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Multiple response optimization of heat shock process for separation of bovine serum albumin from plasma

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ABSTRACT

Bovine serum albumin produced by heat shock from bovine plasma has been researched for the effects of sodium caprylate concentration [Cap], temperature (*T*), and pH on yield (Yield%) and purity (BSA%). Response surface methodology and desirability function approaches were applied to optimize its process. The best compromise solution was found with BSA% = 95.0 and Yield% = 28.5 for a [Cap] = 2 mM, *T* = 67.9°C, and pH = 5. A Monte Carlo simulation showed that it is possible to obtain excellent values for each individual response. However, a techno-economic feasibility study must be carried out to determine which one is the best option.

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KEYWORDS
Bovine serum albumin; multiple response optimization; response surface methodology

Introduction

Bovine serum albumin (BSA) is the most abundant protein in blood plasma, accounting for about 60% of the total protein.[1] Its molecular weight is 66.300–69.000 Da,[2] it is highly soluble in water, and its isoelectric point is 4.78.[3] It has numerous commercial uses, among which ELISAs (enzyme-linked immunosorbent assay), immune blots, and immunohistochemistry can be mentioned. It is also commonly used as protein standard, electrophoresis (M.W. standard), molecular biology, protein base or filler, RIA systems, and serology.[4,5]

One of the first methods of isolation involved extensive dialysis of serum, precipitation with ammonium sulfate, electrophoresis, and affinity chromatography.[4,6] None of these methods are applicable to large-scale production. Initial isolation is prepared by alcohol precipitation (Cohn's method)[7] or by heat treatment.[4] Cohn's method is widely used for the production of albumin and has several disadvantages, such as refrigeration system, protein denaturation, protein losses, and requirement of high-quality starting material.[8] The process of heat shock consists of the adjustment of temperature and pH and the addition of an albumin stabilizer, the sodium caprylate.[9–13] This addition allows the albumin to tolerate temperatures over 65°C without denaturation. Most of the other plasmatic proteins are denatured due to heating and precipitation, leaving BSA in solution. This method became widely used because it is practical, fast to be applied, and can be easily reproduced. The literature available shows different conditions of heat shock, but how these conditions influence on the BSA purity and yield has not been analyzed.

The low cost of BSA, as compared with other proteins, and its diverse biotechnological applications have generated an important increase on its demand.[14] Few manufacturers in the world control the technology needed for BSA production. Thus, the possibility of its production in Argentina is very attractive. Argentina has a very important bovine meat market that ensures the availability of serum or plasma at very competitive prices, and this raw material presents excellent conditions for bovine spongiform encephalopathy (BSE) in relation to the USA and Europe.

In this study, on the basis of the availability of raw material at a low cost, some variables that have an impact on BSA extraction from bovine plasma by heat shock were analyzed. Thermal coagulation was carried out in several laboratory studies, as this is the determinant stage of the heat shock process, since the percentage of purity (BSA%) and yield (Yield%) of the final product depend on it. A high purity with a high yield allows obtaining a higher-quality product at a lower cost.

The response surface methodology (RSM) is a tool that enables the study of the effects of two or more continuous factors over one or more responses and the optimization of the processes.[15,16] The optimization implies to finding the level of the factors that obtain the best compromise solution.

In this research, for the first time, the influence of three factors was studied: sodium caprylate

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concentration [Cap] as a thermal stabilizer of the BSA, pH, and temperature (T). Additionally, the effects of the interaction among these factors on BSA% and Yield% in its extraction from plasma by heat shock were considered. The study was performed using RSM and desirability function to achieve the multiple response optimization.

The aims of this study were to analyze the effect of [Cap], pH, and T on BSA% and Yield% in the first stage of the heat shock method, and to find appropriate models to describe BSA% and Yield% behavior in relation to the studied factors.

Materials and methods

Materials

Table 1 shows the characteristics of dehydrated bovine plasma powder (Yeralbum, Yeruba S.A., Sante Fe, Argentina).

The stock solution of sodium caprylate (AS from Sigma-Aldrich, St. Louis, MO, USA) was 50 mM with a pH of 7.2. To adjust the pH, HCI (AR from Sigma-Aldrich, St. Louis, MO, USA) was used.

Table 1. Bovine plasma characteristics (Yerubá S.A.).

Manufacturing process	Powder dried up by spray
Proteins content	≥76%
Ashes	<10%
Humidity	<6%
Solubility	≥96%
Bacteriological analysis	*Escherichia coli* absent, Salmonella absent

Methods

Experimental design and analysis

The design and analysis of the experimental runs were carried out by RSM. This technique has been widely described from both theoretical and practical points of view.[15–20] RSM has enabled us to study the effects of [Cap], pH, and T on BSA purity (BSA% w/w) and BSA yield (Yield% w/w). In this way, it was possible to find the best compromise solution.

The experimental design was Box–Behken type for three levels and three factors (13 runs) with three replications in the center of the plan, considering (BSA%) and (Yield%) as responses. In Table 2, the factors with coded levels and at real scale are shown.

Table 2. Factor levels and coded values used in the experimental design.

Factors	Coded	−1	0	+1
[Cap] (mM)	X_1	2	11	20
Temperature (T) (°C)	X_2	64	68	72
pH	X_3	5	6	7

Thermal coagulation experiments

Plasma concentration in solution was 6.43% w/v, which is equivalent to 5.14% w/v of protein concentration. This concentration was determined in previous tests with variable concentrations between 6% and 12% w/v. When concentrations were higher than the chosen one, solutions significantly increased their viscosity after heating, and turned into gel in some cases.

Sodium caprylate was added to plasma solution in order to obtain variable concentrations of samples (between 2 and 20 mM). Thermal treatment was applied for 90 min at the chosen T (between 64°C and 72°C). The system was then cooled abruptly to stop reaction, and pH was adjusted at 4.2 with a solution of 0.5 N HCl. It was left standing at 4°C for 12 h to produce better coagulation. The values of pH, T, and [Cap] were chosen in accordance with the values found in the bibliography. The time allotted for the procedure is sufficient to inactivate potential pathogens. Preliminary studies have shown that the more heating time was allowed, the less amount of yield was obtained. Afterward, the solution was centrifuged at 4000 rpm for 20 min, the supernatant was collected and BSA purity and quantity were determined.

