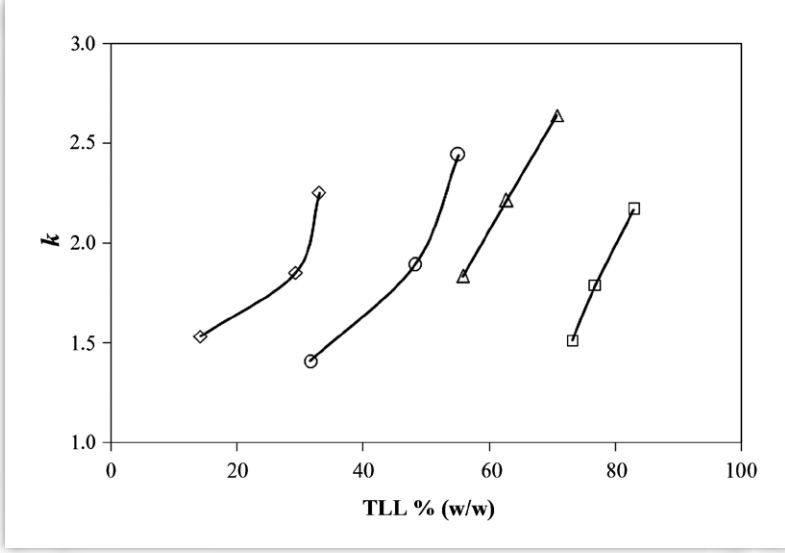

FIGURE 8. Effect of tie line length on partition coefficient in PEG 2000/tripotassium citrate at 303.15 K: ◇ 8 % salt; o 12 % salt; △ 16 % salt; □ 20 % salt.

TABLE 6. ANOVA for the quadratic models predicted for the partition coefficient *k* and *% recovery*.

	Source	DF	SS	MS	*F* Value	Probability > *F*
Partition Coeffient	Regression	14	115.465	8.2475	343.62	0.0001
	Linear	4	64.847	16.2170	675.45	0.0005
	Square	4	105.659	26.4140	1100.55	0.0002
	Interaction	6	7.188	1.1980	49.91	0.0008
	Lack-of-Fit	10	0.382	0.0382	31.98	0.0004
	Pure Error	6	0.002	0.0004	—	—
	Source	**DF**	**SS**	**MS**	***F* Value**	**Probability > *F***
% Recovery	Regression	14	13640.00	974.29	197.89	0.0001
	Linear	4	8815.64	2203.91	447.64	0.0000
	Square	4	4908.14	1227.04	249.23	0.0001
	Interaction	6	6493.60	1082.26	219.82	0.0005
	Lack-of-Fit	10	78.37	7.84	106.75	0.0003
	Pure Error	6	0.40	0.007	—	—

SS: sum of squares; DF: degree of freedom; MS: mean square

$$\% \, recovery = -2539.68 + 59.65A + 103.54B + 81.85C + 2222.81D - 0.68A^2 - 1.78B^2 - 3.87C^2 - 536.68D^2 - 0.29AB - 1.20AC - 7.78AD - 4.00BC - 59.63BD - 126.99CD \quad (12)$$

The ANOVA in Table 6 (previous page) shows that both models explained the process with significant values of *p* at 99% confidence level. From this, Table 6 proves evidence that the *F* values of linear and squared regression were much higher than the lack-of-fit. These large values imply that the partition coefficient and *% recovery* can be adequately explained by the model equation. Generally, *p* values lower than 0.001 indicate that the model is considered to be statistically significant at the 99% confidence level. Both the predictive models were statistically validated since the *F* value of each model was higher than the *F* value of the corresponding lack-of-fit. The partition coefficient and *% recovery* predicted through the model is also reported in Table 5. Further, the *F* and *p* values of the individual terms (Equations 11 and 12) reveal that all the individual variables had significant influence in the partition coefficient and *% recovery*. However, the interaction

TABLE 5. Experimental CCRD runs using four independent variables and corresponding results.

