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Response surface methodology for the evaluation of guanidine hydrochloride partitioning in polymer-salt aqueous two-phase system

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Abstract−The current study employed response surface methodology (RSM) with a face-centered central composite design (CCD) to indicate the essential variables on the partition coefficient of guanidine hydrochloride (GuHCl) in the poly (ethylene glycol) (PEG)-phosphate aqueous two-phase system (ATPS). To evaluate the partition coefficients of GuHCl in the mentioned ATPS, the pH (7.0, 8.5 and 10.0), GuHCl concentration (1.0, 3.5 and 6.0% w/w), PEG molecular weight (2,000, 4,000 and 6,000 $gmol^{-1}$) and PEG/potassium phosphate concentrations ratio were selected as independent variables. A quadratic model is suggested to find the impact of these variables. The suggested model has a strong harmony with the experimental data. The results of the model display that the GuHCl concentration and weight percent of the salt in feed have a large and small influence on the GuHCl partitioning.

Keywords: Guanidine Hydrochloride, Aqueous Two-phase Systems, Partitioning, Response Surface Methodology

INTRODUCTION

Guanidine hydrochloride (GuHCl), as an important denaturant, is necessary to the recovery of the active protein from inclusion bodies by solubilization of aggregated protein during the production of recombinant proteins [1]. One of the useful methods for recovery and refolding of protein from inclusion bodies is an aqueous two-phase system (ATPS), a liquid-liquid extraction strategy in the presence of denaturant [2,3]. ATPS substantially minimizes the number of initial downstream steps, and clarification, concentration, and partial purification can be unified in one unit [4]. Many advantages have been reported in the related studies for employing ATPs, such as simplicity [5], having a continuous steady state [6-12], biologic compatibility [13], low interfacial tension [14], decreased processing time [15], economic efficiency [16,17], good resolution, low energy consumption [18,19], high yield [20], relatively high load capacity [21] scaling up feasibility [22-24] and selective extraction [25].

Despite the considerable advantages, ATPSs have not been extensively adopted for either industrial or commercial applications because the design of an industrial scale process needs to focus on determining phase diagrams, partition coefficients, and other thermodynamic data and also understanding of the mechanism of the process [26]. For conceptualizing the structure of solution partitioning in the ATPS of the previous study [27], they developed an artificial neural network to predict the partition coefficients of GuHCl in poly (ethylene glycol) (PEG) 4000+ phosphate ATPS, under a wide range of conditions of PEG/phase forming salt (% w/w) ratio, GuHCl concentrations, and pH. In the current study, the number of independent parameters were determined by means of central composite design (CCD) and response surface methodology (RSM) to find a more comprehensive model.

The RSM comprises a group of empirical techniques to evaluate the relationship between a cluster of controlled experimental factors and measured responses based on some criteria [28-30]. For this purpose, the system is described by linear or square polynomial functions, and these functions are used to explore (modeling and displacing) experimental conditions until the optimization [31].

Although a number of studies have investigated the use of RSM in the optimization and investigation of the effectiveness of the parameters on the process of biomolecules in ATPS (Table 1), to our knowledge, there is no report on partitioning of denaturants in ATPS using RSM. Consequently, the current study investigated the effect of parameters such pH, temperature, polymer molecular weight in feed, salt and polymer weight percent's in feed ratio and GuHCl concentration on partitioning of GuHCl in PEG (2,000, 4,000, 6,000 $gmol^{-1}$) and potassium phosphate ATPS's by using with CCD. According to the results of the experimental design, a regression analysis was conducted and a model for GuHCl partitioning was proposed.

EXPERIMENTAL

1. Materials

The material used in the experiment consisted of polyethylene glycol, with a mass average 2,000, 4,000 and 6,000 $gmol^{-1}$, dipotassium hydrogen phosphate and potassium dihydrogen phosphate (anhydrous GR for analysis, >99%), sodium hydroxide (NaOH; mass purity >0.99) and sulfuric acid ((95 to 97) % H_2SO_4, GR for analysis, >95.0%) obtained from Merck (Darmstadt, Germany) and used without further purification. Distilled and deionized water

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Table 1. A brief review related to the application of RSM in ATPS