Measurement of BSA quantity and purity in the supernatant was carried out by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad Mini-Protean 3 Cell equipment.

Sample bands were compared with a BSA pattern (from Sigma-Aldrich A-3059) and with untreated plasma. Gels were analyzed using Gel-Pro Analyzer 4.0 software. Protein content was determined by Kjeldahl method.[21] The BSA% and Yield% were calculated using Eq. (1) and (2), respectively.

$$\mathrm{BSA\%} = \frac{\text{BSA band density}}{\text{Total sample density}} \times 100 \tag{1}$$

$$\mathrm{Yield\%} = \frac{\text{BSA final}}{\text{BSA initial}} \times 100 \tag{2}$$

Model fitting

Taking into account the experimental design that was chosen, the complete second-order models were adjusted using linear regression.[17] The quality of the model adjustments was evaluated through the coefficient of determination R^2, analysis of variance (ANOVA), and graphical analysis of residuals.

Multiple response optimization

In order to achieve multiple response optimization, a variation of desirability function approach[22] developed by Derringer and Suich[23] was used. This is one of the most commonly used methods in industry. It consists of transforming each response y_i into an individual desirability function $d_i(y_i)$ that varies within the range $0 \leq d_i(y_i) \leq 1$, where zero (0) is the less desirable value and the most desirable is 1.

Depending on whether a particular response y_i is to be maximized, minimized, or assigned to a target value, different desirability functions $d_i(y_i)$ can be used. In this study, the equation available for the *larger is better* case was chosen, since both responses must be maximized.

$$di(yi) = \begin{cases} 0 & if\ (x) < L_i; \\ \frac{y_i(x)-L_i}{T_i-L_i} & L_i \leq y_i(x) \leq T_i \\ & if\ y_i(x) > T_i \end{cases}$$

where x is the factor, L_i is the lowest acceptable value of y_i, and T_i is the target value desired for ith response. At this point, r is the parameter that determines the shape of $d_i(y_i)$, $0 \leq r \leq 1$. For this case, $r = 1$ was used, which means that the desirability function is linear.

The individual desirabilities are then combined using the geometric media, which gives the overall desirability D:

$$D = (d_1(y_1).d_2(y_2).d_m(y_m))^{1/m}$$

where m is the number of responses. In this way, the problem of multi-response restricted optimization is reduced to a problem of a single response optimization.

The limits and target for both responses were: for BSA(%), $L_1 = 95$ and $T_1 = 100\%$ w/w, and for Yield (%), $L_2 = 20$ and $T_2 = 30\%$ w/w. The value for L_1 was established considering BSA purity as a key quality characteristic for the expected use, which must be ≥95% w/w. This is important since this value avoids a later stage of purification, increasing production costs. For L_2 it was necessary to select a lower yield value, since the desired compromise solution could not be reached at high values.

Results and discussion

Table 3 shows the experimental runs with the values of the measured responses. A simple inspection of the table reveals that the run of major BSA% (run 5) presents one of the lowest Yield% values. On the other hand, the run with highest Yield% (run 8) has a relatively low BSA% value (Fig. 1). This tendency suggests the existence of a certain grade of negative correlation between the responses, which makes it more difficult to maximize both of them simultaneously.

Table 3. Box–Behnken experimental design with measured responses.

Run	[Cap] (mM)	*T* (°C)	pH	BSA (%w/w)	Yield (%w/w)
1	2	64	6	76.0	31.9
2	20	64	6	69.9	57.5
3	2	72	6	81.2	30.7
4	20	72	6	66.0	53.8
5	2	68	5	97.2	27.8
6	20	68	5	85.5	68.4
7	2	68	7	84.7	40.9
8	20	68	7	72.5	77.8
9	11	64	5	82.8	22.5
10	11	72	5	25.3	32.2
11	11	64	7	88.3	37.1
12	11	72	7	65.7	16.1
13	11	68	6	79.7	28.7
14	11	68	6	77.3	29.8
15	11	68	6	75.3	27.0
16	11	68	6	81.2	30.2

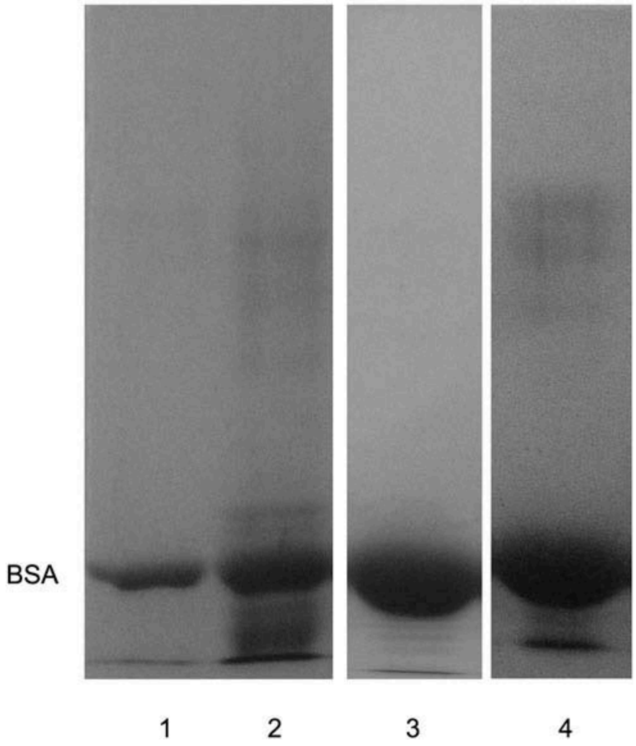

Figure 1. SDS-Page assay: (1) BSA pattern, (2) untreated plasma, (3) run 5, (4) run 8.