Feed		pH	Salt Molality	Top Phase		Bottom Phase		TLL	Phase Volume Ratio (*VR*)	k_{exp}	$k_{predicted}$	Recovery	
PEG 2000 % (w/w)	$C_6H_5K_3O_7$ % (w/w)			% w_p (w/w)	% w_s (w/w)	% w_p (w/w)	% w_s (w/w)					% exp	% predicted
32	16	6.0	0.25	0.5715	0.0302	0.0365	0.3082	0.6029	1.8333	6.0190	6.0253	91.90	91.76
36	14	7.0	0.35	0.5803	0.0286	0.0304	0.3099	0.6177	2.4400	1.4080	1.4744	78.63	78.31
28	14	7.0	0.35	0.4991	0.0336	0.0334	0.2639	0.5196	1.7742	2.1309	2.3290	70.29	71.53
24	16	6.0	0.25	0.4791	0.0360	0.0360	0.2672	0.4998	1.3889	0.1628	-0.1650	33.51	30.15
36	14	7.0	0.15	0.5747	0.0290	0.0445	0.3028	0.5968	2.5417	1.6009	1.5403	89.63	88.16
36	18	5.0	0.15	0.6330	0.0236	0.0190	0.3712	0.7056	2.4000	3.6821	3.5730	83.83	83.19
32	16	6.0	0.25	0.5716	0.0302	0.0375	0.3074	0.6017	1.8333	6.0203	6.0253	91.92	91.76
32	16	6.0	0.25	0.5720	0.0298	0.0373	0.3078	0.6026	1.8333	6.0246	6.0253	91.97	91.76
32	12	6.0	0.25	0.4911	0.0340	0.0324	0.2614	0.5120	2.7500	1.6153	1.5229	70.63	69.61
32	16	6.0	0.25	0.5727	0.0294	0.0374	0.3074	0.6032	1.8333	6.0190	6.0253	91.90	91.76
32	20	6.0	0.25	0.6286	0.0232	0.0232	0.3625	0.6940	1.6129	2.4300	2.4047	59.70	56.98
36	18	5	0.35	0.6521	0.0211	0.0294	0.3703	0.7140	2.2800	2.2198	2.2118	76.20	76.43
28	14	5	0.35	0.5023	0.0340	0.0322	0.2643	0.5235	2.6957	0.8015	0.8868	68.36	69.15
28	18	7	0.15	0.5431	0.0323	0.0324	0.3256	0.5889	1.2857	2.1708	2.3026	75.69	75.08
36	14	5	0.35	0.6047	0.0253	0.0343	0.2887	0.6284	3.2500	2.0474	2.0046	93.99	95.20
28	14	5	0.15	0.4854	0.0331	0.0457	0.2631	0.4962	2.4000	0.1901	0.2901	16.33	15.77
32	16	6	0.05	0.5633	0.0294	0.0299	0.3086	0.6020	2.1482	2.8400	2.9369	74.22	72.38
40	16	6	0.25	0.6661	0.0203	0.0258	0.3616	0.7256	3.1000	0.3032	0.5133	66.55	66.17
28	18	5	0.35	0.5497	0.0286	0.0366	0.3264	0.5933	1.3611	1.2961	1.4457	57.55	59.63
28	14	7	0.15	0.4851	0.0344	0.0382	0.2701	0.5053	1.8667	1.5914	1.6281	66.04	68.94
32	16	8	0.25	0.5692	0.0282	0.0307	0.3032	0.6047	1.8333	2.3153	2.1671	79.74	78.41
28	18	7	0.35	0.5609	0.0286	0.0247	0.3152	0.6080	1.4286	1.7028	1.8123	28.83	29.97
36	14	5	0.15	0.5911	0.0257	0.0282	0.2970	0.6249	2.6250	2.2554	2.1747	52.27	54.25
32	16	6	0.25	0.5722	0.0298	0.0363	0.3082	0.6038	1.8333	6.0104	6.0253	91.75	91.76
32	16	6	0.25	0.5715	0.0302	0.0371	0.3078	0.6021	1.8333	6.0169	6.0253	91.69	91.76
32	16	6	0.45	0.5764	0.0286	0.0314	0.3099	0.6133	1.8966	2.4913	2.2766	70.12	68.21
36	18	7	0.15	0.6233	0.0240	0.0207	0.3732	0.6965	2.2400	1.9198	1.8632	82.72	85.06
28	18	5	0.15	0.5459	0.0290	0.0278	0.3289	0.5986	2.5417	2.0778	2.0401	50.49	53.95
32	16	4	0.25	0.5693	0.0286	0.0398	0.3066	0.5980	2.4000	2.4044	2.4349	76.58	74.16
32	16	6	0.25	0.5722	0.0298	0.0384	0.3070	0.6015	1.8333	6.0668	6.0253	91.22	91.76
36	18	7	0.35	0.6531	0.0207	0.0283	0.3712	0.7164	1.9286	0.6172	0.6061	26.34	27.51

between the PEG and salt concentrations and pH and NaCl molality terms had minimal influence on the responses.