Type of ATPS	Application	Reference
PEG-potassium phosphate	To identify the key factors governing xylanase partitioning	[32]
PEG-potassium phosphate	To optimize the conditions of partitioning of xylanolitic complex	[33]
PEG-potassium phosphate	To optimize the recovery of the chymosin and pepsin in the top phase	[34]
PEG-potassium phosphate	To optimize the refolding and recovery of active recombinant bacillus halodurans xylanase	[35]
PEG-potassium phosphate	To optimize the human antibodies extraction process	[36]
PEG-potassium phosphate	To optimize the conditions for acid protease partitioning and purification	[37]
PEG-potassium phosphate	To optimize the recovery of monoclonal antibodies from unclarified transgenic tobacco extract	[38]
PEG-potassium phosphate	To evaluate the effects of the parameters involved in the purification of clavulanic acid from fermentation broth	[39]
Ethanol-potassium phosphate	To examine the partition coefficient of 1,3-propandiol in synthetic solutions	[40]
PEG-potassium phosphate	To determine a separation of recombinant β-glucuronidase from transgenic tobacco	[41]
PEG-potassium phosphate	To optimize whey protein partitioning	[42]
Ethanol-potassium phosphate	To optimize the extraction yield of lactic acid in aqueous solutions	[43]
PEG-potassium phosphate	To optimized the extraction of protease produced from bacillus licheniformis NCIM 2042	[44]
Ethylene oxide-propylene oxide-potassium phosphate	To evaluate the influence parameters on the partition coefficient and extraction yield of lysozyme	[45]
PEG-potassium phosphate	To evaluate pDNA distribution in a range of ATPS	[46]
PEG-potassium phosphate	The optimize the bromelain extraction	[47]
PEG-potassium phosphate	To investigated the effects of PEG content and the additions of tripotassium phosphate on protopectinase partition	[48]
PEG-potassium phosphate	To purify α-toxin from clostridium perfingens	[49]
PEG-potassium phosphate	To examine the partition and partial characterization of a collagenase from penicillium aurantiogriseum	[50]
PEG-potassium phosphate	To examine the partition and recovery of Canavalia brasiliensis lectin	[51]
PEG-potassium phosphate	To examine extraction of alkaline fungal xylanase	[52]
PEG-dextran	To investigate the effects of pH and NaCl concentration on partitioning of bovine serum albumin	[53]
PEG-dextran	To obtained the optimal conditions of bovine serum albumin	[54]
PEG-dextran	To investigated partition behavior of pure bovine serum albumin	[55]
PEG-dextran	To optimize of the process variables for the extractive fermentation of 2,3-butanediol	[56]
PEG-sodium sulfate	To optimize the conditions for bacteriocin extraction	[57]
PEG-sodium sulfate-sodium chloride	To optimize the conditions for Lysozyme Extraction	[58]
PEG-sodium sulfate	To determine optimal conditions for fibrinolytic enzyme production by bacillus subtilis DC-2	[59]
PEG-sodium sulfate	To optimize the pectinase partitioning	[60]
PEG-ammonium sulfate	To achieve higher purification factor values and greater bromelain recovery	[61]
PEG-ammonium sulfate	To determine the optimal conditions for propionic acid extraction	[62]
Ethanol-ammonium sulfate	To determine the optimal extraction conditions of anthocyanins from purple sweet potato	[63]
PEG-sodium sulfate	To improvement in endo-mannanase yield as compared to un-optimized growth conditions	[64]
PEG-ammonium sulfate	To optimize the extraction of luciferase from fireflies (Photinus pyralis)	[65]
PEG-ammonium sulfate	To optimize the conditions the recombinant D-galactose dehydrogenase partitioning	[66]
PEG-sodium citrate	To optimize of penicillin acylase purification	[67]
PEG-citrate	To modeling of α-amylase purification	[68]

Table 1. Continued

Type of ATPS	Application	Reference
PEG-citrate buffer	To identify the factors affecting separation of α-amylase from a culture supernatant of aspergillus oryzae CBS 819.72	[69]
PEG-sodium citrate	To investigate the optimum level of pH and NaCl concentration for the partitioning of pure bovine serum albumin	[70]
PEG-citrate	To determine the more significant effects in the purification of lactate dehydrogenase	[71]
PEG-potassium citrate	To optimize of aqueous two-phase extraction of lysozyme from crude hen egg white	[72]
PEG-potassium citrate	To optimize the bovine serum albumin partitioning	[23]
PEG-sodium citrate	To optimize the pancreatic trypsin extraction	[73]
PEG-sodium citrate	To achieve partition of lectin from canavalia grandiflora benth	[74]
PEG-sodium citrate	To investigate partition and recovery of phytase from absidia blakesleeana URM5604	[75]
PEG-sodium citrate	To determine extraction of proteases from fermented broth	[76]
PEG-sodium carbonate	To optimize the rhizopus niveus lipase partitioning	[77]
PEG-calcium chloride	To optimize the continuous extraction of α- and β-amylases from zea mays malt	[7]
PEG-ammonium sulfate and sodium chloride	To optimize the affinity partitioning conditions of papain	[78]
PEG-sodium citrate, potassium phosphate, sodium sulfate and ammonium sulfate	To study the effects of different factors on ConBr extraction and to optimize its isolation from a plant extract	[51]
PEG-sodium sulfate PEG-potassium phosphate	To optimize the thaumatin extraction	[79]

were used for the preparation of solutions. All other materials were obtained through analytical grades.