BSA% modeling and the analysis of factors' effects

Table 4 shows an adjusted model for BSA% in coded variables, after eliminating the statistically non-significant terms ($p > 0.05$). The model had a determination coefficient R^2 of 0.993, meaning that about 99% of the variation observed around the average of the response can be explained by the model. The graphical analysis of residuals did not show any violation of the assumptions of least-squares method. Since it was not possible to achieve a good adjustment with a second-degree polynomial, responses and factors were transformed

Table 4. Adjusted model for BSA%; $R^2 = 0.993$; R^2 adjusted = 0.983.

	Coefficients	SE	t (6)[a]	p-Value
Intercept	78.3	0.8	95.2	0.000000
X_1	−5.6	0.7	−8.4	0.000153
X_1^2	6.8	0.9	7.2	0.000361
X_2	−19.7	0.9	−20.8	0.000001
X_2^2	−11.9	0.9	−12.6	0.000015
X_3	2.3	0.9	2.4	0.051639
$X_1^2.X_2$	20.0	1.3	14.9	0.000006
$X_1^2.X_3$	−6.4	1.3	−6.4	0.000657
$X_1.X_2$	−2.2	0.9	−2.4	0.055330
$X_2.X_3$	8.9	0.9	9.4	0.000084

SE, standard error

[a] Statistic t of student calculated.

in order to simplify the model.[18] Because results of these transformations were ineffective, it was necessary to use a third-degree polynomial.

The values of regression coefficients shown in Table 4 indicate that $T(X_2)$ is the factor of major effect on BSA%. Linear and quadratic coefficients of this factor approximately double the coefficients of [Cap] (X_1). The quadratic/linear interaction of greater coefficient involves [Cap] and T, showing a strong interaction between these factors.

The results of ANOVA show that the model does not present lack of fit (Table 5). On this basis, it was considered that the adjusted model was appropriate to describe BSA% behavior in function of the studied factors ([Cap], T, and pH).

Figure 2 shows response surface constructed with the adjusted model for BSA%. From this graphic, the effect of [Cap] and T at a fixed level of pH = 5 can be analyzed. The increase of T at low [Cap] produces an increase in BSA% until reaching a maximum at about 66°C; over this value, BSA% decreases. This behavior is similar for high levels of [Cap]. At the lowest T level, [Cap] increase produces a moderate

Table 5. Analysis of variance (ANOVA) for BSA%.

	SS	df	MS	F[a]	p-Value
Regression	3044.9	7	435.0	64.0	0.002918
Lack of fit	42.7	5	8.5	1.2	0.469632
Pure error	20.4	3	6.8		
Total	3107.9	15			

SS, sum of square; df, degrees of freedom; MS, mean square.

[a] Statistic F of Fisher calculated.

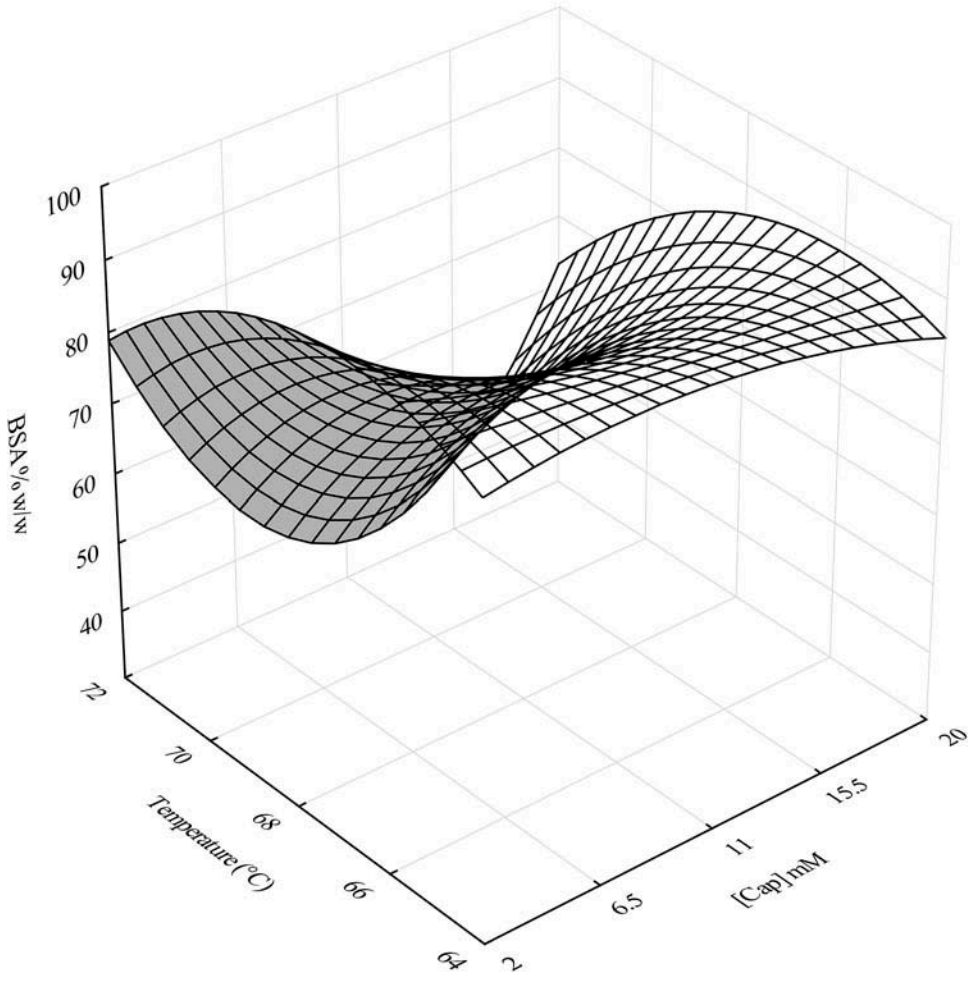

Figure 2. Response surface for BSA%. Effect of [Cap] and T at pH = 5.

approximately linear decrease in BSA%. Nevertheless, due to a strong interaction between both factors, the effect of [Cap] at high T is different, showing a pronounced curvature which passes through a minimum around [Cap] = 11 mM.