The partition coefficient of the present ATPS over different combinations of independent variables were visualized through contour plots (Figures 9 and 10), obtained based on the model equations. The plots (Figures 9A and 9E) are represented as a function of two factors at a time, holding other factors at a fixed level of zero. All of the response plots revealed that at low and high variables levels, the partition coefficient was minimal. However, it was noted that the higher values of the partitioning coefficient represented the middle regions of the contours. This phenomenon

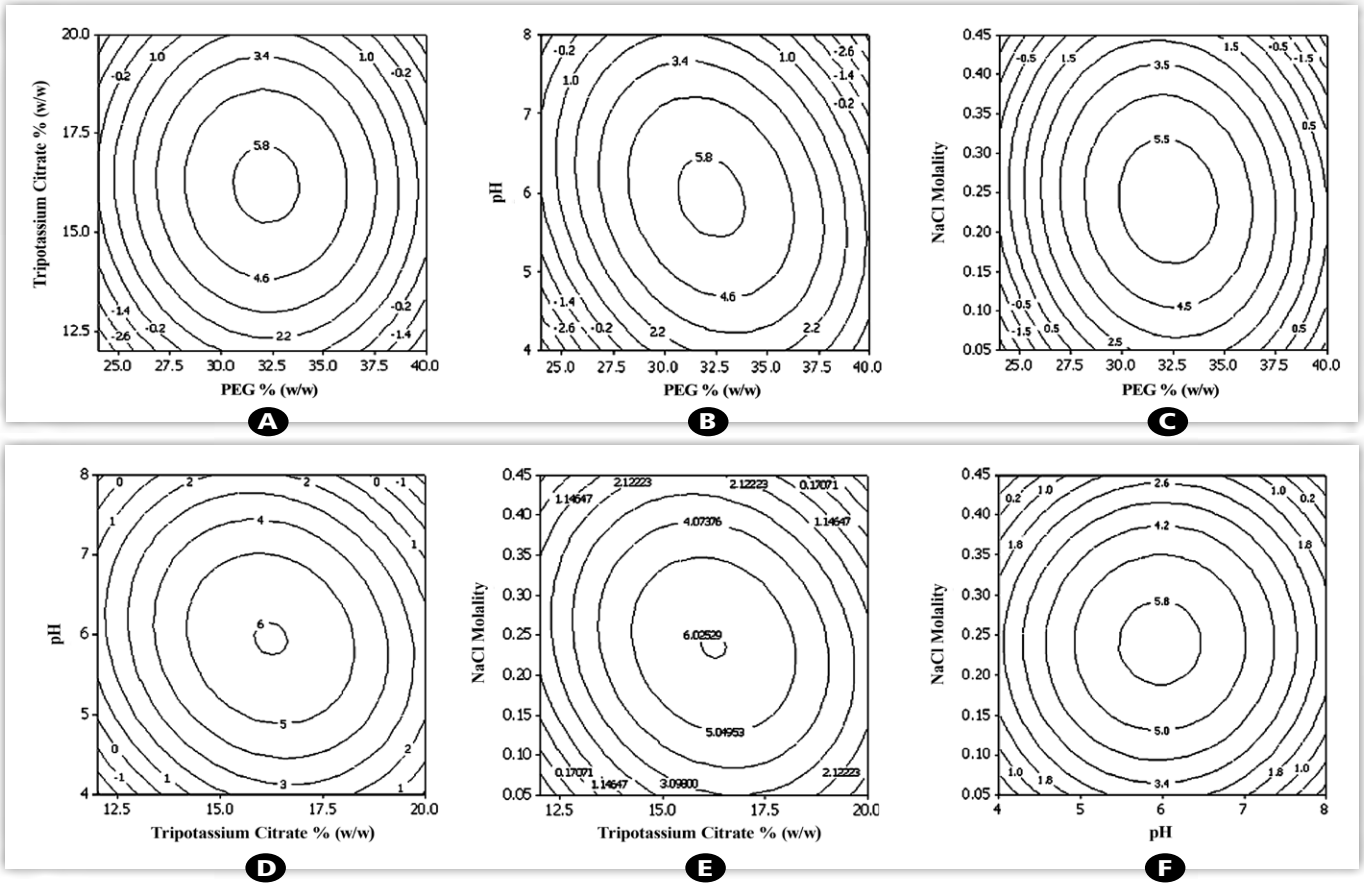

FIGURE 9. Contour plots for the partition coefficient of BSA in the top phase as a function of: (A) tripotassium citrate and PEG concentration; (B) pH and PEG 2000 concentration; (C) NaCl and PEG 2000 concentration; (D) pH and tripotassium citrate concentration; (E) NaCl and tripotassium citrate concentration; and (F) NaCl concentration and pH (0 level values of the other variables were considered as a constant for all figures [*i.e.*, 32 % PEG 2000/16 % tripotassium citrate/0.25 M NaCl, pH 6.0]).