2. Preparation of Aqueous Two-phase Systems for Partitioning

To prepare the biphasic systems, the PEG, dipotassium hydrogen phosphate, potassium dihydrogen phosphate and GuHCl were mixed at the desired pH, after which the solute composition of the mixture (mass) was determined. Then 10 g of the samples was prepared with the suitable weight of the polymer, salt stock, GuHCl, and water mixing in 15 ml graduated cylinders by utilizing an analytical balance (A&D GF 300, Japan) with a precision of $\pm 10^{-4}$ g at 25 °C. To achieve the desired temperature for each system with an uncertainty of 0.05 °C, the tubes were situated in a thermostatic bath (Memert INE 400, Germany). The pH of the salt solutions was adjusted through mixing the appropriate ratio of dipotassium hydrogen phosphate, potassium dihydrogen phosphate, sodium hydroxide, and sulfuric acid. Finally, the pH of the solutions was measured precisely using a Metrohm 827 pH lab meter (Switzerland).

The contents of the test tubes were then rigorously put vortexes for ten minutes before being placed in the thermostatic bath for two hours at the desired temperature, the test tubes were centrifuged (Hermle Z206A, Germany) at 6,000 rpm for five minutes to separate the resulting phases. These phases did not indicate any turbidity and the top and bottom samples could be easily separated.

Sullivan was employed to measure the GuHCl concentration in the separate phases based on the colorimetric assay [80]. Rahimpour and Pirdashti [81], and Pirdashti et al. [27] used 1,2 naphthoquinone-4-sodium sulfonate, which leads to the development of a rose-red color due to the formation of 4-guanido-1,2-naphtoquinone in an acidic medium.

3. Experimental Design and Regression Analysis

The partitioning of GuHCl in ATPS depends on several factors, such as polymer and salt concentrations, polymer molecular weight and pH [27,81]. To study the effect of these factors and their interactions on the GuHCl partitioning, an experimental design was employed.

CCD was used to analyze the partitioning of GuHCl in ATPS of PEG-potassium phosphate. Table 2 presents the independent variables, experimental ranges and statistical levels for this system. The parameters of pH (X_1), the PEG molecular weight (X_2), the GuHCl concentration (X_3), the ratio of PEG weight percent in the feed to the salt weight percent in the feed (X_4) and temperature (X_5) were the independent variables. Each of these parameters was coded at three levels: −1, 0 and +1. The dependent variable was the partition coefficient of GuHCl in ATPS. In addition, the ranges of the independent variable were selected based on the preliminary experiments [81,82], and a two-phase region was formed in the weight

Table 2. The experiment range and level of independent variables in coded units

Independent variable	Range and level		
	−1	0	+1
X_1 (pH)	7.0	8.5	10.0
X_2 (PEG molecular weight)	2000	4000	6000
X_3 (GuHCl concentration)	0.62	1.15	1.67
X_4 (PEG/salt concentration)	10	15	20
X_5 (Temperature)	20	30	40

Table 3. Experimental design based on the central composite design for five independent variables along with the observed response

Run	pH	Mw ($gmol^{-1}$)	GuHCl concentration (w/w%)	Polymer/salt concentration (w/w%)	Temperature (^oC)	K
1	7.00	6000	1.00	1.67	40	0.79
2	7.00	6000	6.00	0.62	40	0.91
3	10.00	2000	1.00	0.62	40	0.56
4	10.00	2000	6.00	0.62	20	0.95
5	8.50	2000	3.50	1.15	30	0.89
6	10.00	2000	1.00	1.67	20	0.82
7	8.50	4000	3.50	1.15	30	0.86
8	8.50	4000	3.50	1.15	30	0.81
9	7.00	2000	6.00	0.62	20	1.14
10	7.00	6000	1.00	0.62	20	0.72
11	7.00	2000	1.00	1.67	40	0.92
12	10.00	6000	6,00	1.67	40	0.88
13	8.50	4000	3.50	1.15	30	0.79
14	8.50	4000	3.50	1.15	40	0.77
15	10.00	6000	6,00	1.67	20	0.98
16	8.50	4000	3.50	1.15	30	0.83
17	7.00	6000	6.00	1.67	20	1.17
18	10.00	2000	6.00	0.62	40	0.85
19	7.00	6000	6,00	1.67	40	1.07
20	10.00	2000	1.00	1.67	40	0.72
21	7.00	4000	3.50	1.15	30	0.90
22	8.50	4000	3.50	0.62	30	0.75
23	8.50	4000	1.00	1.15	30	0.66
24	8.50	4000	3.50	1.67	30	0.91
25	10.00	6000	6.00	0.62	20	0.82
26	7.00	6000	1.00	1.67	20	0.89
27	10.00	4000	3.50	1.15	30	0.71
28	8.50	4000	3.50	1.15	30	0.85
29	10.00	6000	1.00	1.67	40	0.60
30	7.00	2000	1.00	1.67	20	1.01
31	8.50	4000	3.50	1.15	30	0.75
32	7.00	6000	1.00	0.62	40	0.62
33	10.00	6000	1.00	1.67	20	0.70
34	10.00	6000	1.00	0.62	20	0.53
35	7,00	2000	1.00	0.62	40	0.75
36	8.50	4000	3.50	1.15	30	0.80
37	10.00	2000	6.00	1.67	20	1.11
38	10.00	6000	1.00	0.62	40	0.43
39	7.00	6000	6.00	0.62	20	1.01
40	10.00	2000	1,00	0.62	20	0.66
41	7.00	2000	6,00	0.62	40	1.04
42	7.00	2000	6,00	1.67	20	1.20
43	8.50	4000	3.50	1.15	30	0.78
44	10.00	2000	6,00	1.67	40	1.01
45	8.50	4000	3.50	1.15	20	0.87
46	10.00	6000	6.00	0.62	40	0.72
47	7.00	2000	6.00	1.67	40	1.30
48	8.50	4000	3.50	1.15	30	0.76
49	8.50	6000	6.00	1.15	30	0.94
50	7.00	2000	1.00	0.62	20	0.85