Figure 3 shows the effect of pH and T over BSA% at a fixed [Cap] of 2 mM, which is calculated as optimum concentration for the software. The increase of pH at low T produces a linear decrease of the BSA%, while at high T, it produces the contrary effect due to the presence of a strong linear interaction between both factors. The increase of T initially produces an increase of BSA% in all the pH range until reaching a maximum point at around 68°C, where it starts decreasing.

Figure 4 shows the effects of [Cap] and pH at a fixed T of 68°C. The increase of the pH produces a linear decrease of BSA% in extreme values of [Cap]. At a low pH, the increase of [Cap] produces a significant decrease of BSA% passing through a minimum at [Cap] = 11 mM; at higher [Cap], BSA% increases. At high pH, the increase of [Cap] produces a small almost linear decrease of BSA%.

From this model, a maximum BSA% of 97.2 at [Cap] = 2 mM, T = 67.8°C, and pH = 5.2 is obtained.

Yield% modeling and the analysis of factors' effects

Table 6 shows the adjusted model for Yield% in coded variables. The model presented an R^2 of 0.996. The graphical analysis of residuals did not show any violation of the assumptions of least-squares method. In Table 7, ANOVA shows that the model does not present lack of fit. Factor [Cap] (X_1) has the greater effect over Yield%, which markedly prevails over the other factors.

Figure 5 shows that the increase of [Cap] initially produces a decrease in Yield%, passing through a minimum from where it increases abruptly. This behavior occurs in all T range. The increase of T in all [Cap] range produces an increase in Yield% until reaching a maximum at around 67°C.

In Fig. 6, it can be observed that the increase of the pH in all [Cap] range produces an approximately linear increase of Yield%, reaching a maximum for [Cap] = 20

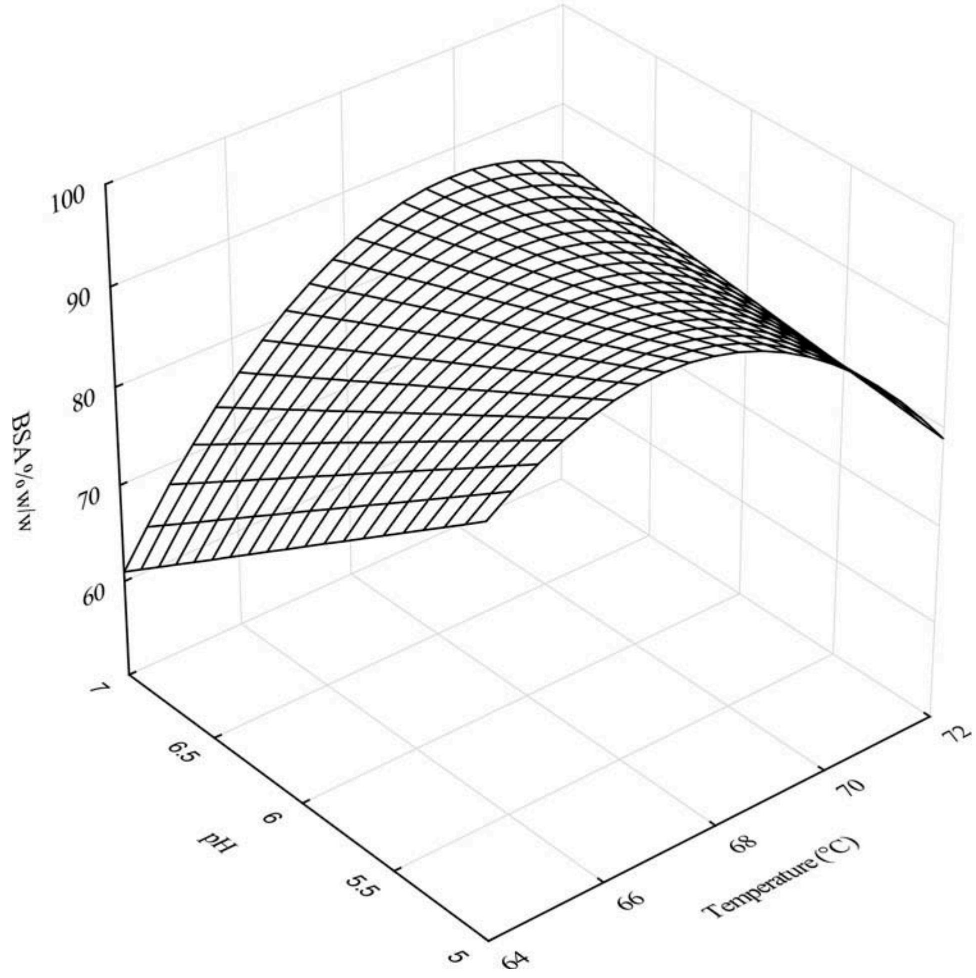

Figure 3. Response surface for BSA%. Effect of pH and T at [Cap] = 2mM.