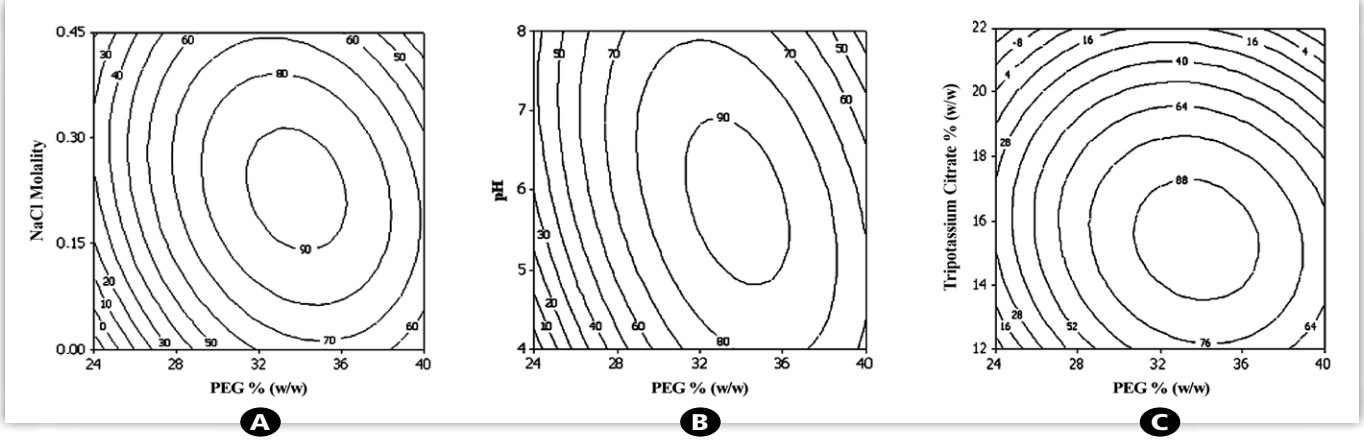

FIGURE 10. Contour plots for the *% recovery* of BSA in the top phase as a function of: (A) % PEG 2000, 16 % NaCl molality, pH 6.0; (B) % PEG 2000, pH range, 16 % tripotassium citrate, 0.25 M NaCl; and (C) % PEG 2000, % tripotassium citrate, 0.25 M NaCl, pH 6.0.

proves that there was an existence of optimization for the independent variables in order to maximize the partitioning and *% recovery*. Also, the contours confirmed the existence of the interaction between the independent variables considered for the optimization.

The maximum predicted partitioning coefficient *k* and *% recovery* for optimum value of the variables were obtained through a point prediction method and contour plots (Figures 9 and 10). The global optimum obtained for maximum partitioning coefficient *k* (6.03) and *% recovery* (91.76) were 32 % PEG 2000 (w/w), 16 % tripotassium citrate (w/w), pH 6.0, and 0.25 M NaCl. Further, three experiments were performed at the optimized process conditions and it was found that the experimental data obtained was well represented by the present model, Equation 6, with an error of ± 1 %.

Conclusion

Aqueous two-phase systems are becoming powerful tools for the purification of biomolecules. In this study, ATPS were employed for the purification of BSA. In addition, the process parameters which govern the partition were optimized using RSM. Basic studies suggested that PEG molecular weight and its concentration affects the partition significantly. Moreover, lower molecular weight PEGs, less salts (including NaCl), and elevated tie lines were preferred for partitioning of BSA in top phase. CCRD was used to design experimental points and develop the model equation. Responses generated were partition coefficient and *% recovery* as a function of the four independent parameters. The experimental data were fit to quadratic polynomial equations through a least square technique, and statistical analyses revealed that the developed model is in agreement with the experimental values. The regression obtained for partition coefficient was 0.997 and *% recovery* was 0.994. The optimal system consisted of 32 % PEG 2000 (w/w), 16 % tripotassium citrate (w/w), and 0.25 M NaCl, pH 6.0, and 303.15 K. A maximum partition of 6.03 and a recovery of 91.76 % in the top phase were obtained under these conditions. Study results show that ATPS can also be used for the recovery and concentration of other proteins by optimizing process parameters.

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