Standard uncertainties: u (GuHCl concentration)=0.001; u (polymer/salt concentration)=0.002; u (pH)=0.001; u (T)=0.05 °C; u (K)=0.01

percent of salt and polymer in the feed for each pH values.

DESIGN EXPERT 7 (Stat-Ease Inc., Minneapolis, MN, USA) software was used for regression and graphical analysis of the data and the most frequently used design based on RSM was CCD. CCD was used to investigate the partition process since it fits well with the quadratic surface. The study design included fifty experiments, and eight-star points were employed to the second-order polynomial model. The optimum values of the selected variables were obtained by solving the regression equation at desired values of the process responses as the optimization criteria [83].

The experimental scheme is shown in Table 3. The ATPS were prepared according to the conditions of Table 3, and the samples of the top and bottom phases were withdrawn and analyzed. A stock solution of GuHCl was added to ATPS as a solute, and it was divided between the phases. Its partition coefficient, K, was a function of the equilibrium concentration of the solute in the upper and lower phases as provided in formula 1.

$$K = \frac{[\text{Solute Concentration}]_{top}}{[\text{Solute Concentration}]_{bottom}} \quad (1)$$

The behavior of system is explained by the following empirical second-order polynomial model Eq. (2):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

where Y is the predicted response, x_i, x_j, ..., x are the input variables, which affect the response Y, x_i^2, x_j^2, ..., x_k^2 are the square effects, $x_i x_j$, $x_i x_k$ and $x_j x_k$ are the interaction effects, β_0 is the intercept term, β_i (i=1, 2, ..., k) is the linear effect, β_{ii} (i=1, 2, ..., k) is the squared effect, β (i=1, 2, ..., k; j=1, 2, ..., k) is the interaction effect and ε is a random error [83-85].

4. Variance Analysis

The analysis of variance was conducted to verify the validity of the model; the results are presented in Table 4. According to the analysis of variance, the F-value for overall regression model (330, 17) was significant at %5 and the lack of feed was insignificant, which indicated that the quadratic model was very adequate in approximating the response surface of the experimental design. The coefficient of determination (R^2) and adjusted R^2 value were 0.9788 and 0.9758, respectively. Accordingly, the model could explain 97.88% of the variability in the response. The regression analysis was carried out on the experimental data to find an optimal region of factors for the studied responses. The analysis allowed the determination of quadratic statistical models that describe the relation among the responses K with pH, PEG molecular weight, GuHCl concentration, PEG/salt concentration, and the temperature. Obtained regression models could be described by the following:

$$K = 4.49089 - 0.063529 * (X1) - 0.0000320588 * (X2) + 0.057294 * (X3) - 0.29797 * (X4) - 0.00438235 * (X5) + 0.19828 * (X4)^2 \quad (3)$$

The normal probability and studentized residuals plot are shown in Fig. 1 for the partition of guanidine hydrochloride. Residual values demonstrated that the model satisfies the assumptions of ANOVA where the studentized residuals measured the number of standard deviations separating the actual and predicted values. It also showed

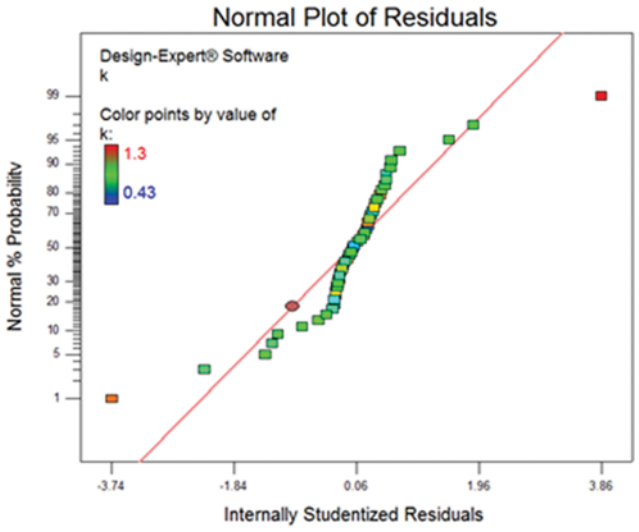

Fig. 1. The studentized residuals and normal % probability plot for guanidine hydrochloride partitioning; red line show predicted value; square is experimental data; circle is normal% probability for zero value of internally studentized residuals.