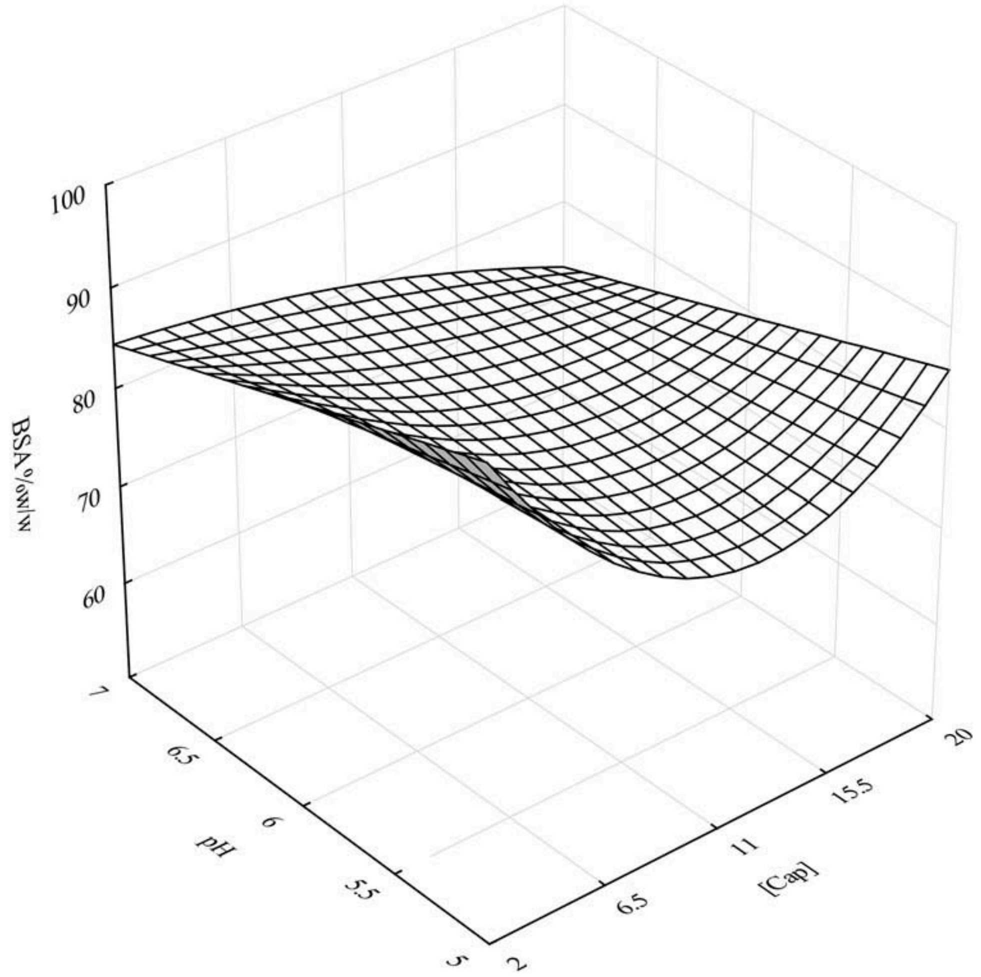

Figure 4. Response surface for BSA%. Effect of pH and [Cap] at T = 68°C.

Table 6. Adjusted model for Yield (%); R^2 = 0.996; R^2 adjusted = 0.992.

	Coefficients	SE	t (3)	p-Value
Intercept	28.9	0.7	40.7	0.000033
X_1	19.4	0.7	27.3	0.000108
X_1^2	20.6	0.7	29.1	0.000089
X_2	−2.0	0.5	−4.0	0.027732
X_2^2	−6.1	0.7	−8.6	0.003290
X_3^2	4.2	0.7	5.9	0.009877
$X_1.X_2^2$	−7.2	1.0	−7.1	0.005634
$X_1^2.X_3$	5.6	0.7	7.9	0.004200
$X_2.X_3$	−7.7	0.7	−10.8	0.001687

Table 7. Analysis of variance (ANOVA) for Yield (%).

	SS	df	MS	F	p-Value
Regression	4411.3	8	551.4	273.6	0.000000
Lack of fit	10.7	4	2.7	1.3	0.431784
Pure error	6.0	3	2.0		
Total	4428.1	15			

mM; T = 66.6°C, and a pH = 7. The increase of [Cap] initially produces a decrease in Yield% passing through a minimum at [Cap] = 7 mM from where it increases abruptly.

Figure 7 shows the effect of T and pH on Yield%. An increase of pH in all the studied range produces an increase in Yield%, and the greater effect starts from pH = 6 where the slope changes. The increase of T initially increments the Yield%, passing through a maximum of around 68°C, the highest values of Yield% are reached at a pH = 7.

From this model a maximum Yield% at [Cap] = 20 mM, T = 66.6°C, and pH = 7 is obtained.

Multiple response optimization of thermal coagulation process

Using desirability function approach, it was found that the following compromise solution between both responses is BSA% = 95.0 and Yield% = 28.5 for [Cap] = 2 mM, T = 67.9°C, and pH = 5. Even though this solution satisfied the BSA% ≥ 95 condition, it produced a low Yield% value that significantly affects the efficiency of the global process. In order to explore possible alternatives, a sensitivity