Table 4. Analysis of variance (ANOVA) for response surface quadratic model

Source of variation	SS	Df	Mean square	F-value	P-value
Model	1.47	6	0.24	330.17	<0.0001
pH	0.31	1	0.31	416.50	<0.0001
PEG molecular weight	0.14	1	0.14	188.55	<0.0001
GuHcl	0.70	1	0.70	940.98	<0.0001
PEG/salt concentration	0.23	1	0.23	304.43	<0.0001
Temperature	0.065	1	0.065	88.08	<0.0001
[PEG/Salt concentration]^2	0.031	1	0.031	42.46	<0.0001
Residual	0.032	43	0.0007		
Lack of feed	0.022	36	0.0006	0.46	0.9404
R^2	0.9788				
R^2_{adj}	0.9758				

%CV 0.9715

that neither response transformation was needed nor was there any apparent problem with normality.

RESULTS AND DISCUSSION

1. Response Surface Experimental Design

One of the most frequently used designs in RSM is the CCD, in which all directions from the center are equally predictable [86]. Several factors should be considered in an ATP system, including polymer molecular weight, concentration, phase-forming salt, ionic strength, and pH [42]. Changing a variable at one time is one of the common methods in experimental design to optimize operating conditions. However, this process has some problems, such as being time-consuming and it cannot guarantee the optimal parameter determination [28,87,88]. RSM is an experimental strategy for seeking the effective parameter in a multi-variable system, which is used in the current study to reveal the true response in a complex system. The results obtained from the model indicated that the GuHCl concentration and weight percent of the salt in feed have a large and small effect on the GuHCl partitioning, respectively. The pH of the ATPS can alter the GuHCl partitioning coefficient through the variation of the GuHCl net charge. The weight percent of polymer in the feed decreases the partitioning coefficients. By increasing the polymer molecular weight, the partitioning coefficient of GuHCl was increased.

2. Effects of pH

The increase in pH reduces the trend of GuHCl in the top phase and decreases the partition coefficient. Following the increase in pH, the interaction between PEG and water decreased [27]. Consequently, increasing pH of the aqueous polymer-salt two-phase system water is transferred from the phase containing high amount of polymer to the phase that includes rich salts; the other effects reported in the studies are the increase in polymer concentration of the polymer-rich phase, decrease in the salt-rich phase concentration, and the increase in the volume of the salt-rich phase [89, 90]. According to Barbosa et al. (2011), isoelectric points (pI) of proteins and enzymes are essential for partitioning them to the phases as the parts of the ATPS system [91]. The other reported effects of the pH of the system are the impact on the charge of the studied protein and composition of ion and the introduction of new type of partitioning to the two phases known as differential partitioning [92]. To avoid potential issues in this regard, Benavides and Rito-Palomares (2008) concluded that there should be a certain level for the initial pH, which is not below the pI of target biomolecules [93]. Accordingly, an appropriate pH level for the PEG/phosphate system is any value above 7, while a pH level below 6.5 is adaptable with different system such as the PEG/sulfate system [94]. The biomolecules including proteins and enzymes have a special feature of stability at pH=7 as a great condition to perform the ATPS partitioning [94]. In this case, according to Fig. 2, the partition coefficient of GuHCl decreased with increasing the pH value. Such behavior was also observed in the study by Zhang et al. (2010), as they found a reduction in partition coefficient of lipase from 7.94 to 4.45 followed by the increase in pH of the ATPS (PEG-4000/K_2HPO_4, 12.13%) from 7.0 to 9.0, and the activity was recovered from 81.1 to 70.6% [95].

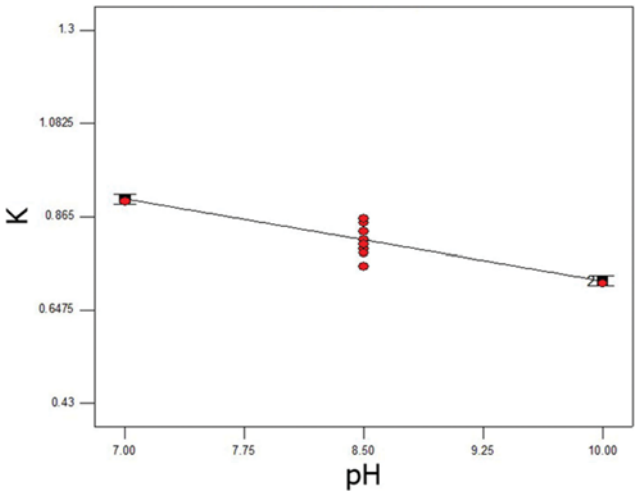

Fig. 2. The solid line show the effect of pH on GuHCl partition coefficient in ATPS of PEG+potassium phosphate+H_2O; circle is design point.

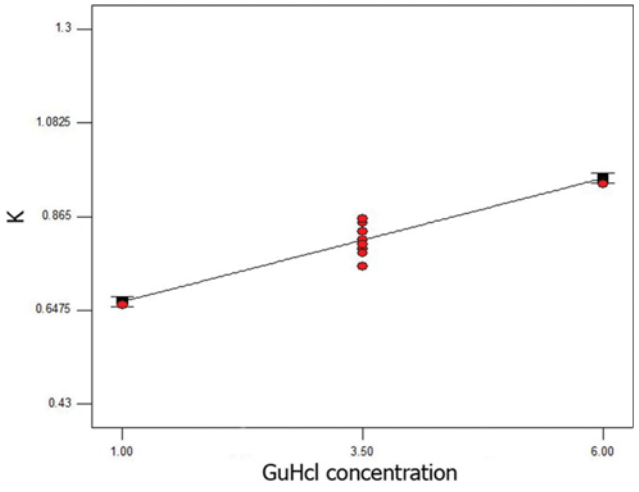

Fig. 3. The solid line show the effects of GuHCl concentration on GuHCl partition coefficient in ATPS of PEG+potassium phosphate+H_2O; circle is design point.

3. Effect of Gunidine Hydrochloride Concentration

The concentration of GuHCl had the paramount effect on the partition coefficient as shown in Fig. 3, the value of GuHCl partition coefficient increased by the increment of the GuHCl concentration. One of the suggested reasons for such increment is the structure that reduces the effect of GuHCl on the water (known as "structure breaking agent") or the distinctive interaction of GuHCL with aqueous interface [96]. Some opposing components were found in these systems with regard to the fact that the so-called structure-making salts include lyotropic series $H_2PO_4^-$ and K^+; however, GuHCl is provided as the structure breaking agent [27].

However, the other feature of GuHCl is in contrast with the structure breaking feature; it is known as the "structure making solute" mainly because of its capability to make hydrogen bounds with the water molecules [27]. Flory (1953) suggested that an intimate connection exists between the "structure breaking co-solute capac-

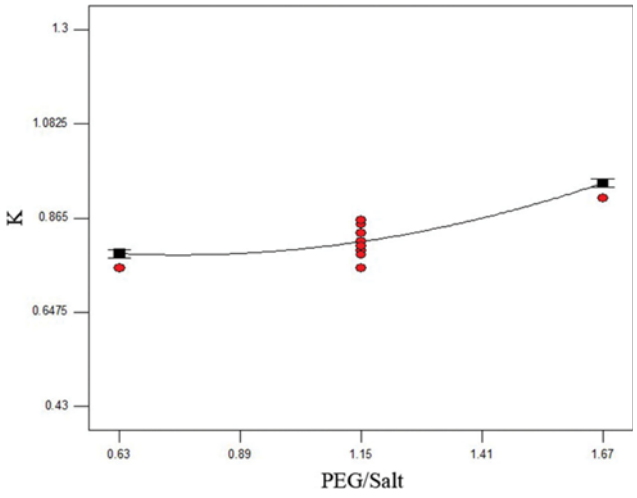

Fig. 4. The solid line show the effect of PEG/salt concentration on GuHCl partition coefficient in ATPS of PEG+potassium phosphate+H_2O; circle is design point.

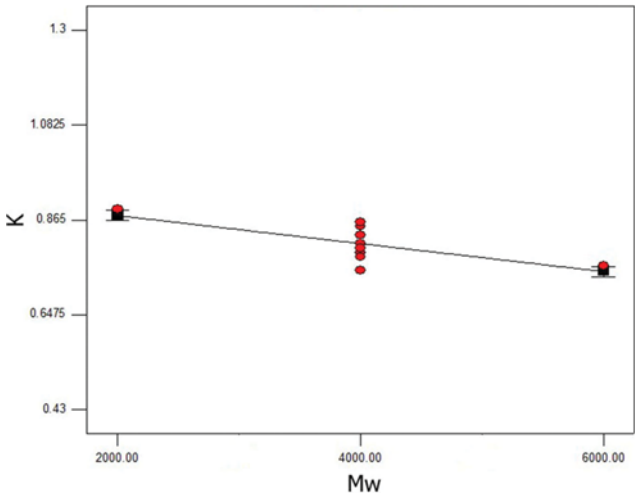

Fig. 5. The solid line show the effects of PEG molecular weight on GuHCl partition coefficient in ATPS of PEG+potassium phosphate+H_2O; circle is design point.

ity" of GuHCl and its concentration [97]. The decrease in the PEG specific partial volume was observed following the decrement in GuHCl, which corresponds to the little water hydration of PEG molecule for structuring of the GuHCl-water with the hydrogen bounds that are placed around the ethylene chains of PEG [98].