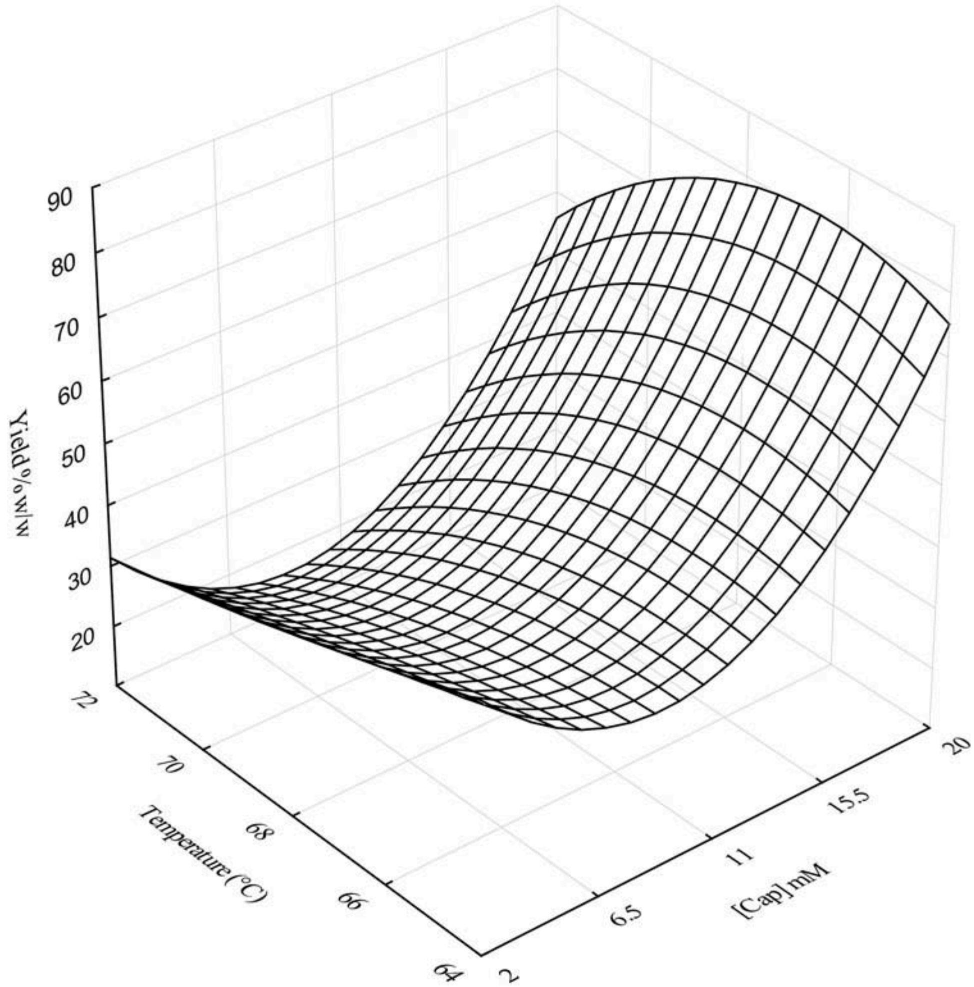

Figure 5. Response surface for Yield%. Effect of *T* and [Cap] at pH = 7.

analysis of both responses to the changes of the three studied factors was carried out. For this, five Monte Carlo[24] simulations were carried out, generating three subsets of 10,000 data in each simulation. Factors follow a normal distribution with media zero (0) and a standard deviation of 0.35, which guaranteed a fluctuation of the factors within the studied range. The results obtained are shown in Table 8, displayed in decreased order and filtered for a BSA% ≥ 95 and Yield% ≥ 80. As it can be observed, optimal values could be obtained independently for both responses (BSA of 99.8% and yield of 99.6%). Even though model predictions are strictly valid within the studied region, these values seem possible considering the appropriate adjustment of the models, and the fact that it extrapolates in experimental limit proximities. Nonetheless, this needs further experimental verification.

Considering the problem of low Yield% (28.5), an alternative could be to operate in a region of high Yield% ≥ 80 and develop a follow-up stage of BSA purification in the global process, though this would increase the total cost of production. The final optimization of the process could be achieved by a techno-economic feasibility study. This would allow to determine which of the following is the best option: (a) to operate in a zone of BSA% ≥ 95 ([Cap] between 0.9 and 4.7 mM; *T* between 66°C and 68°C; pH between 4.7 and 5.2) and to accept a low Yield%; or (b) to work in a zone of Yield% ≥ 80 ([Cap] between 19.3 and 21.9 mM; *T* between 66.7°C and 69.4°C; pH between 5.8 and 7.2) incorporating a follow-up stage of BSA purification.

Conclusions

This research was developed regarding the effects of [Cap], *T*, and pH at laboratory scale on Yield% and BSA% of BSA extracted by heat shock from bovine

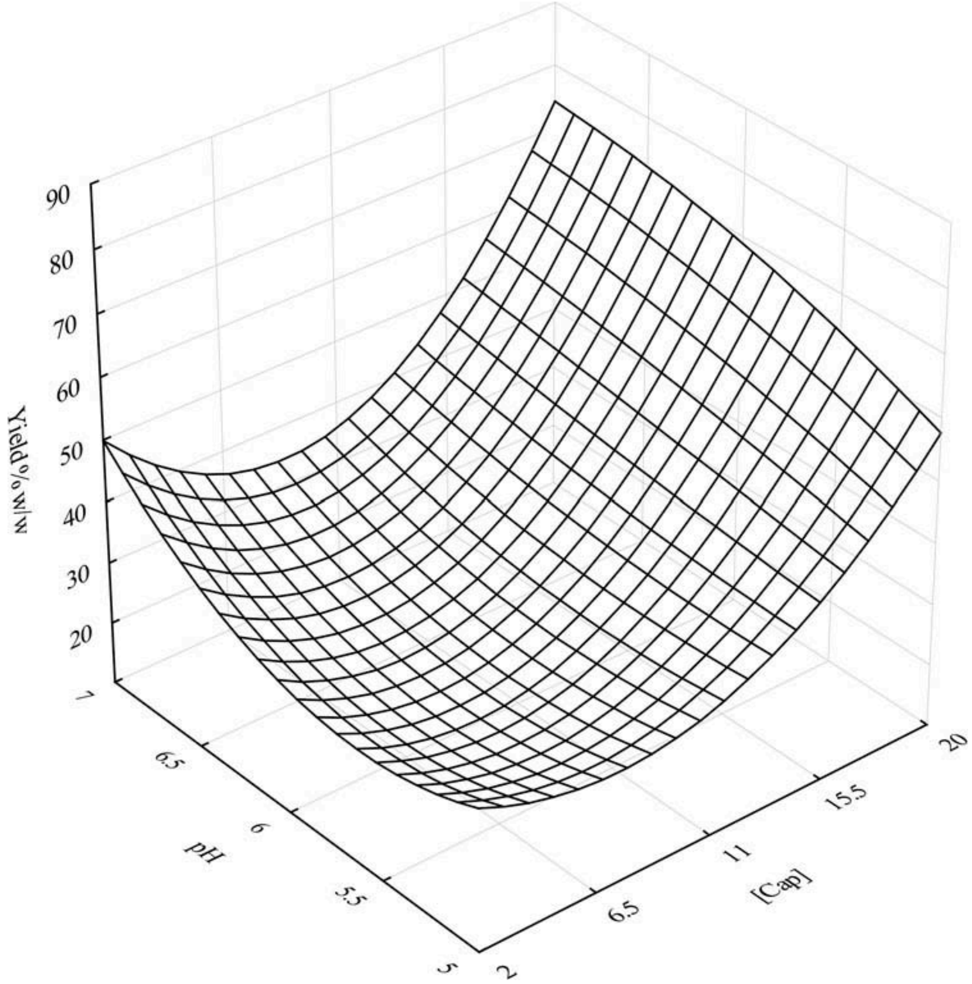

Figure 6. Response surface for Yield%. Effect of pH and [Cap] at T = 66.6°C.