4. The Effects of PEG/Salt Concentration

A number of studies reported the effect of PEG concentrations with various values (7-21%, w/w) on protein partition coefficient and enzyme partition coefficient from various sources [99,100]. Accordingly, there was a consensus of the findings that the PEG concentration had substantial impact on the partitioning of biomolecules in the ATPS. Fig. 4 illustrates the influence of PEG/salt weight on the partition behavior of GuHCL in the ATPS composed of PEG+potassium phosphate+H_2O. In addition, the results of the experimental design indicated that the weight percent of PEG/salt in the feed had the lowest impact on the GuHCl partitioning.

5. The Effects of PEG Molecular Weight

One of the recent studies focused on the molecular mechanism controlling the phase partition in aqueous polymer systems; the direct molecular interaction between monomeric units of the two polymers was found to be an important factor [101]. This analysis was conducted with regard to the Flory-Huggins theory [102] for polymer solutions, as developed by Scott [103] for phase separation in organic solvents. To separate the phases in one solvent system (component 1) and two polymer systems (components 2 and 3), there should be an effective interaction between the units of these two polymers [104]. In other words, considering the time and effort, it is more efficient to make one 2 : 2 and one 3 : 3 pair than having two 2 : 3 pairs [104]. There is a low contribution in the polymer molecules to the overall unbound energy originating from the entropy of combining for long polymers, so that the only requirement for the separation of phase is a "weak repulsion" between the units of the polymer.

Two factors play fundamental roles in the partitioning of biomolecules: the molecular weight of polymers and the other components constituting the phase [94]. The first factor has a high effect on the biomolecules partitioning [99,105]; however, the higher molecular weight of PEG has lower coefficient, a high separation demands for lower polymer concentration [106]. On the other side, the low molecular weight of PEG resulted in a decreased hydrophobicity following the shorter polymer chains in a hydrophilic end group [107], while better partitioning may result from the decreased interfacial tension of low molecular weight [60,104,106]. Moreover, an increment in the PEG molecular mass led to a reduction in free volume by increasing the chain length of the PEG polymer [65,105, 108] The influence of PEG molecular weight on the partition behavior of GuHCL expressed as the partition coefficient and a function the PEG molecular weight, is illustrated in Fig. 5. The results of the model indicated that the increase in the polymer molecular slightly decreased GuHCl partition coefficient.

6. The Effects of Temperature

A decrease in temperature led to the increase in the viscosity, causing difficulty in controlling the system (flow transport rate) [109]. The effect of temperature on protein partitioning was complicated due to the sensitivity of some processes to the temperature such as the phase composition, the electrostatic interactions, and the hydrophobic interactions [110]. Furthermore, the temperature had an indirect effect on the entropy of the water molecules in their interaction with PEG, which drive partition of the protein [111]. In addition, some studies observed an increase in the partition coefficient with the temperature [112,113], whereas others found that the partition coefficient was independent from the temperature [114,115]. The hydrophobic side chains were shielded from water as they were covered inside the proteins during the low temperature. Then the increase in the temperature resulted in the exposure of hydrophobic side chains to the solvent and the water molecules in the solvent formed clusters around these hydrophobic residues. Therefore, there was a higher chance to form water clusters around these exposed side chains, resulting in a decrease in partition coefficient of the protein [116]. Fig. 6 shows the influence of temperature on the partition behavior of GuHCl in the ATPS composed of PEG+potassium phosphate+H_2O. According to the results

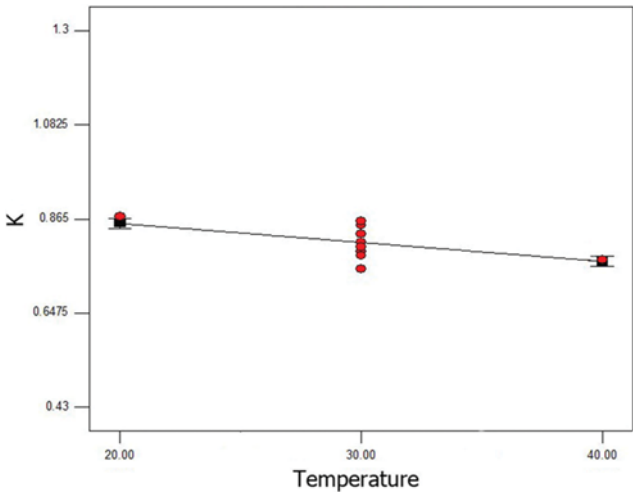

Fig. 6. The solid line show the effects of PEG molecular weight on GuHCl partition coefficient in ATPS of PEG+potassium phosphate+H_2O; circle is design point.

of the experimental design, the temperature had the smallest impact on the GuHCl partitioning. However, by increasing feed temperature, GuHCl partition coefficient was decreased.

7. The Effects of Interaction Between Two Parameters on the Guanidine Hydrochloride Partitioning

Three-dimensional (3D) surface plots for GuHcl partition were constructed as representative (Fig. 7). The 3D surface plots showed the effects and the interaction of the two independent variables on the responding variable as the third independent variable was fixed at the central experimental level of zero. Accordingly, each of these plots allowed the visualization of the significant factors derived from the statistical analysis.