plasma powder. From the results obtained, it can be stated that the adjusted models were appropriate to describe BSA% and Yield% behavior in function of studied factors ([Cap], T, and pH).

RSM and desirability function approach were used for the first time to study multiple response optimization of the process.

Important interactions among the studied factors and a negative correlation between both responses were detected. It was found that T is the factor of greater influence over BSA%. The quadratic/linear interaction of greater coefficient involves [Cap] and T, showing a strong interaction between these factors. The increase of T produces an increase in BSA %, reaching a maximum at around 66–68°C. At this T, the higher values of BSA% were achieved at low values of pH and [Cap].

The factor that has the greater effect over Yield% is [Cap]. The higher values of Yield% were obtained at high values of pH and [Cap], and at a T around 66–68°C.

Using desirability function approach, the best compromise solution was reached between both responses: [Cap] = 2 mM, T = 67.9°C, and pH = 5 being the BSA = 95.0% w/w and yield = 28.5% w/w, which achieved the target for BSA% (≥95%) at the expense of a very low Yield%.

The level of the factors that maximize Yield% and BSA% independently could also be determined. A Monte Carlo simulation study showed that it is possible to obtain excellent values for each individual response (BSA% = 99.8 and Yield% = 99.6) in the proximities of the experimental limits.

An alternative to improve the Yield% could be to operate in a region of high Yield% ≥ 80 and develop a follow-up stage of BSA purification in the global process.

However, the final optimization of the process must be achieved through a techno-economic feasibility study that determines which one is the best option: (a) to operate in a region of BSA% ≥ 95

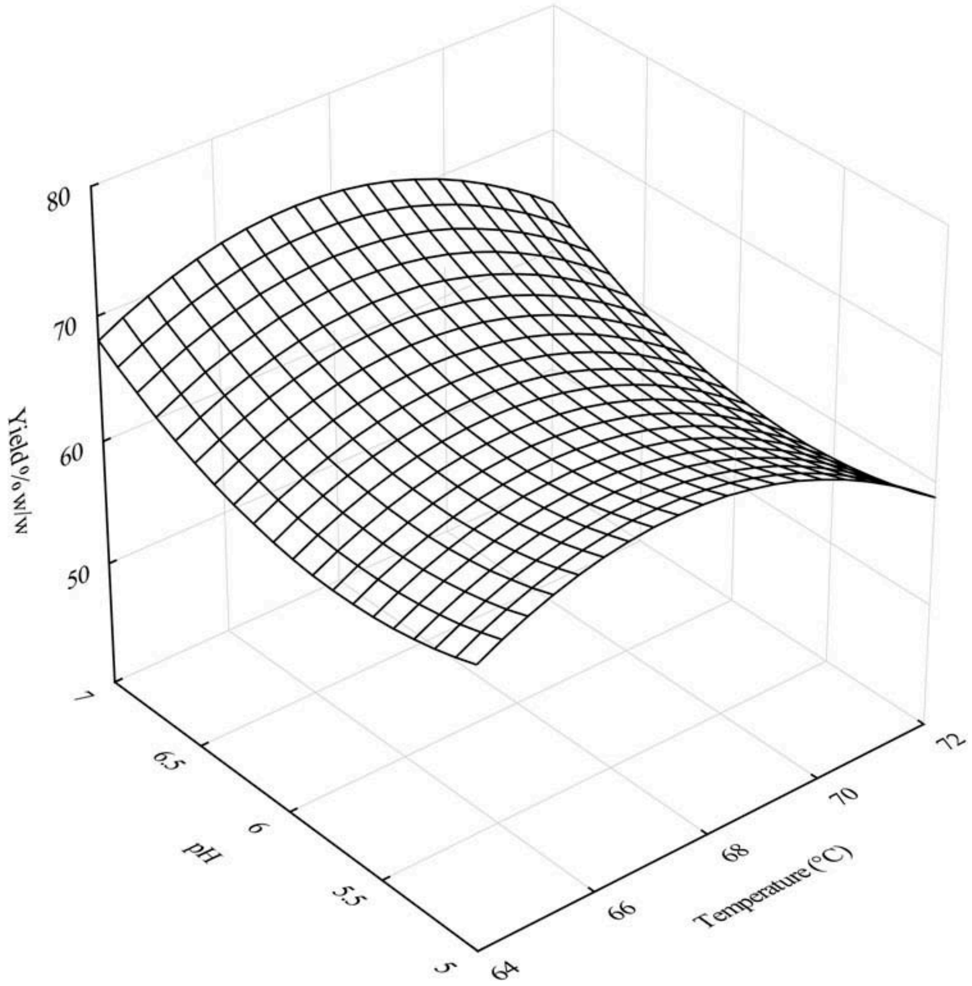

Figure 7. Response surface for Yield%. Effect of *T* and pH at [Cap] = 20 mM.

Table 8. Best solutions obtained through simulation.