CONCLUSION

The partition coefficients of GuHCl in ATPS are crucial to design the separation and purification units in the production of recombinant proteins. In this work, a predictive quadratic statistical model was used that describes the relation among the responses of the partition coefficients of guanidine hydrochloride in the PEG-phosphate aqueous two-phase system respect to pH, PEG molecular weight, GuHCl concentration, PEG/salt concentration, and the temperature. A factorial experimental design consistent in 50 experiments was used to produce the statistical model. ANOVA showed the validity of the results and the model could explain 97.88% of the variability in the response. To study the effect of these parameters on GuHCl partition coefficient, the CCD, based on the full factorial experimental design, was employed to fit a quadratic model for the ATPS. The proposed model had a good agreement with the experimental data. The results of the model indicated that the increase in the polymer molecular weight decreased the GuHCl partition coefficient. However, the increment of the PEG/salt con-

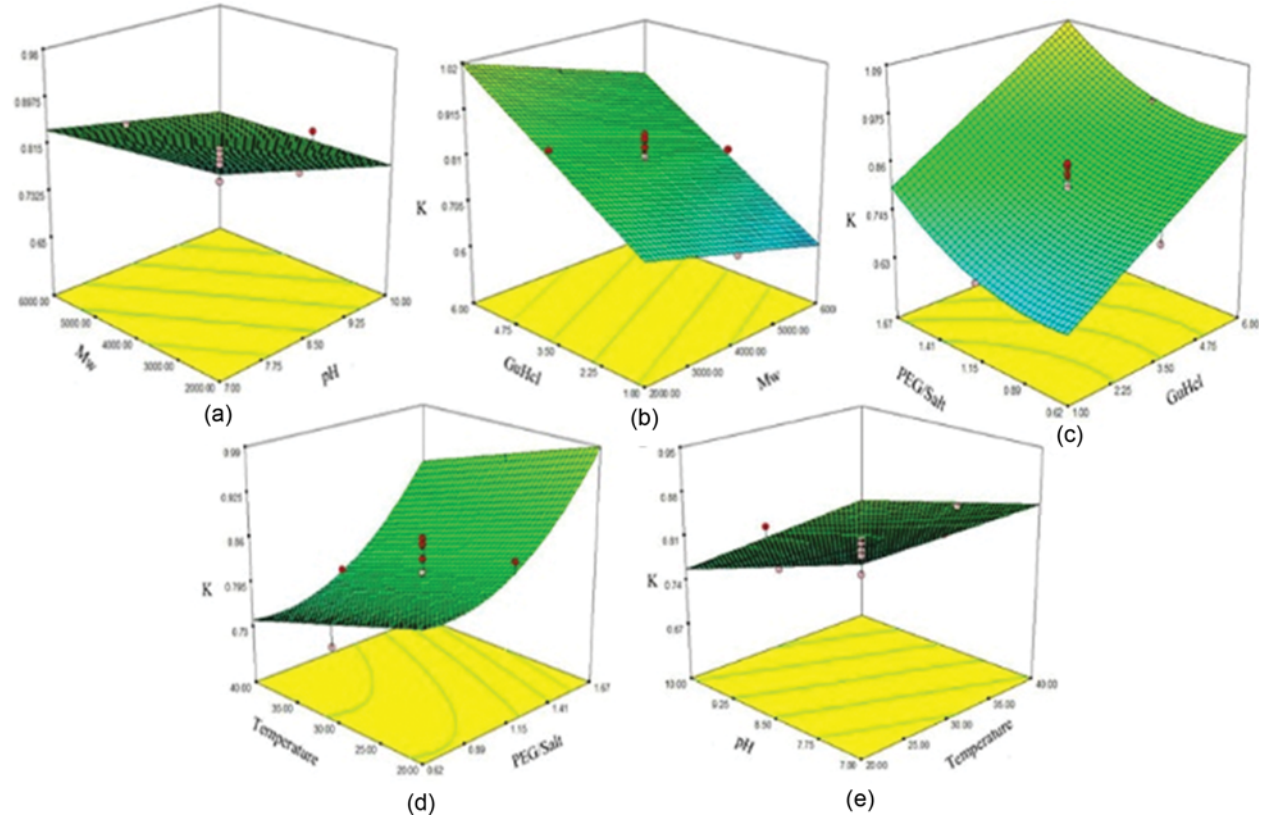

Fig. 7. Response surface plots for the effects of (a) molecular weight and initial solution pH; (b) GuHCl concentration and molecular weight; (c) GuHCl concentration and polymer/salt concentration; (d) polymer/salt concentration and temperature; (e) initial solution pH and temperature; circle is design point.

centration in the feed increased the partition coefficient of GuHCl. The systematic predication capability of this model can be efficient and practical for the related experiments, since they may be an alternative for experiment planning. Furthermore, the model provides a significant opportunity for the researchers and scholars in the field to acquire the knowledge related to the process of separation in the ATPSs to enhance the products of protein refolding.

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