[Cap]	*T*	pH	BSA	Yield
(mM)	(°C)		(%w/w)	(%w/w)
0.9	66.9	4.8	99.8	28.9
2	67.9	4.7	99.4	29.7
1.7	67.6	5.2	97.2	28.4
2.4	65.2	5.1	96.0	25.1
2.0	66.9	5.0	95.7	27.3
2.1	68.3	5.1	95.5	28.7
4.7	64.7	5.1	95.0	21.7
21.8	66.7	7.2	65.5	99.6
19.3	66.7	7.9	68.4	92.0
20.9	66.7	7.1	68.7	89.5
22.6	67.8	6.0	81.2	88.1
22.6	69.4	6.0	85.5	85.2
22.4	68.6	5.8	85.2	84.4
21.9	67.3	6.2	78.1	84.3
21.7	68.3	6.2	80.0	82.8
20.4	67.5	5.8	70.5	80.7
20.0	66.6	7.0	71.4	80.4

accepting a low yield, or (b) to work in a region of Yield% ≥ 80 and develop a later stage of purification to improve BSA%.

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Conflict of interest

The authors declare that they have no conflicts of interest.

References

[1] Salgin, S.; Takac, S.; Ozdamar, T.H. (2006) Adsorption of bovine serum albumin on polyether sulfone ultrafiltration membranes: Determination of interfacial interaction energy and effective diffusion coefficient. *Journal of Membrane Science*, 278 (1–2): 251–260.

[2] Tanaka, K.; Sawatani, E.; Shigueoka, E.M.; Dias, G.A.; Nakao, H.C.; Arashiro, F. (2001) Isolation of bovine plasma albumin by liquid chromatography and its polymerization for use in Immunohematology.

Brazilian Journal of Medical and Biological Research, 34 (8): 977–983.

[3] Avalos, M.A.; Hernández, M.A.; Cabeza Salinas, M. (1997) Método fácil y rápido para obtener cristales de Albúmina Sérica Bovina. *Bioquímia*, 22 (2): 660–664.

[4] A2153 Sigma. (2016) Albumin from bovine serum, Product information sheet. *sigma-aldrich.com*.

[5] Quinlan, G.J.; Greg, G.S.; Martin, S.; Evans, T.W. (2005) Albumin: Biochemical properties and therapeutic potential. *Hepatology*, 41 (6): 1211–1219.

[6] Zhao, K.; Chen, T.; Lin, B.; Cui, W.; Kan, B.; Yang, N.; Zhou, X.; Zhang, X.; Wei, J. (2015) Adsorption and recognition of protein molecular imprinted calcium alginate/polyacrylamide hydrogel film with good regeneration performance and high toughness. *Reactive and Functional Polymers*, 87: 7–14.

[7] Cohn, E.; Strong, E.; Hughes, L.; Mulford, J.; Ashworth, N. (1946) Preparation and properties of serum and plasma proteins. *Journal of the American Chemical Society*, 68: 459–475.

[8] Denizli, A. 2011 Plasma fractionation: conventional and chromatographic methods for albumin purification. *The Journal of Biological Chemistry*, 39 (4): 315–341.

[9] Reid, A.F. Method of Purifying Albumin. U.S. Patent 2,705,230, Mar. 29, 1955.

[10] Porsche, J.D.; Lesh, J.B.; Myron, D.; Grossnickle, M.D. Recovery of serum albumin. U.S. Patent 2,765,299, Oct. 2, 1956.

[11] Moya, A.; Paz, O.; Joó, L.; Gutiérrez, E.; Rodríguez, Z.; Cádiz, A. (2000) Estabilización de la albúmina con caprilato de sodio durante su obtención y pasteurización. *Vaccimonitor*, 9 (4): 10–15.

[12] Anraku, M.; Tsurusaki, Y.; Watanabe, H.; Maruyama, T.; Kragh-Hansen, U.; Otagiri, M. (2004) Stabilizing mechanisms in commercial albumin preparations: octanoate and N-acetyl-L-tryptophanate protect human serum albumin against heat and oxidative stress. *Biochimica et Biophysica Acta*, 1702 (1): 9–17.

[13] Faroongsarng, D.; Kongprasertkit, J. (2014) The role of caprylate ligand ion on the stabilization of human serum albumin. *Journal of Pharmaceutical Science and Technology*, 15 (2): 466–471.

[14] Ribeiro Alves, M.R.; Giraldo Zuñiga, A.D.; Superbi Sousa, R. de C.; Carmelita Zacchi Scolforo, C.Z. (2016) The process of separating bovine serum albumin using hydroxyapatite and active babassu coal (Orbignya martiana). *The Scientific World Journal*, 2016: 9.

[15] Box, G.E.P.; Wilson, K.B. (1951) On the experimental attainment of optimum conditions. *Journal of the Royal Statistical Society, Series B*, 13: 1–45.

[16] Box, G.E.P.; Behnken, D. (1960) Some new three-level designs for the study of quantitative variables. *Technometrics*, 2 (4): 455–475.

[17] Draper, N.R.; Smith, H. (1998) *Applied Regression Analysis*, 3rd Ed.; John Wiley & Sons, New York.

[18] Box, G.E.P.; Draper, N. (1987) *Empirical Model-Building and Response Surfaces*, 1st Ed.; John Wiley & Sons, New York.

[19] Box, G.E.P.; Hunter, J.; Hunter, W. (2005) *Statistics for Experimenters, Design, Innovation and Discovery*, 2nd Ed.; John Wiley & Sons.

[20] Hoerl, A.E. (1959) Optimum solution of many variables equations. *Chemical Engineering Progress*, 55 (11): 67–78.

[21] AOAC (Association of Official Agricultural Chemists). (1995) Official methods of analysis (16th ed.), Vol. II, AOAC International, Arlington.

[22] Harrington Jr., E. C. (1965) The desirability function. *Industrial Quality Control*, 21: 494–498.

[23] Derringer, G.; Suich, R. (1980) Simultaneous optimization of several response variables. *Journal of Quality Technology*, 12: 214–219.

[24] Guerrero, H. (2010) *Excel Data Analysis, Modeling and Simulation*, Springer-Verlag, Berlin Heidelberg, Germany.